Journal of

DAIRY SCIENCE

Vol. 45 June, 1962 Number 6

Dedicated to the Centennial Commemoration of the Legislation Establishing the United States Department of Agriculture and the Land Grant Colleges, 1862

Be It Enacted by the Senate and House of Representatives of the United States of America in Congress assembled,

That there is hereby established at the seat of government of the United States a Department of Agriculture, the general designs and duties of which shall be to acquire and to diffuse among the people of the United States useful information on subjects connected with agriculture in the most general and comprehensive sense of that word, and to procure, propagate, and distribute among the people new and valuable seeds and plants.

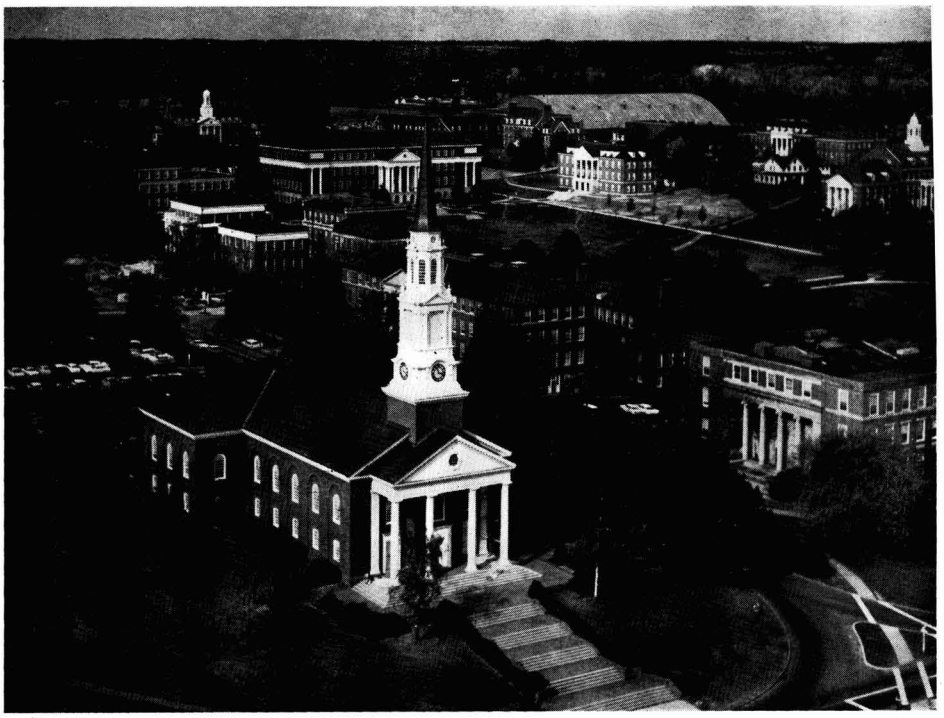
Act Approved May 15, 1862, by

PRESIDENT ABRAHAM LINCOLN

"... maintenance of at least one college where the leading object shall be, without excluding other scientific and classical studies and including military tactics, to teach such branches of learning as are related to agriculture and the mechanic arts, in such manner as the legislatures of the States may respectively prescribe, in order to promote the liberal and practical education of the industrial classes in the several pursuits and professions in life."—From the Morrill Act approved July 2, 1862, by

PRESIDENT LINCOLN

U. S. Department
of Agriculture
Washington, D. C.



Aerial View of the
University of Maryland Campus
College Park, Maryland

The 57th Annual Meeting of the American Dairy Science Association, jointly hosted by the University of Maryland and the United States Department of Agriculture, was held on the College Park campus of the University of Maryland, June 17 to 21, 1962.

The Maryland State College of Agriculture was established at College Park in 1856. In 1920, an Act of the Maryland State Legislature merged it with the University of Maryland professional schools (founded in 1807) in Baltimore. The University has grown rapidly, increasing from 5,500 students in 1935 to 18,500 in 1962. The University of Maryland covers over 2,500 acres, which includes campuses in College Park, Baltimore, and Princess Anne and numerous research and service installations throughout the state. Its staff consists of 3,300 full-time and part-time instructional and research personnel. At the College Park campus are located eight undergraduate colleges, the graduate school, and various institutes and bureaus. The Baltimore campus consists of six professional schools, Psychiatric Institute, and the University Hospital. The University, through the University College, operates a self-supporting overseas academic program in 23 countries for armed service personnel and their dependents. Annual enrollment in this program is approximately 29,000. In terms of size and enrollment, the University of Maryland ranks 13th in the nation. It truly represents the land-grant university ideals.

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JOURNAL OF DAIRY SCIENCE

OFFICIAL PUBLICATION OF THE
AMERICAN DAIRY SCIENCE ASSOCIATION SINCE 1917

E. O. HERREID, Editor-in-Chief, University of Illinois
Editorial Address: Station A, Box 250, Champaign, Illinois
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SUMMARY

TERRAMYCIN IN MILK PRODUCTION
AVERAGE OF 17 TRIALS (186 DAYS)

	Control	Treated
Number of cows	1193	1177
Av. days on experiment [1]	186	186
Av. daily milk prodn./cow, lb.	30.98	31.85
Increase in daily production, lb.	—	0.87
Increase, %	—	3
Extra milk per cow, lb.	—	161.8
Value of extra milk, $[2]	—	8.09
Cost of Terramycin per cow, $[3]	—	1.26
Extra return per cow, $	—	6.83

[1]Range 119-280 days [2]Milk valued at $5 per cwt.
[3]Terramycin 13.95 gm./cow; value $0.09/gm.

There was no effect of Terramycin feeding upon the bacterial content of milk, the antibiotic did not appear in the assayed milk, and cheese cultures were not inhibited by this milk.

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ORGANIZATION OF PAPERS

1. Title should appear at the top of the first page, be as brief as possible, and be indicative of the research, followed by the author(s) name(s) and affiliation(s).
2. Summary and its preparation.
   a. There are three reasons for the summary: first, convenience to readers; second, reduce costs and expedite work of abstracting journals; and third, to disseminate scientific information.
   b. The summary should be brief, specific, and factual. It should not exceed 200 to 225 words.
   c. The opening sentence should state the research objectives, but the title *should not be repeated.*
   d. It should be intelligible without reference to the original paper and contain complete sentences and standard terminologies. It should be assumed that the reader has some knowledge of the subject.
   e. The author(s) should emphasize newly discovered facts and observations, unique apparatus and techniques, numerical data with statistics, physical-chemical constants, and new methods and their accuracy.
   f. References to earlier work should be omitted, except in most unusual cases.
3. Statement of the problem, pertinent investigations, and reasons for the study.
4. Experimental procedures.
5. Results.
6. Discussion. (5 and 6 may be combined.)
7. Conclusions.
8. Acknowledgments.
9. References. All references must have author(s) name(s), name of periodical, volume, page number, and year of publication. If a book, publisher's name and address must be added.
10. Manuscripts must be typed double-spaced[3] on 8½- by 11-inch bond paper. Lines on each page should be numbered from 1 to 26 or 28, to make it easier for the Editorial Board to review papers. The side margins should be one inch wide. Clipped-to, pasted-on, and written insertions are not acceptable. Do not staple pages together.
11. Figures (graphs) should be made with black India ink on white drawing paper, tracing paper, or blue linen and the sheets should not exceed 8½ by 11 inches. Graph papers with yellow, green, and red lines should not be used, because the lines cannot be filtered out. Curves should be identified with the symbols ○, ⊙, ●, □, ■, △, ▲, ▼, +, or ×, and they should be about 0.8 mm thick, for the axes about 0.5 mm thick, and for grid lines about 0.4 mm thick. Grid lines are necessary only if readings are to be made from the curves. Letters on the abscissae, ordinate, and the figure should be in upper case and be about 4 by 4 mm and about 0.5 mm thick, to be readable when graphs are reduced to column width. Titles for figures (graphs) must be on separate sheets. Following is a well-made figure reduced to size of the printed page:

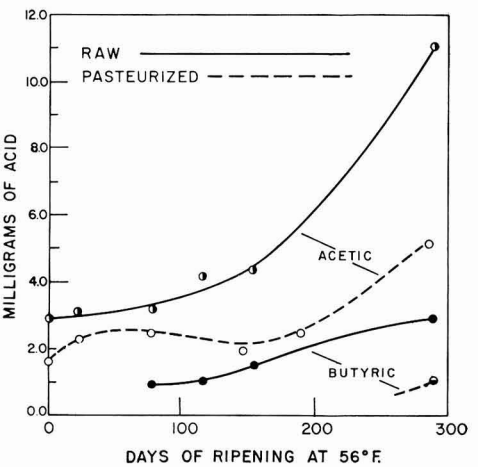

FIG. 1. Acetic and butyric acids in raw and pasteurized milk Cheddar cheese during ripening (milligrams in distillate obtained from 150 g of cheese oil).

12. Tables should be numbered on the center of the page with the title immediately below, and each table should be typed on a separate sheet of 8½- by 11-inch bond paper. They should be placed together at the end of the manuscript.

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[2] J. Dairy Sci., 44: 1788. 1961.

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Titles should indicate the content of tables and facilitate comparisons, show relationships clearly, be self-explanatory, and save space. Label heading and subheadings accurately and concisely with the data centered under them. Use correct abbreviated dimensions. Data should be referred to and discussed but not repeated in the text, and they should be presented in only significant digits within the accuracy of the methods. Use the metric system whenever possible. Do not use vertical lines and only a minimum of horizontal ones.

Tables are costly. Use graphs whenever possible.

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15. Terms such as Cottage cheese, Cheddar cheese, Limburger cheese, etc., should be capitalized as indicated. Butteroil, skimmilk, buttermilk, etc., should be written as one word. Milk fat has replaced butterfat.
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17. All manuscripts should be submitted to the Editor-in-Chief.
18. Receipt of manuscripts will be acknowledged. Authors will be notified within 30 to 60 days of the action taken by the Editorial Staff.

PEOPLE AND EVENTS

MEMORIALS

Portrait of Dr. Otto Frederick Hunziker Presented to Purdue University

A portrait of the late DR. OTTO F. HUNZIKER, Head of the Purdue University Dairy Department from 1905 to 1916, was unveiled at a memorial dinner held at Purdue University March 16, 1962. The portrait, and a beautifully inscribed plaque, were presented to the University by former students and friends of Dr. Hunziker. GLEN OGLE of the Ideal Pure Milk Company, Evansville, Indiana, was Chairman of the Indiana Hunziker Memorial Committee and made the presentation. The portrait was accepted by DR. F. N. ANDREWS, Head of the Department of Animal Sciences at Purdue University. The portrait and plaque will hang in Smith Hall, the building in which the Dairy Manufacturing group is located and which contains the Purdue Creamery.

MRS. O. F. HUNZIKER, who resides in LaGrange, Illinois, attended the memorial dinner and spoke briefly about her family and the devotion of Dr. Hunziker to his family and to his profession. DR. G. MALCOM TROUT represented the American Dairy Science Association at the unveiling and mentioned the numerous activities of Dr. Hunziker in the Association, including the Presidency in 1910-1911. Others taking part in the program were DR. J. W. HICKS, Executive Assistant to the President of Purdue University, DEAN E. L. BUTZ, Dean of Agriculture, DR. F. J. BABEL, Professor in charge of the Dairy Manufacturing Section of the Department of Animal Sciences, VIRGIL SCHWARZKOPF, a student of Dr. Hunziker's and later associated with Dr. Hunziker in the Laboratories of the Blue Valley Creamery Company, and W. K. HOLM, Executive Secretary of the Indiana Dairy Products Association, who served as toastmaster at the memorial dinner.

In addition to Mr. Ogle, the members of the Indiana Memorial Committee were: REED SHAFER, VIRGIL SCHWARZKOPF, HARVEY BEHLMER, and PROFESSOR H. W. GREGORY, all former students of Dr. Hunziker.

As Dr. Otto F. Hunziker's portrait was unveiled. Left to right: Virgil Schwarzkopf, La Grange, Ill., a 1918 Purdue graduate and long-time friend of Hunziker; Glen Ogle, Sr., Evansville, chairman of the Hunziker memorial committee; Mrs. Hunziker, La Grange, Ill.; and Dr. F. N. Andrews, head of Purdue's animal sciences department.

Enos J. Perry Honored by Alma Mater

E. J. PERRY, retired dairy specialist, was honored by Pennsylvania State University as a distinguished alumnus because his personal life and professional achievements best exemplify the objectives of the University. He served 33 yr on the staff of Rutgers University, and was largely responsible for the development of the artificial insemination program as a method of improving dairy herds in this country.

E. J. Perry

On leave from Rutgers in 1937, he studied dairy farming and herd management practices in Europe, chiefly in Denmark, and brought back plans for the cooperative artificial insemination of cattle. The first United States organization began operations in New Jersey in 1938.

Mr. Perry was born on a farm near Stewartstown, Pennsylvania. After graduating from Dallastown high school, he taught rural school for 2 yr before entering Pennsylvania State College, where he graduated in 1916 with a Bachelor of Science degree in dairy husbandry. His Master of Arts degree, with a major in economics, was conferred by Columbia University in 1928.

Mr. Perry began his career in agricultural extension at Pennsylvania State College, serving from 1916 until 1920 as a county agricultural agent in Tioga County. He was a dairy extension specialist on the staff of West Virginia University from 1920 until 1923, when he joined the dairy husbandry extension staff at Rutgers, remaining there until his retirement in 1956.

Following retirement, he served 3 yr as livestock adviser for the International Cooperation Administration, working in Egypt, Lebanon, and Brazil, as well as in Washington, D. C.

He is presently working part-time for the American Dairy Association and for the Dairy Council of New York in their program of milk promotion. He served for 3 yr as a director of the American Dairy Science Association and was chairman of the Breeding Committee of the Association for 10 yr. He is a member of the American Society of Animal Production and a Fellow of the American Association for the Advancement of Science.

For his service to agriculture, Mr. Perry received the Superior Service Award of the U. S. Department of Agriculture in 1949; DeLaval Achievement Award for Dairy Extension of the American Dairy Science Association in 1951; Certificate of Recognition of Epsilon Sigma Phi in 1951; Service to the

Livestock Industry of New Jersey Award from the Co-operative Inter-Breed Cattle Association of New Jersey, also in 1951; Merit Award of the New Jersey Milk Industry Association of New Jersey in 1953; Meritorious Service to 4-H by the 4-H Clubs of New Jersey in 1954; and Distinguished Service to Agriculture Award of the New Jersey Department of Agriculture in 1961.

In his home community, he has served as president of the Board of Health of Highland Park, New Jersey, and as president of the New Brunswick Exchange Club. He is also a member of the F. and A. Order of Masons and the Presbyterian Church.

G. M. Werner Honored by USDA

A University of Wisconsin extension specialist in dairy production, G. M. WERNER, was given a superior service award by the U. S. Department of Agriculture at a ceremony in Washington on May 18.

Werner was cited for outstanding achievements and leadership in the field of dairy science and especially for his pioneering efforts in developing an artificial breeding program and for his leadership in improving dairy cattle feeding.

Professor Werner has been at Wisconsin since 1920, when he started work at the College of Agriculture as an official test supervisor. From 1923 to 1939 he was chief herdsman with the University dairy herd. In 1939 he was named instructor in dairy extension. He was appointed full professor in 1951.

G. M. Werner

He was among the first to recognize the practical application of artificial breeding for dairy cattle. In spite of skepticism by some scientists and many prominent dairy farmers, he provided leadership and technical knowledge to establish artificial breeding cooperatives in 1938 and 1939.

Much of the equipment he devised for these first organizations provided the basic ideas used by the industry now. During World War II years he devised the system of artificial insemination technique, now widely used.

He has been very active in disease prevention programs in dairy cattle. He has helped set up numerous disease control clinics with the University veterinary science department. Working with agronomists, he established the widely used forage clinics to encourage proper dairy cattle feeding of quality feeds

produced on the farm. He has also been active in such programs as grassland farming, sure-fire alfalfa, more milk from pasture, and grass silage. He has developed rating cards for silage and hay that are widely used.

In 1960 Professor Werner received the American Dairy Science Association DeLaval Extension Dairyman Award and was chairman of that group's extension section in 1956. He was president of the Wisconsin Extension Workers' Association in 1952 and has been secretary of the Wisconsin Purebred Dairy Cattle Association since 1950. He has served on numerous industry committees, including the Governor's Advisory Committee on Milk Production and Marketing. He is a member of Alpha Zeta and Epsilon Sigma Phi, agricultural honorary fraternities.

Dr. R. E. Erb Leaves Washington State University

Dr. R. E. Erb, Professor of Dairy Science at Washington State University, resigned April 11, to become Assistant Head of the Department of Animal Sciences at Purdue University, where he will be in charge of Dairy Science work. He came to WSU from Purdue in 1947, where he was a member of the Dairy Science staff.

Dr. Erb received in 1961 the annual $1,000 Borden award and a gold medal at the annual meeting of the American Dairy Science Association.

Dr. Erb has been a pioneer in developing artificial breeding in dairy cattle. For the past 10 yr his main research work has been on female dairy reproduction, in relation to the significance and physiology of female sex hormones. He has developed methods for the chemical determination of these hormones. Open-shed calf housing, developed by Dr. Erb as part of his research work at WSU, is now widely used. He has also worked on improved dairy cattle management. He has analyzed data on milk composition obtained through electronic computation of DHIA records to determine the effect of heredity, age, season of calving, length of dry period, and other factors on protein and nonfat solids content of milk. Dr. Erb has also been sum-

marizing data obtained from 15 yr of measuring and classifying animals in WSU's dairy herds at various ages to develop criteria for predicting type at an early age. He has published some 200 research papers and technical and popular publications. A native of Illinois, he obtained his B.S. degree from the University of Illinois, and M.S. and Ph.D. degrees from Purdue.

Dr. L. O. Luedecke Appointed to Washington State Staff

DR. L. O. LUEDECKE has joined the dairy science staff at Washington State University as dairy bacteriologist.

L. O. Luedecke

He will hold a joint teaching-research assignment, with the rank of Assistant Professor of Dairy Science.

The new staff member comes to WSU from Michigan State University, where he has been a graduate assistant since 1956. He was awarded an M.S. degree in 1958 and the Ph.D. degree this year by Michigan State. He graduated from Montana State, Bozeman, in 1956.

Dr. Luedecke's research at Michigan State dealt with the effect of early lactation on skimmilk used in making Cottage cheese, and the influence of milk fat on heat-resistant bacteria.

Dr. J. C. Knott's picture installed in building bearing his name.

PERFECT LIKENESS, JOE! This is the considered opinion of the new photograph of DR. J. C. KNOTT, installed in the reception

room of Washington State University's J. C. Knott Dairy Center recently. Expressing the general opinion to Dr. Knott, center, are DR. T. H. BLOSSER, left, chairman of the department of dairy science, and DR. L. L. MADSEN, director of the University's Institute of Agricultural Sciences.

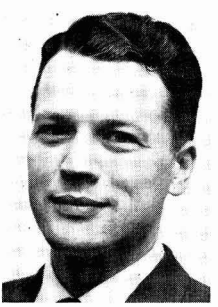
The enlarged tinted photograph of Dr. Knott was presented to the Center by IVER YOUNGQUIST for the donors: the United Dairymen's Association and Consolidated Dairy Products Company, Inc., Seattle.

The University's new $265,000 research-teaching center located 5½ miles from Pullman was named and dedicated last July. The center is located on land willed to the University by the late MRS. TULA HASTINGS YOUNG. Construction and equipment were financed by legislative appropriations.

Dr. Knott, dairy professor emeritus, is former director of the University's Institute of Agricultural Sciences and Agricultural Extension Service. He retired in 1958 after 30 years of service to Washington agriculture.

L. V. Estergreen Appointed to Staff at Washington State

L. V. ESTERGREEN has been appointed to the dairy science position left vacant by the resignation of R. E. Erb at Washington State University. He will hold a joint research-teaching appointment with the rank of assistant professor. His teaching and research assignment will be in the area of dairy cattle physiology. He has been doing research on the secretion of hormones by endocrine glands in dairy cattle for the past 8 yr.



L. V. Estergreen

A native of Lynden, Washington, Dr. Estergreen was graduated from WSU with a B.S. degree in dairy science in 1950, and an M.S. in 1956. He was awarded a Ph.D. degree by the University of Illinois in 1960. He spent the following year doing post-doctoral study at the University of Utah's College of Medicine.

Dr. Estergreen is a member of the Endocrine Society, the American Dairy Science Association, and of the following research or scholastic honoraries: Sigma Xi, Alpha Zeta, Phi Kappa Phi, and Alpha Tau Alpha. The new appointee is married and has one son, Martin, aged 5.

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WAYNE REGINALD GOMES—Assay for Progestins in Peripheral and Ovarian Venous Blood from Cows.

DONALD E. WALDERN—The Effect of Diet on the Volatile Fatty Acid Production and Absorption in the Bovine.

Ohio State News

Two events in April culminated the year's recruitment activities of Ohio State's Department of Dairy Technology, conducted with the support of the Education Committees of Ohio Dairy Technology Societies, dealer groups, and interested individual plants.

On April 14, 60 dairy industry men and carefully selected students attended a Department-sponsored Dairy Technology Career Day. The program consisted of tours of the Department designed to illustrate the varied nature of career opportunities in the dairy field and the presentation of a slide sequence narrated by students and staff members, which further illustrated industry opportunities and the functions of the Department. DR. A. E. RITCHIE, Assistant Dean of the College of Agriculture, and DR. I. A. GOULD, Chairman of the Department, reviewed the needs of Agriculture in general and Dairy Technology in particular for technically educated personnel. Following a lunch provided by the Ohio Dairy Products Association, those in attendance were divided into small groups, where they were encouraged to seek answers to individual questions from panels made up of Department faculty members, graduate and undergraduate students, and Industry personnel.

On April 28, 23 high school seniors assembled in Vivian Hall, home of the Department of Dairy Technology at Ohio State, to compete for the scholarships available for Dairy Technology students next fall. These potential Dairy Technologists were given a general scholastic aptitude examination and interviewed by representatives of the Department and the Industry. Industry representatives included B. FORBES, Cleveland, M. SWINE-

HART, Akron, G. JOHNSON, Cleveland, R. SCHAFER, Barnesville, and T. TISHER, Warren (see attached photograph).

H. E. Randolph discusses the diversity of milk products with Ohio State Career Day attendees.

W. L. SLATTER, Professor of Dairy Technology, The Ohio State University, has returned from a 4-yr assignment in India, serving as a member of the Ohio State's Agriculture Education Mission which is assisting in the development of an educational system patterned after the Land-Grant College system in the United States.

Dr. Slatter was stationed at the National Dairy Research Institute in Northern India, and helped to develop a college for training students in Dairy Technology and instituted agricultural operations on the 1,800-acre site of the Institute. During his tour of duty, a modern dairy plant was completed and considerable progress was made toward conducting agricultural operations on a modern basis.

STUDENT CHAPTER NEWS

W. W. SNYDER, Editor
A Section Devoted to News of Student Members

Louisiana State News

The Dairy Science Club of Louisiana State University climaxed their year of activities with the annual club banquet on May 5, 1962. The affair was scheduled in connection with Spring Alumni Day, which is an annual affair on the campus of Louisiana State University. More than 125 former students, faculty members, and guests were in attendance. Mr. Leon Kleinpeter of Kleinpeter Farm's Dairy, Inc., of Baton Rouge, Louisiana, was recognized as the Outstanding Louisiana Dairyman of the Year by the club. Honorary membership in the club was extended to Mr. E. W. Neasham, who recently retired as head of dairy extension work in Louisiana.

Mr. Robert C. Force, who served as manager of the L.S.U. Creamery for more than 30 yr, was honored by a group of former students who had worked in the creamery under his supervision.

The incoming officers, consisting of Ronald Blanchard, President, Frank Millican, Vice-President, Lynn Boddie, Secretary-Treasurer, and D. L. Evans, Adviser, were presented to the group.

Washington State University

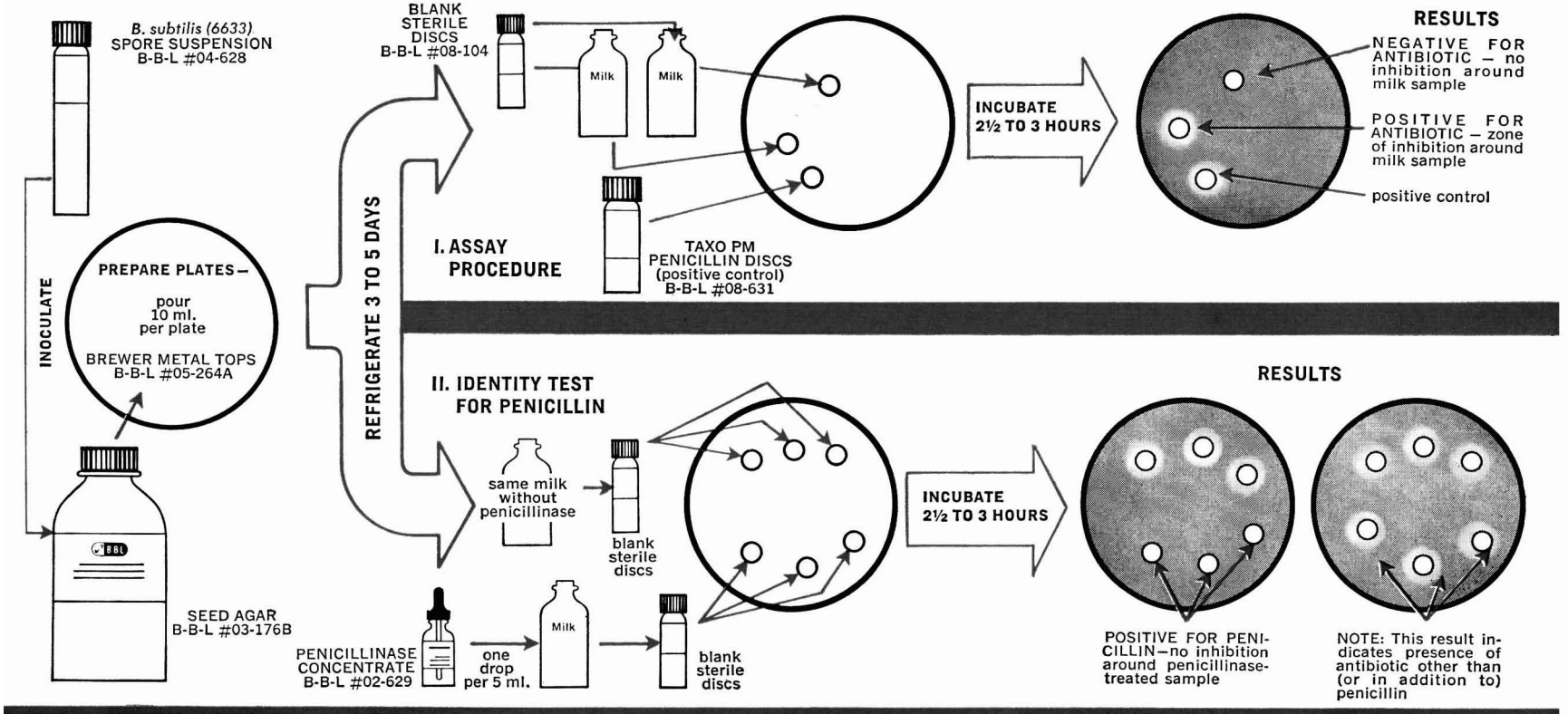
Student Chapter of A.D.S.A., Washington State University, on March 6 sponsored a banquet for the 31st Annual Institute of Dairying.

Glenn Betz was presented with the Virginia Dare Award for excellence in the judging of ice cream. Betz, a junior in Dairy Science (Manufacturing) at Washington State University, was presented with a plaque and a check for $25. The award is furnished by the Virginia Dare Extract Co., New York, for the Dairy Science junior or senior student excelling in the judging of ice cream, scholarship and leadership abilities. Betz placed fourth in the judging of vanilla ice cream in the 27th Collegiate Students' International Contest in the Judging of Dairy Products. Betz is the son of Mr. and Mrs. J. Edward Betz of Cheney, Washington.

Larry Pickering, son of Mr. and Mrs. Vern M. Pickering of Monroe, Washington, is the 1962 recipient of the Yakima Valley Holstein Scholarship. This is an award presented by

B·B·L techniCHART

DETECTION OF PENICILLIN IN MILK



The presence of antibiotics in milk following mastitis therapy in cows has created serious public health problems and caused technical difficulties within the dairy industry. A rapid, practical laboratory procedure to assist regulatory agencies and the dairy industry in solving these problems was described by Arret and Kirshbaum.* This procedure employs rapid growth of a sensitive strain of *B. subtilis* for assaying the presence of antibiotics in milk and for determining its identity with penicillin. Inhibition of growth by the presence of as little as 0.05 unit of penicillin per ml. of milk sample is detectable within 2½ hours.

In answer to many requests for information about the availability of B-B-L products for this simplified procedure, the B-B-L Development Laboratory has prepared this TECHNICHART. It graphically illustrates the basic procedure, showing the materials necessary—**all of which are available from B-B-L.** A complete brochure with detailed technique and product listing is available upon request. No. 13

*Arret, B., and Kirshbaum, A.: J. Milk and Food Technol. 22:329, 1959.

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the Yakima Valley Holstein Club to a student interested in Holsteins. Larry is a freshman majoring in Dairy Science at Washington State University.

In 1961 he was FFA Chapter President, Tolt High School, Carnation, Washington. He was named a State Farmer in 1960 and was president of the sophomore class in high school. He carried a Holstein project in vocational agriculture and still has Holsteins in his parents' herd.

J. F. Kendrick Takes New Assignment

DR. J. F. KENDRICK left on May 1 his position in charge of the USDA National Cooperative Dairy Herd Improvement and Sire Proving Programs to take a new position in the Statistical Reporting Service, where he will develop IBM data processing programs for economic information.

Dr. Kendrick has been the recipient of many honors and has been a recognized leader in Dairy Herd Improvement work. He has been honored twice by the Secretary of Agriculture for Superior Service.

At the 56th Annual Meeting of the American Dairy Science Association at the University of Wisconsin he was the recipient of the DeLaval Extension Award, which included a framed citation and a check for $1,000.

INDUSTRY NEWS

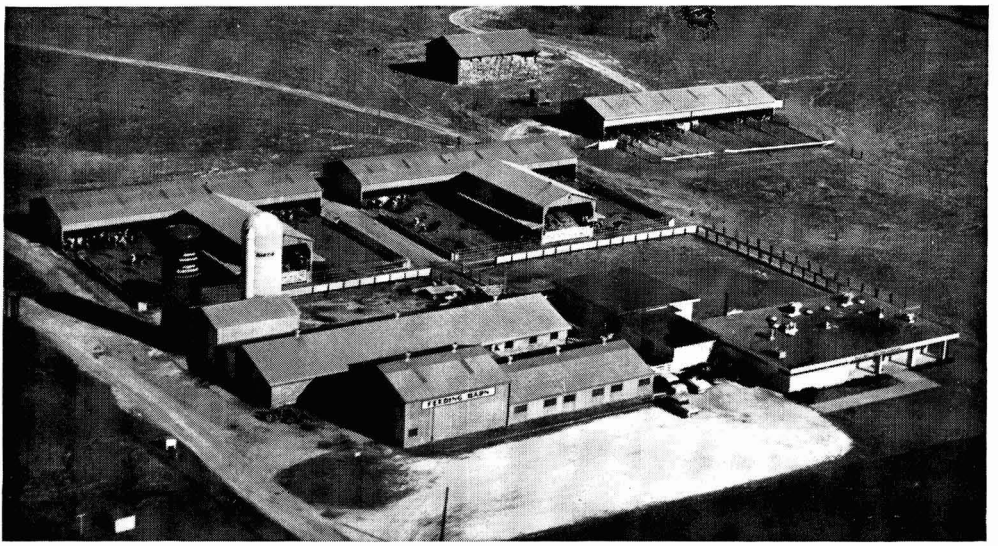
Beatrice Foods Dedicates New Plant

Built at a cost of $3,000,000, Beatrice Foods Company's Meadow Gold plant recently completed in Champaign, Illinois, is the newest and believed to be one of the most efficient milk and Cottage cheese facilities in the nation.

The ultra-modern plant, constructed on a 20-acre site northwest of the city, can produce 144,000 gallons of milk per day—enough to provide one 8-oz glass for 2,304,000 youngsters. When completed, the Cottage cheese capacity will be 25,000 lb daily. Other products of the plant for distribution throughout Central Illinois include cream, skim, chocolate and butter milk, fruit ades and sour cream.

In anticipation of future growth, the one-story plant, which now has 65,000 sq ft under roof, is designed to permit expansion in every department without materially interrupting operations.

The plant is the result of more than a year's study and planning by Beatrice Foods' engi-



Part of Dairy Research Unit

Continuous research adds to knowledge of dairy nutrition

For many years competent MoorMan scientists have been building a backlog of research knowledge. This experience has helped the Moorman Manufacturing Company produce dairy feeds, calf feeds and fly sprays that help the dairyman obtain GOOD RESULTS. The research staff has developed a four-step research program that gives consistent results in improving dairy nutrition and management:

1—*Nutritional Research*—In the laboratory and on the 1,280 acres on the three Research Farms, nutritionists are working continuously testing a wide variety of feed ingredients and additives. These tests help develop feed formulas that will produce milk at lower cost.

2—*Veterinary Research*—The control of insects and parasites—internal and external—has become an important part of dairy management. It is the responsibility of the Veterinary Research Division.

3—*Field Research*—Any new development in nutrition or management must be carefully proved in the field under normal farm conditions. This is where the Field Research team takes over to make the new development prove itself in practical use. Tests are often conducted on hundreds of customer farms before important changes are made in formulations.

4—*Quality Control*—Only after extensive development and testing, is a new or improved product offered for sale. It is then placed under constant Quality Control supervision. For regardless of the adequacy of the formula, a feed is only as good as the raw materials that go into it.

We would be honored to have any or all members of the Dairy Science Association visit with us in Quincy, Illinois. We would like to show you our research facilities and have you meet some of our research staff.

MoorMan's*

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Good Results Through Research and Service

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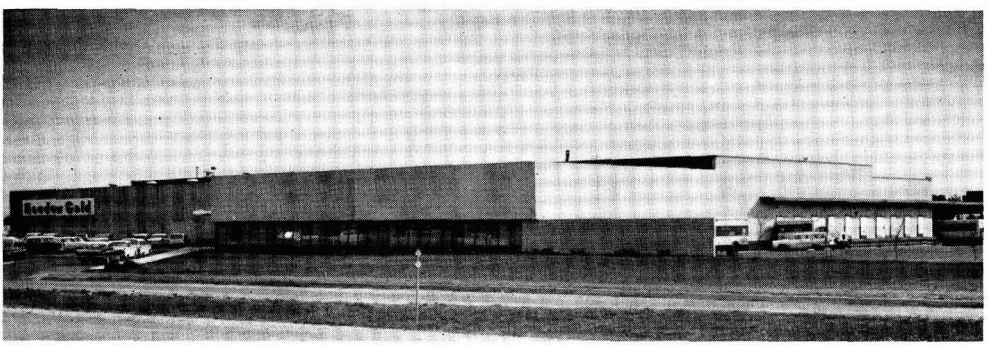
neering and production departments and Johnson and Johnson, Chicago specialists in dairy architecture. Production flow charts were designed to obtain the most efficient processing system. The latest high-speed equipment was combined with automation to make the plant, largest ever built by Beatrice Foods, a model of efficiency and sanitation.

Those taking part in the milk toast at the dedication ceremonies for the new Beatrice Foods Plant at Champaign, Illinois are, left to right: Jay Neubauer—Regional Vice-President; Dean Huxtable—Manager, Champaign Ice Cream Division; Delos Huxtable—retired Manager, Champaign Ice Cream Division; Cecil Bair—Manager, Champaign Dairy plant; Vernon Janes—Illinois District Manager; Carl N. Hansen—Vice-President; E. B. Carter—Manager, Decatur Dairy plant; William B. Karnes—President.

BEATRICE FOOD'S NEW MILK PLANT IN CHAMPAIGN, ILLINOIS



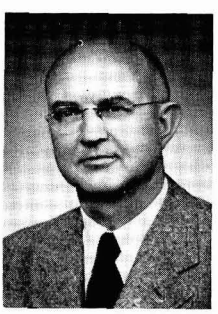
Klenzade Appoints New General Manager

Officials of the Klenzade Products Division, Economics Laboratory, Inc., Beloit, Wisconsin, have announced the appointment of S. K. MAHOOD to the position of General Manager.

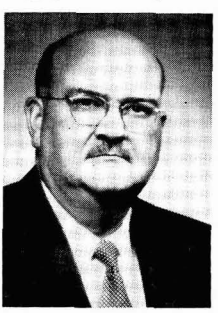
S. K. Mahood

Mr. Mahood will immediately assume his new duties directing the activities of Klenzade's research, manufacturing, and sales departments. A. L. and C. B. SHOGREN, Vice-Presidents of the Klenzade Products Division, will continue in senior advisory capacities and assist Mahood in the development of his duties.

A native of Canada, Mahood started as a sales representative with International Harvester Company, rising to the position of President and General Manager of DeLaval Company, Ltd., Peterborough, Ontario, the



A. L. Shogren



C. B. Shogren

Canadian distributors of Klenzade Sanitation Products. He played an important part in the development of today's modern milking and dairy processing equipment which has helped to give the consumer clean, quality dairy products. It is in this vital area that Mahood brings a wealth of experience in dairy sanitation to Klenzade Products.

Microbiology Congress to Meet at Montreal

The Eighth International Congress for Microbiology will be held at Montreal, Canada, August 19 to 25, 1962, under the auspices of the Canadian Society of Microbiologists. Headquarters of the Congress will be at the new Queen Elizabeth Hotel.

There will be five Sections: Structure and Function; Agricultural Microbiology; Industrial Microbiology; Medical and Veterinary Microbiology; and Virology. Two or more symposia are being planned for each Section. All speakers at the symposia will be especially invited. There will be sessions for contributed papers in all Sections.

Back Numbers of Journal Needed

Certain numbers of the Journal of Dairy Science are needed to maintain an inventory and to take care of orders. If any of the numbers listed are available and of no further use to the owners, please send them to the Garrard Press, 510 North Hickory Street, Champaign, Illinois. Seventy-five cents plus postage will be paid for each number.

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RESEARCH PAPERS

INFLUENCE OF SODIUM CHLORIDE ON THE STABILITY OF THE CALCIUM CASEINATE MICELLE

CHARLES A. ZITTLE AND LENORE B. JASEWICZ
Eastern Regional Research Laboratory,[1] Philadelphia, Pennsylvania

SUMMARY

Sodium chloride of relatively low concentration (0.17 M) destabilizes colloidal calcium caseinate (whole casein) so that part of it precipitates. This occurs also with colloidal calcium caseinate prepared from calcium-sensitive (α_s)-casein and kappa-casein. In the first system the optimal concentration of sodium chloride for destabilization is independent of the calcium chloride concentration, whereas in the second system the optimal concentration of sodium chloride increased with calcium chloride concentration. The dissociation of the α_s-casein–kappa-casein complex, manifest by precipitation of the dissociated α_s-casein by calcium chloride, was increased by decreasing the temperature from 30 to 7 C, and increased further by the addition of sodium chloride (0.15 M).

Sodium chloride (at saturation, about 5 M) will not precipitate sodium caseinate, but it will precipitate calcium caseinate. Investigation of the influence of calcium concentration and pH on this precipitation revealed that casein is partly precipitated at much lower concentrations of sodium chloride (0.17 M). Details of this observation are reported. In addition, the influence of sodium chloride on the formation of the calcium caseinate micelle at 7 C is reported, a reaction that presumably involves the association between the calcium-sensitive and the kappa-caseins. These latter results have been reported briefly (3).

MATERIALS AND METHODS

Whole casein. This was acid-precipitated casein prepared in the usual way (6). Neutral solutions of sodium caseinate were prepared by adding NaOH to an aqueous suspension of casein, avoiding an excess of the alkali.

Kappa-casein. Prepared by fractionation in ethanol, essentially by the method of McKenzie and Wake (1).

Calcium-sensitive (α_s) casein. Prepared by fractionation in urea (5).

The influence of sodium chloride on calcium caseinate was determined at room temperature (22 to 25 C). The calcium caseinate was prepared by the addition of calcium chloride to a neutral solution of sodium caseinate and the pH adjusted to the required value of pH 6 or 7 with 0.1 N HCl. For the higher concentrations of sodium chloride, the solid was added to the 10-ml volume of the test solutions. Since the sodium chloride contributed very little in volume, the molarities were calculated from the weight of sodium chloride and the volume of the test solution. For the lower concentrations of sodium chloride, the required volume of a 30% solution was added and the total volume adjusted to 10 ml.

The test mixtures were held at 30 C for 15 min, then centrifuged at about 3,000 × G for 5 min. A portion of the supernatant solution was withdrawn for determination of the protein remaining in solution. After dilution, one drop of 0.5 N NaOH was added for clarification, and the protein concentration was determined from the light absorbance (optical density) at 280 mμ with a 1-cm light path. A factor of 1.0 was used for converting light absorbance to milligrams of casein per milliliter.

Other experimental conditions are described under each experiment.

RESULTS

The influence of sodium chloride on the calcium caseinate micelle was determined for 0.02, 0.03, and 0.04 M calcium chloride. The results are shown in Figure 1 for pH 6.0. Results were the same at pH 7.0. The concentration of calcium chloride determined the level of solubility with no sodium chloride. In each case, the addition of sodium chloride destabilized the micelle. Maximum destabilization of the calcium caseinate micelle occurred with a sodium chlo-

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[1] Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

ride concentration of 0.17 M, irrespective of the calcium chloride concentration. This was also found with a calcium chloride concentration of 0.010 M, not shown in the figure. Somewhat larger concentrations of sodium chloride brought about solubilization of the casein in nonmicellar form, that is, the solutions were clear. Whole casein precipitated (not shown) quantitatively, as did α-casein, at near-saturated concentrations of sodium chloride (about 5 M) with the concentrations of calcium chloride used in the above experiments. Precipitation did not occur with sodium caseinate. Kappa-casein with 0.020 M calcium chloride precipitated slowly and incompletely with 5 M sodium chloride; with 0.20 M calcium chloride precipitation with sodium chloride was rapid and complete.

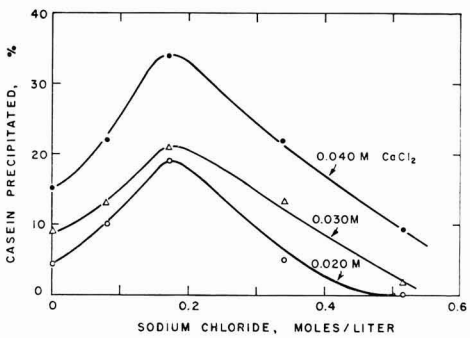

FIG. 1. Destabilization of calcium caseinate micelles by sodium chloride. Concentration of whole casein was 1%.

Similar experiments were performed with the reconstituted casein complex obtained by mixing α_s-casein and kappa-casein. Calcium ions were added to obtain micelles and the influence of sodium chloride determined. The results are shown in Figure 2. Sodium chloride destabilized this simplified casein system also. In this instance the concentration of sodium chloride giving maximum destabilization was influenced by the calcium chloride concentration.

The influence of sodium chloride on the dissociation of the α_s-casein and kappa-casein complex also was studied. The results shown in Figure 3 for 7 and 30 C were obtained with a relatively crude preparation of kappa-casein containing considerable of the λ-casein fraction. These results have, however, been confirmed with the purified kappa-casein used for the other experiments in this paper. The complex dissociates strongly at the lower temperature which makes possible the precipitation of the α_s-casein by the calcium ions. This process is

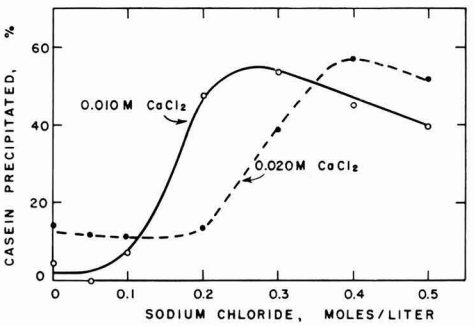

FIG. 2. Destabilization of caseinate micelles, formed from calcium-sensitive (α_s)-casein, kappa-casein and calcium chloride, by sodium chloride. Concentration of α_s-casein 0.3%, of kappa-casein 0.03%, concentration of calcium chloride as indicated.

not reversed at higher temperatures, for the test is completed by warming to 30 C (4). The presence of sodium chloride increases the dissociation at 7 C, and this is manifested by increased precipitation of the α_s-casein. Addition of sodium chloride at 30 C, however, had a negligible effect on the results. The slight tendency to enhance the casein solubilization shown here was less pronounced in other experiments. This appeared to be contrary to the destabilization results reported in Figure 2. The methods differed in detail, but the principal difference seemed to be the order in which the sodium chloride and the calcium chloride were added. Consequently, the type of experiment shown in Figure 3, in which the calcium chloride was added last, was performed with the sodium chloride (final concentration 0.15 M,

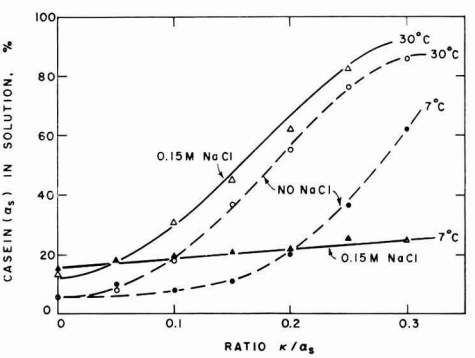

FIG. 3. Influence of sodium chloride concentration (none, and 0.15 M), and temperature (7, 30 C) when mixed, on the stabilization of calcium-sensitive casein (α_s) by a preparation of kappa-casein. Concentration of calcium chloride was 0.02 M.

added in a volume of 0.35 ml) added last. In this case a destabilization did occur. With a K/α_s ratio at which 75% of the α_s-casein was soluble, addition of the sodium chloride decreased the solubility to 53%.

DISCUSSION

Colloidal calcium caseinate is negatively charged. This charge helps to maintain the calcium caseinate as a physically stable colloid. Presumably, the low concentration of sodium chloride (0.17 M) reduces the stabilizing repulsive effects between the negatively charged ions and destabilization leads to precipitation of a part of the casein. Electrophoretic examination of the precipitate from whole casein showed that no fractionation had occurred. With the addition of still larger concentrations of sodium chloride a considerable ion atmosphere forms about the casein molecule, which leads to almost complete solubilization of the casein. At concentrations of sodium chloride approaching saturation the salt ions compete with the casein for water molecules, so that a total precipitation of the calcium caseinate occurs. That this occurs with calcium caseinate and not with sodium caseinate emphasizes the strong binding of calcium to casein and its marked influence on the properties of the product. It is expected that the observed destabilizing action of low concentrations of sodium chloride on calcium caseinate will also be evident in more concentrated systems and would have a marked influence on the physical state of milk products.

The destabilizing action of sodium chloride on calcium caseinate is also obtained with a mixture of calcium-sensitive (α_s)-casein and kappa-casein. Since in this case the concentration of sodium chloride giving maximum destabilization shifted with the concentration of calcium chloride, other components of whole casein, particularly β-casein, probably have a role in the stability of the total system also. The fact that the results are influenced by the order in which the sodium chloride and the calcium chloride are added suggest that there are time (aging) effects or that the ionic components are not in equilibrium.

The influence of sodium chloride on the stability of the α_s-casein–kappa-casein mixture at 7 C when calcium chloride is added reflects its influence on the dissociation of the α_s-casein–kappa-casein complex. When the complex is dissociated the calcium ion precipitates the α_s-casein, an interaction that is not reversed at higher temperatures. The results in Figure 3 show that a decrease in temperature from 30 to 7 C facilitates the dissociation and this is enhanced by the presence of sodium chloride. Methods for the separation of the α_s-casein from the kappa-casein utilize this effect of low temperature; the solution of whole casein is chilled (1, 2) before the calcium chloride is added; subsequently, the temperature is raised to obtain complete precipitation of the α_s-casein.

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STUDIES ON CASEIN. IV. THE SIALIC ACID CONTENT OF CASEIN[1]

M. N. CAYEN, G. O. HENNEBERRY, AND B. E. BAKER
Macdonald College of McGill University, Macdonald College, Quebec, Canada

SUMMARY

A study has been made of the effects of conditions of hydrolysis and color reagent (resorcinol, orcinol) on the apparent concentrations of sialic acid in whole casein. Consistently higher values were obtained with hydrolysates prepared by hydrolysis of the protein with a Dowex 50 × 12—hydrochloric acid mixture for 0.5 hr at 95 C, and with use of the resorcinol reagent.

The sialic acid content of whole casein, alcohol washed casein, α-casein, β-casein, γ-casein, and two fractions prepared from α-casein (Fraction A and α-casein—Fraction A) were 3.84, 3.53, 4.10, 1.36, 0.82, 22.2, and 0.64 mg/g, respectively.

The preparation of a calcium-sensitive casein and a casein fraction designated Fraction A have been described in a previous report (11). The behaviours of the materials resembled those of the second-cycle casein and the Fraction S described by Waugh and Von Hippel (18). The total sugar content (anthrone) of Fraction A was about six times that of the calcium-sensitive fraction, and the galactose, mannose, and glucose contents of Fraction A did not account for its total (anthrone) sugar content.

The present paper deals with the determination of sialic acid in the above-mentioned fractions, as well as in other fractions of the casein complex.

MATERIALS

Whole casein was prepared from a composite sample of milk (Macdonald College herd) by the method of Warner (17).

Alcohol-extracted casein was prepared by extraction of whole casein with 95% ethanol in a Soxhlet extractor for 96 hr (12).

α-Casein was prepared from whole casein by the method of Warner (17), and β-casein and γ-casein were prepared by the methods of Hipp et al. (7, 8).

The α-casein, Fraction A and α-casein—Fraction A gave only one electrophoretic component in barbital-acetate buffer at pH 7.4 (Antweiler-Microelectrophoresis apparatus equipped with a Philpot-Svennson optical system for the schlieren analysis). However, the β-casein preparation contained 19.2% of a protein with the same mobility as α-casein, and the γ-casein preparation contained 27.3% of a protein with the same mobility as β-casein.

The sialic acid had been prepared from biological material and was obtained from Dr. L. Svennerholm, University of Gothenburg, Sweden.

The Dowex resins (2 × 8; 50 × 2; 50 × 8; 50 × 12) were purchased from Dow Chemical Company, Midland, Michigan.

EXPERIMENTAL PROCEDURE

Sialic acid was determined by the method proposed by Svennerholm (15). In the preliminary experiments an attempt was made to find conditions for hydrolysis of the protein which would liberate the sialic acid from the protein, and at the same time would not lead to destruction of sialic acid.

Whole casein (0.020 g) and 5 ml of the hydrolytic agent [(a) 0.05 N sulfuric acid; (b) suspensions containing one part of the following resins (acid form) in four parts 0.05 N hydrochloric acid: Dowex 50 × 2, Dowex 50 × 8, Dowex 50 × 12; (c) suspension containing one part Dowex 50 × 8 in four parts water] were placed in a soft glass test tube (15 × 150 mm). Each tube was sealed and then heated at 95 C in a rotary oven for a prescribed interval of time. At the end of the heating period the tube was cooled and opened and the solution was transferred to the chromatography tube containing Dowex 50 × 8 resin (15). This tube was placed immediately over a Dowex 2 × 8 column so that the effluent from the first chromatography tube dropped directly into the second (15). The effluent was collected in a volumetric flask and the volume was adjusted at 10 ml with distilled water. Two-milliliter

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[1] Macdonald College Series No. 487.

portions of the diluted effluent were pipetted into each of four test tubes. Two milliliters of orcinol reagent, blank orcinol reagent, resorcinol reagent, and blank resorcinol reagent were added to the first, second, third, and fourth tubes, respectively. Following Svennerholm's procedure, the absorbances of the isoamyl alcohol phase were read on a Beckman DU spectrophotometer at 575 mμ for the samples containing orcinol, and at 580 mμ for the samples containing resorcinol, against pure isoamyl alcohol taken at 100% transmission. The readings were completed within 1 hr after the heating period. The quantity of sialic acid in the sample was ascertained by reference to a standard curve prepared with N-acetylneuraminic acid supplied by Dr. Svennerholm. Aqueous solutions containing 0, 50, 100, 150, 200, and 250 μg of N-acetylneuraminic acid were treated with the orcinol and resorcinol reagents under the same conditions as were the casein samples. The absorbances at 575 mμ (orcinol) and 580 mμ (resorcinol) were used to construct standard curves. Following the recommendations of Blix (3), Gottschalk (4), and Klenk (10), no corrections were made for the absorbances at 450 mμ and 580 mμ when the orcinol and resorcinol reagents, respectively, were employed. Table 1 shows the effect of conditions of hydrolysis and color reagent (orcinol, resorcinol) on the apparent concentration of sialic acid in whole casein. The values reported are averages of two independent determinations. It will be noted that the highest value for the sialic acid content of whole casein was 3.90 mg/g and that this value was obtained after hydrolysis of the casein sample for one-half hour, with a mixture of Dowex 50 × 12 and hydrochloric acid, and with use of the resorcinol reagent. Svennerholm (14) found that the resorcinol method was about 50% more sensitive than the orcinol method and that the interference due to carbohydrates, other than sialic acid, was considerably lower.

In another series of experiments sialic acid was determined (resorcinol reagent) in various fractions of the casein complex and in whole casein after different periods of hydrolysis (95 C) with a mixture of Dowex 50 × 12 and 0.05 N hydrochloric acid. Table 2 shows the results of the analysis of the separate hydrolysates of each casein preparation.

DISCUSSION

In the preliminary experiments, an attempt was made to find conditions of hydrolysis which would liberate sialic acid and at the same time not lead to its destruction. The highest apparent concentration of sialic acid (resorcinol reagent) in whole casein was found in hydrolysates prepared by hydrolysis of the casein for one-half hour at 95 C, with a mixture of Dowex 50 × 12 and 0.05 N hydrochloric acid. The highest value obtained with the Dowex 50 × 8—hydrochloric acid mixture was slightly lower, and that obtained with Dowex 50 × 2—hydrochloric acid was less than half the maximal value obtained with the Dowex 50 × 12—hydrochloric acid mixture. Anastassiadis (2) has found that the apparent concentration of hexosamine was higher in heparin hydrolysates prepared with either Dowex 50 × 8 or Dowex 50 × 12 than with Dowex 50 × 2.

It will be noted that all the protein prepara-

TABLE 1

Effects of conditions of hydrolysis and color reagent (resorcinol, orcinol) on the apparent concentration of sialic acid in whole casein

Hydrolytic agent	Apparent concentration (mg/g) of sialic acid in whole casein								
	Time of hydrolysis (hours)								
	0.25	0.5	1	2	3	6	9	12	24
0.05 N H_2SO_4	.....	1.96[a] (1.00)	2.08 (1.78)	2.40 (2.73)	2.70 (1.98)	2.19 (1.29)	1.72 (0.99)	1.47 (0.85)	1.10 (0.74)
Dowex 50 × 2 in 0.05 N HCl	.....	0.53 (0.51)	0.75 (0.78)	1.11 (1.16)	1.46 (1.32)	0.87 (0.86)	0.66 (0.67)	0.56 (0.56)	0.39 (0.44)
Dowex 50 × 8 in 0.05 N HCl	2.71	3.30 (2.18)	3.22 (2.43)	2.41 (2.83)	1.72 (2.40)	1.10 (1.62)	0.88 (1.33)	0.78 (1.19)	0.55 (1.02)
Dowex 50 × 12 in 0.05 N HCl	3.11	3.90 (1.24)	3.44 (1.41)	2.80 (2.08)	2.46 (2.29)	2.13 (2.38)	1.87 (1.47)	1.68 (1.22)	1.40 (0.77)
Dowex 50 × 8 in water	.....	2.40 (1.98)	2.68 (2.23)	1.61 (2.39)	1.39 (1.41)	1.27 (1.17)	1.17 (0.90)	1.08 (0.74)	0.92 (0.55)

[a] Resorcinol values without parentheses, orcinol values in parentheses.

TABLE 2

Effects of period of hydrolysis on the apparent concentration of sialic acid in fractions of the casein complex

Protein	Period of hydrolysis (hours)	Apparent concentration of sialic acid (mg/g) Hydrolysate No. 1	Hydrolysate No. 2	Hydrolysate No. 3	Average
Whole casein	0.5	3.85	3.90	3.79	3.84
Whole casein (alcohol extracted)	0.5	3.65	3.40	3.44	3.50
α-Casein	0.5	4.08	4.00	4.22	4.10
	1.0	1.64	1.46	1.66	1.59
	3.0	0.73	0.82	0.82	0.79
β-Casein	0.5	1.45	1.29	1.35	1.36
	1.0	1.27	1.11	1.22	1.20
	3.0	1.30	1.16	1.10	1.19
γ-Casein	0.5	0.90	0.76	0.80	0.82
	1.0	0.75	0.70	0.60	0.75
	3.0	0.70	0.66	0.70	0.69
Fraction A	0.5	22.15	21.85	22.55	22.2
	1.0	11.24	11.77	12.66	11.9
	3.0	5.51	6.85	8.27	6.8
α-Casein—Fraction A	0.5	0.65	0.66	0.61	0.64
	1.0	0.50	0.60	0.66	0.59
	3.0	0.56	0.60	0.60	0.59

tions contained some sialic acid. Assuming that the contaminant (19.2%) of the β-casein preparation is α-casein (4.1 mg/g sialic acid), then pure β-casein should contain approximately 0.71 mg/g sialic acid. Similarly, if it is assumed that the contaminant (27.3%) of the γ-casein preparation is β-casein (0.71 mg/g sialic acid), then pure γ-casein should contain approximately 0.86 mg/g sialic acid.

Sullivan et al. (13), who studied the distributions of kappa-casein in skimmilk, assumed that kappa-casein was the only casein component containing sialic acid. They calculated the concentration of sialic acid in kappa-casein on the basis of previous reports that whole casein contains 3.9% sialic acid (9) and that kappa-casein makes up 15% of the casein complex (16). Their calculated value was 28.7 mg of sialic acid per gram of kappa-casein, which is 6.5 mg per gram higher than the analytical figure presented in the present paper.

Alais and Jollés (1) reported values of 3.6, 3.8, and 24 mg/g for the sialic acid content (thiobarbituric acid method) of whole casein, alpha-casein, and kappa-casein, respectively. These values are in fair agreement with those reported in this paper.

The authors are not aware of previous reports on the linkage of sialic acid to casein. Gottschalk and associates (5, 6) have found that the disaccharide α-D-N-acetylneuraminic (2 → 6) N-acetylgalactosamine is the prosthetic group of ovine submaxillary gland mucoprotein and that it is attached to the polypeptide chain by an ester-type linkage. As far as the authors are aware, D-galactose or D-galactosamine has been found in all the mucosubstances of animal origin that contain the neuraminic acids. Previous work (11) has shown that Fraction A contains total sugar (anthrone) 3.94 mg/g, galactose 2.75 mg/g, mannose 0.51 mg/g, hexosamine 4.58 mg/g, and no glucose. Calculations based on these values show that if each molecule of galactose, mannose, and hexosamine in Fraction A had attached to it one molecule of sialic acid, the fraction would contain 13.5 mg sialic acid per gram of protein. If it is assumed that the sialic acid is not present in a polymeric form, it would appear that 8.68 mg/g of sialic acid per gram of Fraction A are bound directly to the peptide chain or through some sugar or other substance not yet identified.

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OCCURRENCE OF CALCIUM PHOSPHATES IN THE PRESENCE OF ORGANIC SUBSTANCES, ESPECIALLY PROTEINS

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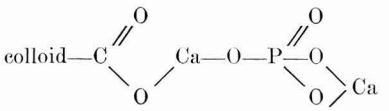
SUMMARY

Different types of phosphates were formed by titrating phosphoric acid with alkali in the presence of calcium and the proteins casein, gelatin, and ovoalbumin; the polysaccharides agar, starch, and dextrin, simple molecules such as organic acids, amines, and alcohols, and polymers such as krilium and carboxymethylcellulose. Casein derivatives depleted of their carboxyl and amino groups were also used in these titrations. Between pH 5 and 12, calcium caseinate and tricalcium phosphate can occur together in a soluble chemical complex, and the formation of this complex is possible between colloids with free carboxyl groups and tricalcium phosphate. The colloidal matrix and the carboxyl groups must be present simultaneously and combined in one substance. The general formula for this complex is as follows:



Tricalcium phosphate will occur combined with milk and blood proteins. The complex in blood makes possible the transport and the deposit of calcium phosphate in the skeleton and in dentine, and in soils this is possible by a calcium phosphate–humic acid complex.

Although it is known that in several biological systems (e.g., milk and blood) the solubility of calcium phosphates is influenced by the presence of organic compounds, little work has so far been done to elucidate the mechanism. On examining the system casein–calcium phosphate, as this occurs in milk, it is found that the casein here is present in a colloidal dispersed state and the calcium phosphate is found to be present in the micellae. Van Slyke and Bosworth (10), the first noteworthy workers on this system, suggested that the casein would serve as a Schutzcolloid for a dicalcium phosphate suspension. At a later date, however, Eilers (1) pointed out that the calculations of Van Slyke and Bosworth were based on incorrect hypotheses and that the presence of mainly tricalcium phosphate should be assumed. Pyne (8) also came to the conclusion that tricalcium phosphate in the tertiary form was linked with calcium caseinate, mainly in the form of a double salt.

De Kadt and Van Minnen (6), however, were of the opinion that the tricalcium phosphate was coupled onto calcium salts of the esterified phosphate present in the casein.

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METHODS

A Cambridge meter was used for the potentiometric pH measurements. Burettes, either with a capacity of 50 ml (accurate to 0.05 ml) or of 10 ml (accurate to 0.01 ml) were used for the titrations. The solutions were stirred continuously by means of an electric stirrer. The initial volume of the solutions was always 400 ml.

RESULTS

(a). *Potentiometric titration of casein.* Figure 1 shows the potentiometric titration curve of 5 g of casein, prepared from skimmed cow's milk, according to the method of Hammarsten (4). To obtain strong enough alkali suitable for the titration, 1 N $Ca(OH)_2$ was used in a 33% cane sugar solution (5), the cane sugar having no influence on the course of the titration. To the casein, 300 mg of sodium chloride was added to cancel out the influence of the Donnan equilibrium on the course of the titration. For a detailed discussion of the shape of the casein curve, we refer to the work of Eilers (1, 2). Suffice it here to remark that the titration curve, between pH 2.5 and 10.5, represents the actual alkali consumption of protein by neutralization.

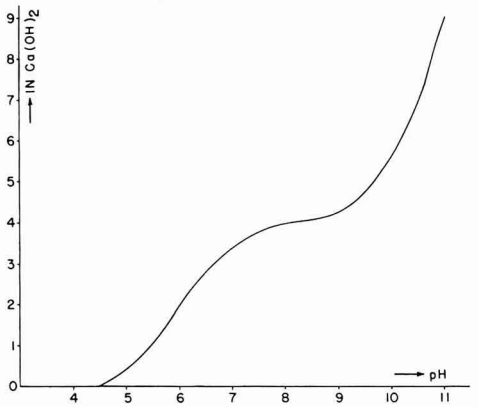


FIG. 1. Potentiometric titration of 5 g casein with 1 N $Ca(OH)_2$.

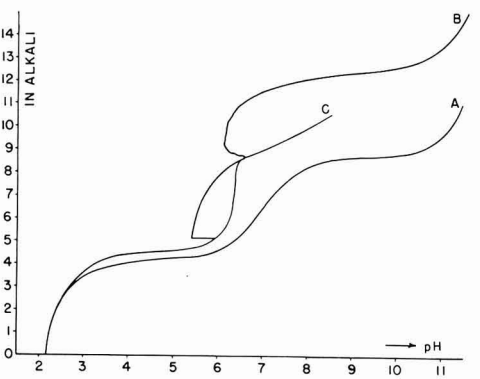


FIG. 2. Potentiometric titrations of 400 mg phosphoric acid with: a. 1 N KOH; b. 1 N $Ca(OH)_2$ (rapid titration); c. 1 N $Ca(OH)_2$ (slow titration).

(b). *Potentiometric titration of phosphoric acid.* Figure 2, Curve a, illustrates the change of pH in a solution where 400 mg of phosphoric acid are titrated with 1 N KOH. Subsequently, the formation of mono- and dipotassium phosphates is observed, while the tertiary salt can obviously exist only in such a strong alkaline environment that its formation below pH 11 does not occur.

Figure 2, Curve b, was obtained by titrating phosphoric acid with 1 N $Ca(OH)_2$. It is evident that both of these curves are almost identical up to the monophosphate stage. After addition of more alkali, however, the formation of the tertiary salt is apparent in Curve b. As Figure 2, Curve c, was obtained when repeating this titration, it was decided to add as little as 0.1 ml of calcium hydroxide at the time, and to record the pH only after it had been constant for 5 min. It then appeared that when the pH reached the initial value of 6.0, it quickly receded to 5.4, with the simultaneous formation of a precipitate. Possibly, a secondary calcium phosphate was present after the addition of approximately 5 ml of 1 N alkali, and occurred in a supersaturated state, but at pH 6 it changed suddenly into the tertiary salt:

$$Ca(H_2PO_4)_2 + Ca^{\cdot\cdot} \rightarrow 2\,CaHPO_4 \text{ (supersaturated)} + 2H^{\cdot} \quad \text{(a)}$$

$$2CaHPO_4 + Ca^{\cdot\cdot} \rightarrow Ca_3(PO_4)_2 \downarrow \text{(supersaturated)} + 2H^{\cdot} \quad \text{(b)}$$

After further addition of alkali, only a very slow change in pH occurred, which probably must be attributed to the conversion of (precipitated) dicalcium phosphate into the tertiary salt, and it follows from the total consumption of alkali up to pH 8 that a mixture of secondary and tertiary salts was eventually obtained.

Equations (a) and (b) indicate that calcium ions also influence the course of the pH changes. Therefore, phosphoric acid was next titrated with KOH, in the presence of a known and constant amount of calcium chloride. Figure 3 shows the potentiometric titration curve, obtained from two such titrations: Curve a illustrates a titration for which twice as much calcium was present as would have been necessary for the formation of the tricalcium phosphate. The drop of the pH after the formation of monocalcium phosphate is more apparent

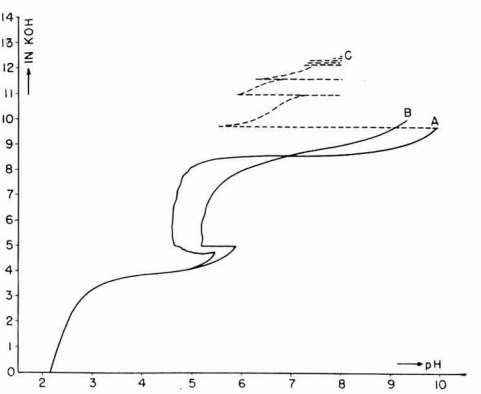


FIG. 3. Potentiometric titrations of 400 mg phosphoric acid with 1 N KOH in the presence of: a. 650 mg $CaCl_2$; b. 1.3 g $CaCl_2$; c. Intermittent re-titration of end product of titration a.

here than in the case of Curve b, obtained with an equivalent amount of calcium.

In both cases dicalcium phosphate is formed nearly exclusively. Thus, the calcium concentration seems to depress the solubility of the dicalcium phosphate to such an extent that the transformation into tricalcium phosphate is not observed. By constantly shaking these suspensions of dicalcium phosphate in a nitrogen atmosphere, and by titrating them at regular intervals (Figure 3, Curve c), it was shown that the dicalcium phosphate does change eventually into tricalcium phosphate. This is, however, an extremely slow reaction (three months in our case).

(c). *Potentiometric titration of casein–phosphoric acid mixtures.* Titrations were next carried out with 1 N potassium hydroxide on a mixture of 5 g casein, 400 mg phosphoric acid, and 1.3 g calcium chloride (Figure 4, Curve a).

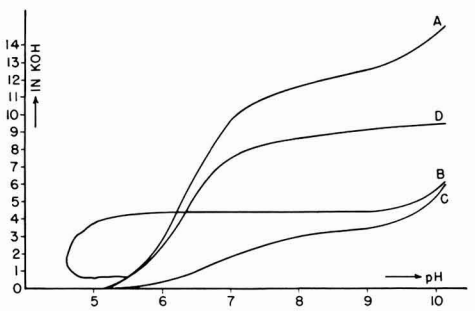


FIG. 4. Potentiometric titrations of phosphoric acid-casein–calcium chloride mixture (curves only drawn for pH region above 5): a. 400 mg H_3PO_4, 5 g casein, 1.3 g $CaCl_2$; b. 400 mg H_3PO_4, 1.3 g $CaCl_2$; c. 5 g casein; d. calculated from a-c.

Whereas it was found previously that when phosphoric acid was present on its own, it formed dicalcium phosphate (Curve b), the formation of tricalcium phosphate took place when casein was present as well (Figure 4, Curve d=a−c). The precipitate which was always noticed above pH 6 in the phosphoric acid solutions of Section b did not appear when casein was also present. From Figure 5, Curve a, it follows that when 5 g of casein is titrated in the presence of as much as 1 g of phosphoric acid and 3 g of calcium chloride, apart from tricalcium phosphate, the secondary salt is also formed (Curve d=a−c). In this case the formation of a precipitate is noticed at pH values over 9. Thus, the amount of tricalcium phosphate which can be formed depends on the quantity of casein present in the system.

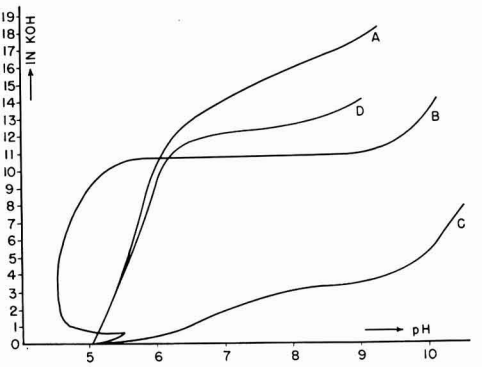


FIG. 5. Potentiometric titrations of phosphoric acid-casein–calcium chloride mixture (curves only drawn for pH region above 5): a. 1,000 mg H_3PO_4, 5 g casein, 3 g $CaCl_2$; b. 1,000 mg H_3PO_4, 3 g $CaCl_2$; c. 5 g casein; d. calculated from a-c.

Eilers (1) calculated that the maximum quantity of tricalcium phosphate formed depends on the number of acid groups which can be neutralized on the casein.

(d). *Potentiometric titrations of phosphoric acid in the presence of various organic substances.* When, in the titrations of Section c, casein is substituted by another protein like gelatin, and if 8 g is titrated in the presence of 400 mg of phosphoric acid and 1.3 g of calcium chloride, then tricalcium phosphate is formed (Curve d), whereas the solution remains clear during the whole of the titration (Figure 6, Curves a, b, c, d). Ovo-albumin (5 g of Ovo-

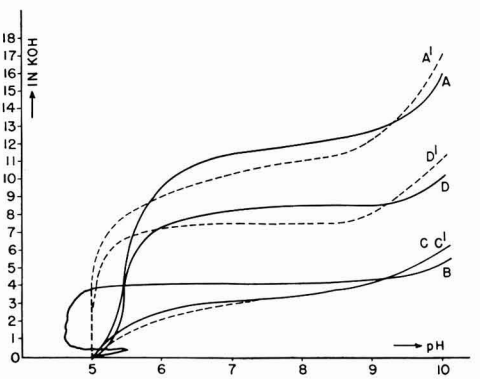


FIG. 6. Potentiometric titrations of phosphoric acid-protein–calcium chloride mixtures (curves only drawn for pH region above 5): a. 400 mg H_3PO_4, 8 g gelatin, 1.3 g $CaCl_2$; b. 400 mg H_3PO_4, 1.3 g $CaCl_2$; c. 8 g gelatin; d. calculated from a-c; a'. 400 mg H_3PO_4, 5 g ovo-albumin, 1.3 g $CaCl_2$; c'. 5 g ovo-albumin; d'. calculated from a'-c'.

albumin titrated in the presence of 400 mg of phosphoric acid and 1.3 g of $CaCl_2$) shows a similar phenomenon (Curves a′, b′, c′, d′). When trying to find an explanation of this phenomenon, three different possibilities should be considered:

a). Tricalcium phosphate is formed as a result of the colloidal properties of the organic matter present; the phenomenon is a physical one.

b). Tricalcium phosphate is formed as a result of certain chemically active groups present in the organic molecule; the phenomenon is, then, a chemical one.

c). A combination of the two possibilities mentioned above. In order to investigate a), the phosphoric acid titration was carried out in the presence of various colloidal polysaccharides like dextrin, agar, and starch. A monosaccharide-like dextrose appeared to have no influence on the course of the reaction, so that in the case of saccharides the possibility mentioned under b) can be ruled out.

It now appeared that when concentrated viscous gels of these were used (about 4-5 g substance in 200 ml water), insoluble tricalcium phosphate was formed, whereas in the tenfold-diluted liquids the insoluble secondary salt was formed. The formation of the tricalcium phosphate in the viscous gels should probably be attributed to temporarily high concentrations in reactants, due either to adsorption by the colloid or to a decrease in diffusion rates caused by the high viscosity of the medium. Both mechanisms, however, are in any case dissimilar to the one mentioned under the proteins as, in the case of the polysaccharides, the formation of an insoluble calcium phosphate was invariably observed during these titrations.

To test the possibility mentioned under b), the end groups present in the casein molecule, mainly carboxyl, amine, and hydroxyl groups, were examined. Consequently, phosphoric acid in the presence of an excess of calcium chloride was titrated in solutions of various carbonic acids (propionic, valeric, oxalic, and malonic acid), amines, (ethylenediamine, diethylamine), phenol, ethanol, the monosaccharide glucose, and of low molecular substances with several end groups combined into one molecule (tartaric acid, glycine, o-aminophenol, tyrosine). None of these substances, however, caused the formation of tricalcium phosphate, so that this phenomenon is probably allied to the combined occurrence of both a colloid and of one or more chemically active groups [possibility c)]. To investigate this, it was decided to eliminate or to mask one at a time the most important end groups in the casein molecule, then to repeat the phosphoric acid titrations with the product obtained.

e). *Elimination of the end groups on the casein molecule.* 1. *The carboxyl end groups.* (a) For the determination of these end groups, Fromageot et al. (3) used a method whereby the free carboxyl groups were reduced with Li-Al-hydride. Although the use of this reagent in the case of proteins will undoubtedly lead to other reductions than only $-COOH \rightarrow -CH_2OH$, it was proved experimentally that these reactions occur only to a negligible extent.

Method: For a period of three days, 45 g of casein were subjected, under constant stirring and under rigorously dry conditions, to a suspension of 15 g of Li-Al-hydride in two liters of water-free dioxan at 40 C. The product obtained was dialyzed to an ash content of 2%. The titration curve of the end product is shown to agree reasonably well with the curve calculated from the reactive groups present in the casein molecule. [Reference (1), page 1011 a.; *cf.* Figure 7, Curves 1 and B.]

(b) A second method used, probably not as drastic as the one with Li-Al-H_4, but likely to result in a less complete reaction, consisted of the esterification of the free carboxyl groups with methanol.

Method: To 20 g of casein suspended in one liter of anhydrous methanol, 10 ml conc. HCl was added and mechanically shaken for ten days at room temperature. The titration curve of the end product appears to be analogous to the theoretical one. (*cf.* Figure 7, Curves 2 and B.)

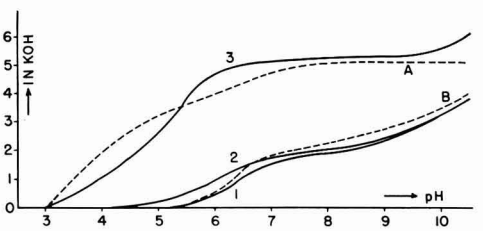


FIG. 7. Potentiometric titrations of casein derivatives:

	Experimental titration curve	Calculated titration curve
$LiAlH_4$ reduced casein	1	B
CH_3OH esterified casein	2	B
D.N.P. casein	3	A

2. *The amino end groups.* These were removed with 1-fluoro-2,4-dinitro benzene (F.D.-N.B.), (7, 9). *Method*: 1 g of F.D.N.B. in 5 ml ethanol was added to 20 g of casein in 50 ml of a saturated $NaHCO_3$ solution and the mixture shaken for 24 hr; after concentration in vacuum and removal of excess reagent, the end product was obtained as a thick oil at pH 2.

The titration curve of this product resembles closely the calculated curve (*cf.* Figure 7, Curves 3 and A).

f. *Potentiometric titrations of phosphoric acid in the presence of various colloidal substances with selected end groups.* The potentiometric titration curves of the mixture containing reduced casein and phosphoric acid and in the presence of excess calcium chloride with 1 N KOH is shown in Figure 8 (Curves

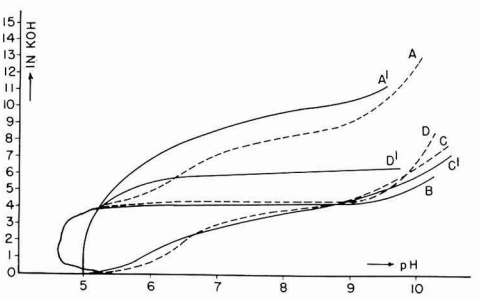


FIG. 8. Potentiometric titration of phosphoric acid with mixtures of $LiAlH_4$ reduced casein or CH_3OH esterified casein and calcium chloride (curves only drawn for pH region above 5): a. 400 mg H_3PO_4, 5 g $LiAlH_4$ reduced casein, 1.3 g $CaCl_2$; b. 400 mg H_3PO_4, 1.3 g $CaCl_2$; c. 5 g $LiAlH_4$ reduced casein; d. calculated from a-c; a'. 400 mg H_3PO_4, 5 g CH_3OH esterified casein, 1.3 g $CaCl_2$; c'. 5 g CH_3OH esterified casein; d'. Calculated from a'-c'.

a-d). The presence of a diphosphate plateau is very clear. Curves a'-d' obtained under the same circumstances for the esterified casein similarly show the formation of secondary calcium phosphate instead of the usual tertiary salt. In both cases, therefore, where the casein does not possess free carboxylic groups, it has no influence on the course of the normal phosphoric acid titration. If next, the D.N.P. casein, again with phosphoric acid and an excess of calcium chloride, is titrated with KOH, it is obvious that tricalcium phosphate is formed (Figure 9, Curves a-d) so that, under these circumstances, the D.N.P. casein reacts like normal casein. Free amino groups, there-

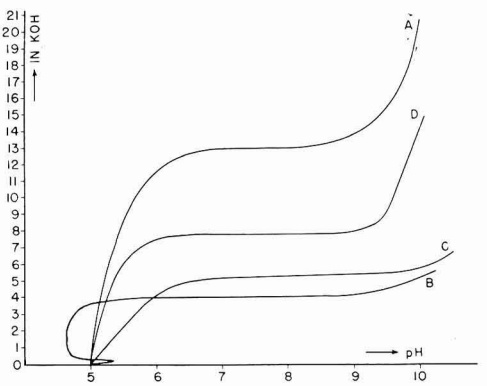
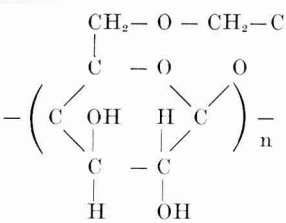


FIG. 9. Potentiometric titration of phosphoric acid with D.N.P. casein and calcium chloride (curves only drawn for pH region above 5): a. 400 mg H_3PO_4, 5 g D.N.P. casein, 1.3 g $CaCl_2$; b. 400 mg H_3PO_4, 1.3 g $CaCl_2$; c. 5 g D.N.P. casein; d. calculated from a-c.

fore, have no influence on the formation of the ultimate calcium salt; it also follows from these results that neither have hydroxyl groups any effect. Because in the above phosphoric acid titrations strong indications were obtained that carboxyl groups influence the type of calcium phosphate formed, although low molecular carbonic acids did not exhibit this influence (page 713), the reaction of substances with high molecular weight and with only carboxylic end groups was next investigated. As suitable substances Krilium, a hydrolyzed polyacrylonitril:

$$-(-CH_2-\underset{\displaystyle COOH}{\underset{|}{CH}}-CH_2-\underset{\displaystyle COOH}{\underset{|}{CH}}-)-_n$$

(used as a soil conditioner and produced by Monsanto), and carboxymethylcellulose were chosen:



In the presence of both substances tricalcium phosphate was formed while the solution remained completely clear: Figures 10 and 11. To summarize these results, Table 1 is drawn up.

Discussion. From the facts obtained, it is obvious that the possibility mentioned under

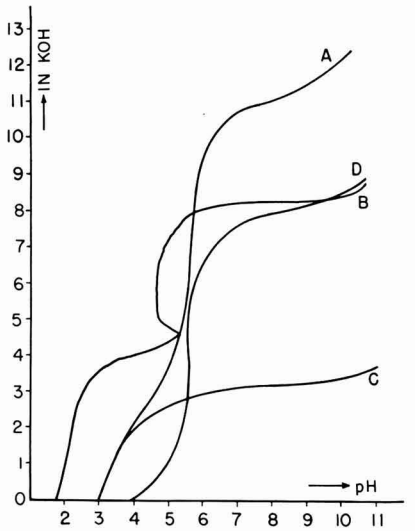


FIG. 10. Potentiometric titration of phosphoric acid with krilium and calcium chloride: a. 400 mg H_3PO_4, 600 mg krilium, 1.3 g $CaCl_2$; b. 400 mg H_3PO_4, 1.3 g $CaCl_2$; c. 600 mg Krilium; d. calculated from a-c.

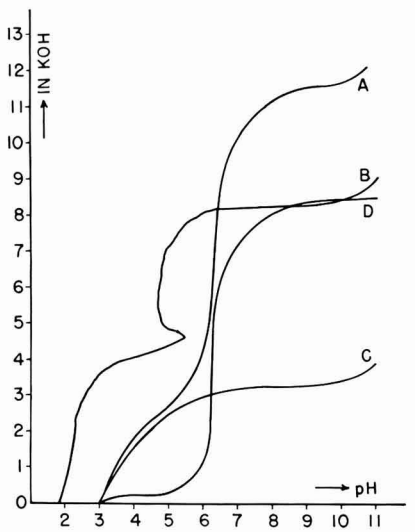


FIG. 11. Potentiometric titration of phosphoric acid with carboxy methyl cellulose (C.M.C.) and calcium chloride: a. 400 mg H_3PO_4, 1.6 g C.M.C., 1.3 g $CaCl_2$; b. 400 mg H_3PO_4, 1.3 g $CaCl_2$; c. 1.6 g C.M.C.; d. calculated from a-c.

c) is the only correct one, i.e., that in a phosphate titration with $Ca(OH)_2$ for the formation of the soluble tricalcium phosphate

TABLE 1

Type of phosphate formed on titration with alkali in the presence of calcium chloride and various organic substances

Conditions	Reactive groups present in organic molecule	Type of calcium phosphate formed
Rapid titration	.....	tri
Slow titration	.....	di
Presence of native casein	Basic, acidic	tri
Presence of reduced casein	Basic	di
Presence of esterified casein	Basic	di
Presence of D.N.P.	Acidic	tri
Presence of gelatin	Basic, acidic	tri
Presence of albumin	Basic, acidic	tri
Presence of krilium	Acidic	tri
Presence of carboxymethyl cellulose	Acidic	tri
Presence of dextrin	.....	di
Presence of low molecular carboxylic acids, amines, alcohols, and phenols	Various	di

the presence of both a colloidal substance and free carboxyl groups is necessary. It appears that these carboxyl groups must form part of the colloidal substance since, in the case of mixtures of carbonic acids as mentioned on page 713 and of colloids as mentioned on page 714, only insoluble dicalcium phosphate was formed. It should be added that the above phenomenon applies only to phosphates that are in solution: on precipitated calcium-phosphate in whatever form, the above-mentioned products, like proteins, krilium, etc., have no solvent action, not even after a few months.

The system investigated is probably the one in which calcium phosphate is present in milk or in which calcium phosphate occurs in blood. In the latter, tricalcium phosphate will be bound to the blood proteins and in this way available for transport. The complex might also play an important role in the transport of calcium to, and the deposit of, calcium in the skeleton and in dentine.

Another possibility of the transport of calcium phosphate in a soluble and tertiary form is in the intestines. The above-mentioned findings might explain why the calcium from milk is so much better absorbed than that from vegetables.

Finally, the binding of calcium phosphate to humic acids should be mentioned. It was found that these acids also form a soluble complex with tricalcium phosphate and this phenom-

enon will most certainly be of importance in the transport and the binding of calcium phosphate in organic soils.

ACKNOWLEDGMENTS

My sincere thanks go to Prof. H. Eilers for suggesting the subject and for the many helpful discussions.

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CELLULOSE ACETATE ELECTROPHORESIS OF MILK SERUM PROTEINS[1]

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SUMMARY

Milk serum proteins were electrophoretically fractionated, using cellulose acetate membrane as a stabilizing medium in a veronal buffer (pH 8.6, ionic strength 0.05), at 200 v for 2 hr at 25 C. The strips were stained with Ponceau S dye and analyzed in a Spinco Analytrol or eluted and read in a Coleman Jr. Spectrophotometer. Choice of buffer, effect of ionic strength and pH of buffer, position of serum application, effect of voltage, effect of temperature, and concentration of serum solution were studied for optimum resolution of the serum proteins. Five distinct fractions were identified as blood serum albumin, beta-lactoglobulin, alpha-lactalbumin, pseudo-, and euglobulin in their decreasing rates of migration.

Standard deviations and coefficients of variation for these five fractions indicated that the procedure was reliable. The dye-binding capacities of the five proteins were approximately the same and a linear relationship was established between dye uptake and protein concentration. Use of the method seems to offer significant advantages for investigating milk serum proteins.

Milk serum proteins contribute considerably to nutrition, flavor, color, body, and keeping quality of milk and its products. Of the major proteins in milk, serum proteins are most easily affected by heat treatment. Their heat-sensitive nature is useful for estimating heat treatment of milk during processing, sterilization, evaporation, and drying. Consequently, methods for quantitative determination of these proteins are of considerable interest to the dairy industry.

Conventionally, moving boundary electrophoresis is used to differentiate the serum proteins. To simplify the procedure and eliminate expensive instrumentation, a number of workers have adopted ionophoretic methods, using filter paper as a medium (13, 15-17). For research purposes, however, incomplete fractionation and adsorption of proteins by the paper, and the probability of artifacts being present, limit the use of zone electrophoresis on paper. To improve fractionation, a number of supporting media other than filter paper have been proposed.

Recently, Kohn (6, 11) has shown that cellulose acetate membrane can be used with considerable success in analytical separation of blood serum proteins. The electrophoretic separation is accomplished in much less time and with greater sensitivity and improved resolution than had been possible with paper or similar media (8-10). Extensive use of the method for blood serum protein analysis was done by several workers (2-4, 7). Grunbaum et al. (7) obtained eight distinct fractions for normal blood sera, using the microelectrophoresis technique. Favorable features of the cellulose acetate membrane are: Only microliter quantities of serum are required; no spreading of serum occurs upon application; sharp separation is obtained without tailing-off; the fractionation takes only 2 hr. To adapt a method for analysis of milk serum proteins incorporating the advantages of the cellulose acetate medium, a study was required of a number of variables, including statistical reliability. This paper describes the result of the study, and the modifications of procedure adopted.

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MATERIALS AND APPARATUS

Supporting medium. Oxoid electrophoresis strips of cellulose acetate (Consolidated Laboratory Inc., Chicago Heights, Illinois).

Dye. Ponceau S (Allied Chemical), 0.2% in 3% aqueous trichloroacetic acid.

Buffer. Veronal buffer, ionic strength 0.05, pH 8.6.

Power supply. Constant voltmeter 0-500 v (Buchler Instruments, Fort Lee, New Jersey).

Electrophoresis cell. A horizontal tank, specially designed for cellulose acetate electrophoresis (Shandon Scientific Co., Ltd., London), holding eight 18- by 2.5-cm strips or four 18- by 5-cm strips, with internal dimensions of 23.5 by 21.5 by 5 cm. Total buffer capacity of the cell is 1,300 ml and provides continuous fluid surface. The strips are stretched horizontally across two bridges, adjustable to allow variation of effective strip lengths. A filter paper wick interposed between strip and holder and partially immersed in the buffer provides a conducting medium between buffer and strip.

METHOD

Preparation of milk serum proteins. Bring the milk sample to pH 4.6 by slow addition of 1 N HCl and remove casein and denatured serum proteins by filtration on Whatman No. 1 paper. Dialyze the clear filtrate against about 6 vol of distilled water at about 5 C 12 hr; remove from the bag and centrifuge at 500 × G 30 min to remove the precipitated proteins. Dialyze the solution two additional times in the same manner. Using any standard lyophilizing equipment, freeze-dry the clear solution. Make either a 5% (densitometer method) or 10% (elution method) (w/v) solution from the lyophilized powder in the buffer used for electrophoresis.

Electrophoretic procedure. Prepare eight cellulose acetate strips 9 by 2.5 cm. With a light pencil, draw a line 2.5 cm away from one end of the strip, to indicate point of application of the sample. Immerse the strips in the buffer solution for at least 1 min, remove, and blot excess buffer from surface. Place 500 ml of buffer solution in the tank, and tilt the tank to allow buffer level in all compartments to equilibrate. Place the eight strips, free of surface buffer film, in position across the bridges. Allow the system to equilibrate by switching on the current and maintaining it at 200 v for 15 min. Apply a sample of 10 μl to each strip from a micropipet. After 2 hr, remove strips and immerse in Ponceau S solution 10 min. Wash strips in several changes of 5% acetic acid until background is clear and no further dye appears in bath. Press between filter paper sheets and allow to dry at room temperature. Analyze the dried strips by either of the following methods:

Densitometric evaluation. Evaluate the stained strips with the Spinco Analytrol apparatus, equipped with the B2 balancing cam, using a neutral density filter in the rear holder and a blue (500 mμ) interfering filter in both front and rear holders. Detailed instructions are provided in the manual (18).

Elution method. From the dried strip, cut out the individual bands. Dissolve each cut section in 2 ml of a mixture of 70% chloroform and 30% ethanol. Fraction *B* (see Figure 1)

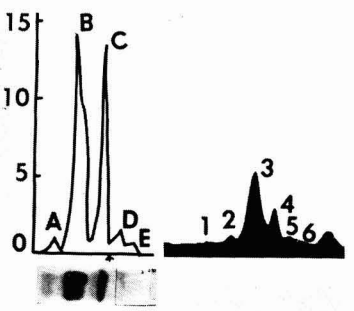

FIG. 1. Membrane electrophoretic pattern (left) compared with moving-boundary electrophoretic pattern (right) for raw milk serum proteins. Units for the peak height are in centimeters. The vertical arrow indicates the point of application. Migration proceeded to the left of the arrow. The moving-boundary pattern was obtained by using veronal buffer of pH 8.6, ionic strength 0.1, at 1.2 C for 9,000 sec, with a field strength of 7.8 v per square centimeter.

should be dissolved in 4 ml of the solvent (the absorbance values are, therefore, multiplied by two). Determine absorbance in a Coleman Jr. Spectrophotometer, using an 8- by 8- by 100-mm cuvette at 520 mμ. Calculate per cent fraction as follows:

$$\text{per cent fraction} = \frac{\text{absorbance of serum fraction}}{\text{sum of absorbance of serum fractions}} \times 100.$$

STUDY OF VARIABLES AND DISCUSSION

Choice of buffer. Aronsson and Gronwall (1) recommended tris (hydroxymethyl) aminomethane buffer, claiming the buffer possessed certain favorable features over the usual veronal buffer because of its lower molar ionic strength and greater buffering capacity. However, only two distinct bands were apparent and migration was very slow. Further work with the buffer was discontinued. Much of the published

work in the field describes preference for the veronal buffer; it was accepted for the entire work.

Effect of ionic strength of buffer. Buffer solutions with ionic strength of 0.100, 0.075, and 0.050 were prepared. Mobility of the fastest-moving fraction and degree of separation of the two slow-moving fractions were noted with each buffer solution. Optimum results were obtained during 2 hr of migration at 200 v, at pH 8.6, and ionic strength 0.05.

Choice of pH of buffer. Since earlier workers have found a pH of 8.6 to be suitable for moving-boundary and paper electrophoresis, this value was accepted.

Position of serum application. Fractions *D* and *E* (see Figure 1) migrate towards the cathode. The migration for a 2-hr period does not exceed 2.5 cm, yet the two fractions separate distinctly. At the same time, the fastest-moving fraction, *A*, is well within the strip under the prescribed conditions, when the point of application is 2.5 cm away from the cathode.

Effect of voltage. Electrophoretic migration was examined, using 100, 150, and 200 v for 2 hr and 0.05 ionic strength buffer at pH 8.6. Strips maintained at 200 v gave maximum separation of individual fractions. Similar results were obtained when constant current at 0.6 ma per centimeter width of strip was employed.

Effect of temperature. When the electrophoresis was carried out at 5 C, the buffer of higher ionic strength was required to obtain sufficient current at 200 v, the migration was extremely slow, and fraction separation was unclear. However, experiments at laboratory room temperature of 22-25 C afforded distinct separation with maximum migration. Higher temperatures produced blurring of the migrating fractions.

Concentration of serum solution. Serum concentration is quite critical in both the methods, viz., elution and densitometer quantification. In the elution method, the range of absorbance should be between 0.05 and 0.50. This is achieved when 10 μl of 10% (w/v) protein solution is applied. Limits of sensitivity for the Analytrol method are set between 4- and 14-cm peak height (see Figure 1). Using 10 μl of 5% (w/v) serum solution, optimum results are obtained when the densitometric method is used.

Reproducibility of results obtained by densitometer and spectrophotometer methods. Raw milk from the University herd was used for preparing serum solution as described. The serum solution was applied to 24 strips and subjected to electrophoresis 2 hr at 200 v in a buffer of 0.05 ionic strength and a pH of 8.6. The strips were analyzed on a Spinco Analytrol apparatus. Similarly, serum samples were applied to another 24 strips and electrophoretic patterns examined in a Coleman Jr. spectrophotometer.

Standard deviation and coefficient of variation were calculated for each fraction, using the following equations:

$$\text{S.D.} = \sqrt{\frac{\Sigma(x - \bar{x})^2}{n - 1}}$$

$$\text{C.V.} = \frac{\text{S D.} \times 100}{\bar{x}}$$

where x is the observation, $\bar{x}$ is the mean of observations, and n is the number of observations.

A close agreement was observed between the two methods. However, the elution method offered some advantage in the reliability of the estimation of Fractions *D* and *E*. It was noted (Table 1) that when a protein fraction migrated further from the point of application, its estimation apparently became more reliable. For example, Fraction *A*, which migrated the most, exhibited lowest values for standard deviation and coefficient of variation. The mean values for each fraction and its order of migration

TABLE 1

Electrophoretic analysis of raw milk serum proteins, using densitometer (1) and elution (2) techniques

Fractions	Means of observations [a]		Standard deviations		Coefficient of variation	
	1	2	1	2	1	2
A	4.61	5.12	0.07	0.06	1.51	1.20
B	51.90	51.71	2.88	2.63	5.54	5.09
C	30.92	25.62	3.10	3.25	10.02	12.68
D	8.04	11.92	1.69	1.89	21.23	15.86
E	4.52	5.75	1.33	0.61	28.76	10.57

[a] Observation is obtained by calculating per cent fraction.

suggested the identification of the individual zones. In order of mobility Fractions *A, B, C, D,* and *E* (see Figure 1) were tentatively ascribed to blood serum albumin, beta-lactoglobulin, alpha-lactalbumin, pseudoglobulin, and euglobulin, respectively. Further, their quantitative estimation fell within the normal range described in (5).

Relationship of dye-uptake to amount of protein. Because the protein concentration is determined from the amount of dye absorbed on the membrane, the proportionality of such a relationship should be ascertained. Using Lissamine Green on cellulose acetate, Brackenridge (2) has shown that the dye uptakes of albumin and four globulin fractions in human blood sera were virtually linear with respect to concentration. Similarly, Scherr (14) described that, in the case of blood serum proteins when stained by Ponceau S dye, Beer's Law was obeyed over a wide range of concentrations when the fractions were eluted and analyzed in a spectrophotometer. This point was studied in the following manner:

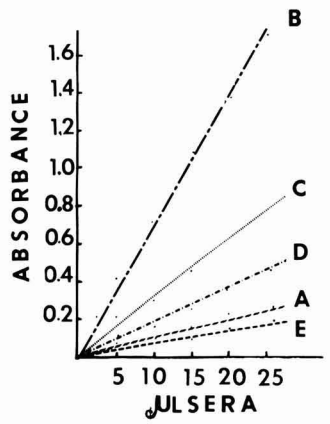

FIG. 2. Protein Fractions A, B, C, D, and E separated from milk sera are plotted at different concentration levels and absorbancy at 520 mμ.

Separate strips were charged with 2.5, 5.0, 10.0, 15.0, 20.0, and 25.0 μl of 10% (w/v) solution of milk serum proteins. The strips were subjected to electrophoresis as described earlier. The fractions were cut out and dissolved in 2 ml of solvent mixture. Four milliliters of eluant were used in higher concentrations (15-, 20-, and 25-μl strips) and absorbance values multiplied by two. Readings were taken in a Coleman Jr. spectrophotometer at 520 mμ. Figure 2 shows that all the serum components obey Beer's Law over the range of volumes of serum samples used. The experiment was repeated four times and the original findings confirmed.

Using the micro-Kjeldahl method described by Whitney et al. (19) for the protein nitrogen determination, the relative dye-binding capacity of each protein fraction was studied. Since a micro-quantity of protein is applied to the membrane for electrophoresis, an extremely small amount of nitrogen would be expected in the euglobulin, pseudoglobulin, and blood serum albumin fractions on a single strip. With this difficulty in mind, the following procedure was used: Four strips were run simultaneously and one of them stained in the usual manner. The remaining strips were immersed in 3% trichloroacetic acid. Excess buffer was removed from the membrane by three 10-min washings in fresh solutions of 3% trichloroacetic acid. The strips were dried and, with the stained strip as a guide, five separated zones were cut out. Zones representing blood serum albumin from the three strips were pooled to bring the nitrogen content in the analytical range (0.00-0.10 mg). A similar procedure was used for eu- and pseudoglobulins. Table 2 describes the results of the nitrogen analysis.

TABLE 2

Comparison of the per cent composition of the electrophoretic components of serum proteins by nitrogen analysis and Ponceau S elution method

Serum protein fraction	Nitrogen recovered	Nitrogen recovered/ strip	Per cent nitrogen/ strip	Per cent fraction by elution
	(mg)		(%)	
Blood serum albumin	0.0093[a]	0.0031	4.49	5.12
Beta-lactoglobulin	0.0388	0.0388	56.23	51.71
Alpha-lactalbumin	0.0183	0.0183	26.52	25.62
Pseudoglobulin	0.0180[a]	0.0060	8.69	11.92
Euglobulin	0.0085[a]	0.0028	4.06	5.75

Nitrogen values of each fraction represent the average of six determinations. Average of per cent total nitrogen recovered was 96%, ranging from 88 to 107%.

[a] Total of three strips.

TABLE 3

Electrophoretic mobility of the proteins of milk serum

Component in Figure 1	Mobility [a]		Protein
	Present study	Larson's (12)	
1	−8.3	−7.9	Proteose-peptone component
2	−6.6	−6.6	Serum albumin
3	−4.4	−5.0	Beta-lactoglobulin
4	−3.5	−3.6	Alpha-lactalbumin
5	−2.6	−2.5	Pseudoglobulin
6	−1.6	−1.7	Euglobulin

[a] Mobilities (μ) expressed in 1×10^{-5} cm^2 v^{-1} sec^{-1} and calculated from descending boundary.

The data show there is good agreement between the protein analysis by the nitrogen method and the elution technique. Additional support was supplied by the following experiment: Different quantities of each known purified protein were applied on the membrane and stained and eluted in the usual manner. The absorbance values at 520 mμ for different concentrations were plotted for the five proteins. The slope of the plot for each protein was found to have approximately the same value.

Ionographic identification of milk serum proteins. Raw milk samples have shown five distinct zones for their serum proteins on cellulose acetate membrane electrophoresis. Figure 1 shows a typical ionograph and Tiselius electrophoretic pattern of the same serum protein sample. In the latter, there are six distinct peaks. Identification of these peaks is accomplished by comparing the mobility values which agree with the published data (5, 12).

Peaks *A*, *B*, *C*, *D*, and *E* in the ionograph (Figure 1) correspond to Peaks 2, 3, 4, 5, and 6 in the moving-boundary electrophoretic pattern, with respect to their per cent composition and order of migration. Such identification was tentative; however, for precise and reliable identification, experiments with purified known components were carried out.

Purified samples of alpha-lactalbumin, pseudoglobulin, and euglobulin were supplied by Dr. R. Jenness;[3] samples of beta-lactoglobulin by Dr. T. L. McMeekin[4] and a commercial laboratory,[5] and blood serum albumin fraction obtained from a commercial company.[6]

A 5% solution (w/v) of each purified known component was prepared using the electrophoresis buffer. Also, a 5% solution (w/v) of raw milk serum was prepared and added separately to the purified component solution in 1:1 ratio. For identification of each component, three solutions were electrophoretically examined, using 5 μl of sample for each strip.

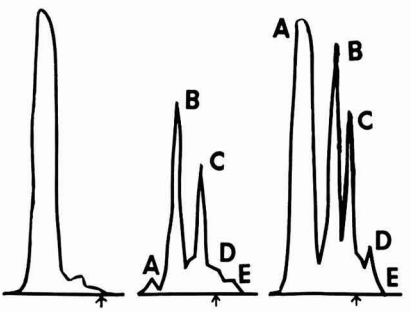

FIG. 3. Identification of blood serum albumin. From left to right, ionographic patterns for purified blood serum albumin, raw milk serum, and a mixture of the two in a 1:1 ratio. The vertical arrows indicate the points of application and the migration proceeded to the left of the arrows.

Figure 3 shows the densitometric pattern for blood serum albumin identification. Peak *A* is enhanced by the addition of the purified fraction to the serum protein solution. Thus, Peak *A* may be attributed to the blood serum albumin. Further, it was noted that the change in the peak area for each component in the mixture corresponded to calculated values. The per cent composition for Peaks *A*, *B*, *C*, *D*, and *E* in the serum protein was 4, 55, 30, 8, and 3, respectively. The mixture analysis showed the peak values in the same order as 54, 26, 14, 4, and 2, respectively. Thus, quantitative recovery in the mixture became evident.

Beta-lactoglobulin studies are shown in Figure 4. Milk from a single cow was used in these studies and a single Peak *b* appeared for beta-lactoglobulin. Whenever milk from a mixed herd was used, two peaks appeared, indi-

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[4] Eastern Utilization Research and Development Division, USDA, Philadelphia, Pennsylvania.

[5] Pentex, Inc., Kankakee, Illinois.

[6] The Armour Laboratories, Kankakee, Illinois.

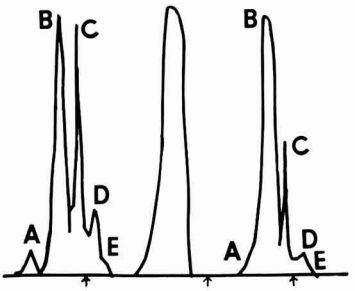

FIG. 4. Identification of beta-lactoglobulin. From left to right, ionographic patterns for raw milk serum, purified beta-lactoglobulin, and a mixture of the two in a 1:1 ratio. The vertical arrows indicate the points of application and the migration proceeded to the left of the arrows.

cating two fractions, beta-lactoglobulin A and B (see Figure 1).

The alpha-lactalbumin sample was found to be approximately 80% pure and gave two peaks, as shown in Figure 5. In the mixture, Peak *c*

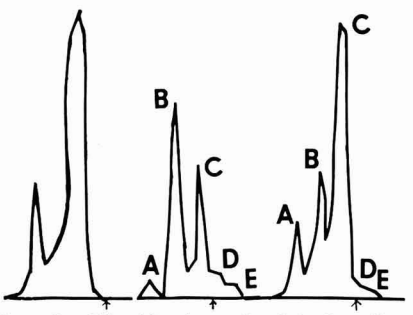

FIG. 5. Identification of alpha-lactalbumin. From left to right, purified alpha-lactalbumin, raw milk serum, and a mixture of the two in a 1:1 ratio. The vertical arrows indicate the points of application and the migration proceeded to the left of the arrows.

is seen to be enhanced by the major peak in the purified fraction solution. The contaminant can be attributed to Peak *A*, previously identified as blood serum albumin.

Figures 6 and 7 show the identification of pseudoglobulin and euglobulin fractions. Each protein showed one major peak. The globulin fractions migrated to the cathode, euglobulin migrating farther than pseudoglobulin. The apparent back migration of globulins was due to electroosmosis.

Finally, a serum protein solution was synthesized by adding all five purified known fractions in a ratio similar to the natural milk serum solution. The ionographic pattern resembled the pattern given by milk serum proteins, as shown in Figure 1. Also, the experimental values for the individual fractions in the synthetic protein solution came close to expected values. In all our studies, five major peaks appeared. However, the peaks observed in the moving-boundary electrophoresis (12) for proteose-peptone fractions did not appear on the cellulose-acetate membrane. It is believed that the dye Ponceau S does not stain these fractions.

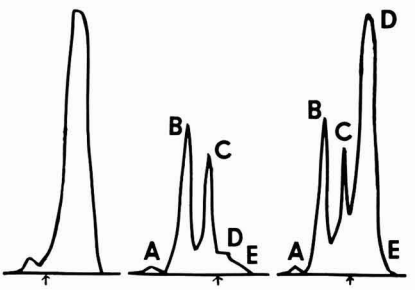

FIG. 6. Identification of pseudoglobulin. From left to right, purified pseudoglobulin, raw milk serum, and a mixture of the two in a 1:1 ratio. The vertical arrows indicate the points of application and the migration proceeded to the left of the arrows.

ACKNOWLEDGMENT

The authors are grateful to Dr. R. Jenness, University of Minnesota, for the samples of alpha-lactalbumin, pseudoglobulin, and euglobulin, and to Dr. T. L. McMeekin, USDA, Philadelphia, for a sample of beta-lactoglobulin. The technical assistance given by Mr. Otto Pollins, E. R. Squibb and Sons, New Brunswick, New Jersey, in carrying out the moving-boundary electrophoresis of the milk serum samples is much appreciated. Thanks are also extended to Dr. John C. Anderson, Rutgers—The State University, for help in statistical analysis of the data.

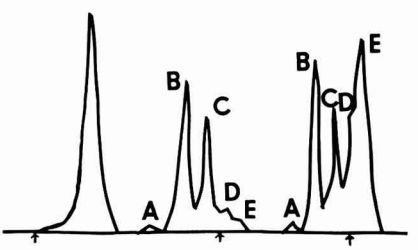

FIG. 7. Identification of euglobulin. From left to right, purified euglobulin, raw milk serum, and a mixture of the two in a 1:1 ratio. The vertical arrows indicate the points of application and the migration proceeded to the left of the arrows.

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IDENTIFICATION OF SOME VOLATILE COMPOUNDS RELATED TO THE FLAVOR OF MILK AND CREAM [1, 2]

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SUMMARY

A low temperature-reduced pressure distillation technique was employed to obtain a total distillate considered to be representative of milk flavor. The carbonyl compounds contained in the aqueous fraction were separated and identified as their 2,4-dinitrophenylhydrazones by paper chromatography, column partition chromatography, ultra-violet spectroscopy, and melting point determinations. Data thus obtained established the presence of formaldehyde, acetaldehyde, acetone, butanone, pentanone-2, and hexanone-2 in milk and cream and presented tentative evidence for heptanone-2 in cream. Quantitative analysis of these individual carbonyl compounds also was performed.

The volatile compounds obtained from liquid nitrogen trapping of milk and cream were also investigated. These volatiles were fractionated by gas chromatography and several of the eluted components trapped in liquid nitrogen for mass spectral analysis. The mass spectra data in conjunction with retention times established the identity of ethyl ether, methyl sulfide, acetone, butanone, ethanol, chloroform, acetonitrile, and ethylene chloride.

Evidence also was obtained for the presence of a waxy, nut-like odor which is highly characteristic of cream. Its identification awaits further study.

PART I. RECOVERY AND IDENTIFICATION OF VOLATILE CARBONYL COMPOUNDS AS THEIR 2,4-DINITROPHENYLHYDRAZONES

The extensive study of the carbonyl compounds in milk products such as Cheddar cheese (1) and dry whole milk (7) has permitted the easy demonstration of their significance in the flavor of the product. Although carbonyls have been identified in fresh milk (3, 6), they have not been comprehensively studied.

EXPERIMENTAL PROCEDURE

The distillation procedure for the collection of volatile compounds was the same as that used previously in this laboratory (10).[4] Eight quarts of raw heavy cream or 5 gal of raw whole milk were distilled in a 12-liter round-bottom flask or a 5-gal Pyrex carboy, respectively, at 35-40 C and 15-20 mm Hg. for 4 hr. Following the distillation period, the contents of the traps were combined, after noting each for characteristic odor.

Since it was of interest to determine which carbonyls are present in the raw milk, an acid solution of DNPhydrazine reagent [5] (2 g of DNPhydrazine per liter of 30% sulfuric acid) was added to the aqueous distillate. The reaction was allowed to proceed for seven days at 4 C, after which the DNPhydrazones were extracted from the aqueous medium with carbonyl-free hexane and the extracts dried over sodium sulfate.

The tentative identification of the carbonyls thus obtained from milk and cream was accomplished through paper chromatography and ultraviolet spectral analysis. The paper chromatographic method employed was that of Huelin (4), which is efficient in separating a homologous series up to eight carbons in the parent compound. The extract from milk distillate separated into six fractions. These corresponded in Rf to hexanone-2, pentanone-2, butanone, acetone, acetaldehyde, and formaldehyde. An additional fraction was observed in

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[3] Present address: Food Division, Food and Drug Administration, Washington 25, D. C.

[4] This apparatus consisted of the distilling flask connected in series to a cold water condenser, a wet ice and two ethanol-dry ice traps.

[5] 2,4-dinitrophenyl is abbreviated DNP throughout.

TABLE 1

Spectral and melting point data of DNPhydrazones from milk and cream

DNP hydrazone [a]	Absorption maximum (mμ)		Color reaction with ethanolic NaOH	Melting point (C)		
	Observed	Reported		Observed	Authentic	Mixed
Heptanone-2 [b]	360	362	Stable after 2 hr	.....	72	.....
Hexanone-2	360	362	Stable after 2 hr	104	106	104–106
Pentanone-2	360	362	Stable after 2 hr	139	143	140–143
Butanone	360	362	Stable after 2 hr	113	116	113–116
Acetone	360	362	Stable after 2 hr	124	125	124
Acetaldehyde	355	356	Faded after 1 hr	152	157	152–155
Formaldehyde	345	346	Faded after 15 min	165	166	165

[a] Tentative identification based on paper chromatography.
[b] Observed only in cream.

the extract from the cream distillate which corresponded chromatographically to heptanone-2.

The column partition method of Day et al. (1) was employed for the separation of the carbonyls, to fractionate larger amounts for further identification. The separated fractions were then paper chromatographed and the light absorption of the fraction extracted from the paper was determined in 95% ethanol. Additional evidence concerning the nature of the compound was obtained by observing the diminution of color after the addition of 0.25 N ethanolic sodium hydroxide. Jones et al. (5) have observed that the color produced by aliphatic aldehyde DNPhydrazones fades more rapidly than the color exhibited by other derivatives.

Conclusive identifications were obtained by melting point and mixed melting points with authentic DNPhydrazones on a Fischer micro melting point apparatus.

For quantitative evaluation, the identified fractions were retained in a definite volume of solvent and an aliquot spotted on filter paper and chromatographed by the method of Huelin. The development bands were cut from the paper, extracted with a definite volume of ethanol, and the optical density at maximum absorption measured. The optical densities were measured at 345 mμ for formaldehyde DNPhydrazone, 335 mμ for acetaldehyde DNPhydrazone, and all other hydrazones at 360 mμ. Calculations for determining the concentrations of the carbonyls were made as described previously (10).

In light of information concerning the identities and concentrations of the carbonyls in milk, it was of interest to determine the flavor threshold value of hexanone-2. Using the spray aspersion technique (9), the average threshold of this compound for five judges was determined to be 0.5 ppm.

TABLE 2

Concentrations of carbonyls obtained from milks and creams [a]

Carbonyl	Milk		Cream	
	(mg per liter)			
Heptanone-2	.000	.000	.007	.004
Hexanone-2	.007	.011	.017	.018
Pentanone-2	.007	.026	.025	.045
Butanone	.079	.077	.177	.154
Acetone	1.152	.847	.508	.354
Acetaldehyde	.016	.013	.004	.001
Formaldehyde	.001	.003	.001	.004

[a] Two different samples of each.

RESULTS AND DISCUSSION

Identification data and quantitative results for carbonyl compounds are given in Tables 1 and 2, respectively.

The evidence presented in Table 1 establishes the identity of formaldehyde, acetaldehyde, acetone, butanone, pentanone-2, and hexanone-2 in milk and the tentative identification of heptanone-2 in cream. Data obtained on the reaction of the DNPhydrazones with ethanolic sodium hydroxide, although relative in nature, do serve to distinguish aldehydes from ketones. The melting points of the hydrazone derivatives listed except acetone, butanone, and pentanone-2 are without benefit of recrystallization, the small quantities available making this impossible. Thus, some minor discrepancies are evident between the melting points for unknown and authentic derivatives. However, mixed melting points with authentic preparations showed no depressions, which establishes the postulated identities of the compounds in this table.

The precise origin of these carbonyl compounds in milk is not known at the present time. The fact that they are found in milk which has received no heat treatment, indicates that they are normal constituents or that the mechanism of their formation is easily initiated.

Spontaneous decarboxylation of β-keto acids, which are intermediates in the β-oxidation of fatty acids, may account for the formation of these ketones and also for the fact that β-keto acids have never been identified in milk.

PART II. INVESTIGATIONS OF THE VOLATILE COMPONENTS OF MILK AND CREAM BY GAS CHROMATOGRAPHY AND MASS SPECTROMETRY

Although methyl sulfide (8) and the carbonyls identified in milk were observed to contribute to the flavor of the product, they did not account for its entire flavor. Since the flavor of the milk after distillation was always more bland than before treatment, it was felt that although volatiles were being removed from the milk by the distillation procedure, they were not being retained in the cold traps. In view of this, it was considered advantageous to increase the trapping efficiency, to investigate the more volatile compounds.

The procedure employed was similar to that of Day (2). Five traps were used after the cold water condenser, a wet ice trap, two dry ice–ethanol traps, followed by two liquid nitrogen traps. The two liquid nitrogen traps filled with glass wool were reverse-connected, which enabled the large amounts of carbon dioxide which normally would condense in the central tube and seal off the flow to be collected on the wall of the test tube.

After the distillation was completed, the last three traps were pinched off and the vacuum maintained. The contents of only the last three traps were analyzed, because the first two contained large amounts of water. To analyze these volatiles conveniently, it was necessary to transfer the volatiles from the other traps into the first liquid nitrogen trap by manipulating the temperature and pressure. This latter trap was prepared so that gas chromatographic samples could be drawn from it by placing an injection system in the entrance tube and closing the other end with a rubber balloon to equalize pressure.[6] This manipulation also permitted release of excessive carbon dioxide by sublimation.

A Barber-Colman Model 10 gas chromatograph with an argon ionization detector was used for the fractionation of the volatiles. The column materials used were 15% Carbowax 1540 on 100-140 mesh Chromosorb W and 20% dioctylphthalate on 80-100 mesh Chromosorb W. The columns were 9 ft by 6 mm glass U-tubes.

A sample was drawn from the trap with a hypodermic syringe fitted with a no. 26, 1½-inch needle, and immediately injected onto the column. Initially, small gas samples were analyzed, but it was found larger samples could be injected if the column pressure was reduced to 12 psi. A typical chromatogram for cream volatiles is shown in Figure 1. Since flavor

[6] Analysis indicated that the balloon material produced no artifacts.

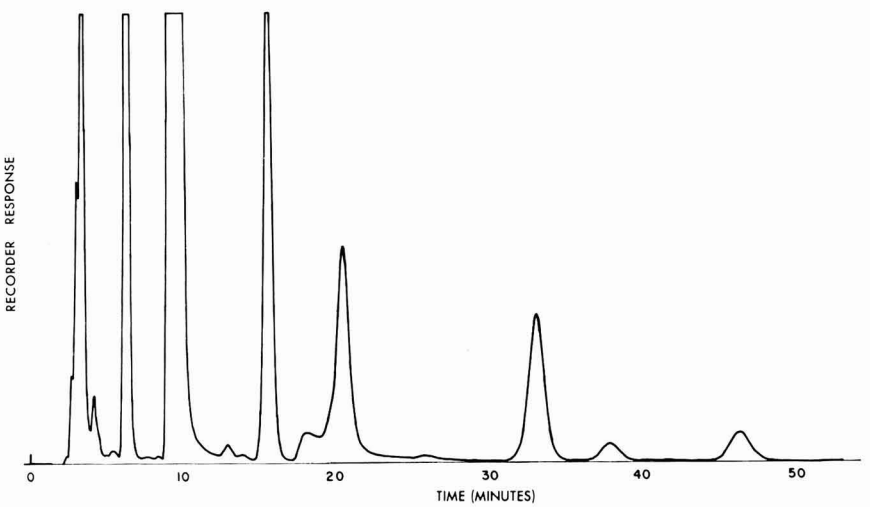

FIG. 1. Gas chromatogram of cream volatiles. Column temperature 65 C, Argon flow 34 ml per minute, Column—Carbowax 1540. The major peaks represent ethyl ether (4 min), methyl sulfide (6 min), acetone (10 min), butanone (16 min), ethanol (21 min), chloroform and acetonitrole (both 33 min).

and aroma increase in the order: skimmilk, whole milk, cream, the bulk of the investigational effort involved cream.[7]

Tentative identifications for components from the volatile mixture were obtained by comparison of their retention times with those of authentic compounds.

To analyze the separated fractions by mass spectrometry, it was necessary to trap the fractions as they were eluted from the gas chromatograph. This required the use of the capillary cell detector, because argon could not be used as a carrier gas when the fractions were to be collected in liquid nitrogen. The capillary cell was arranged so that helium could be used as a carrier gas and argon as the ionizing gas.

The fractions trapped were selected because they were consistently the largest appearing on the chromatogram. Although the size of the peak produced on a chromatogram by a compound is not necessarily related to flavor significance, this method served as a guide in determining the areas to be trapped. A chromatogram obtained under trapping conditions showing the areas trapped is presented as Figure 2.

Each of the fractions were collected individually in a liquid nitrogen cooled trap and analyzed in a General Electric Mass Spectrometer. The mass spectra obtained were qualitatively evaluated by conventional procedures for analyzing such data. The catalog of Mass Spectral Data published by the American Petroleum Institute was used as the principal source of reference data.

A control experiment also was performed in which distilled water was low temperature-reduced pressure distilled using the identical conditions and equipment employed for milk and cream. A chromatogram of the volatiles obtained under these conditions revealed peaks in trace quantities which corresponded in retention to acetone, ethanol, and chloroform. A possible explanation for the recovery of these compounds in the control experiment is that they were residual and not completely removed from the apparatus, since all of them were obtained in the distillation of cream and in much greater quantities.

RESULTS AND DISCUSSION

The identification of several of the fractions was greatly facilitated by evidence accumulated by gas chromatographic analysis. In the case of Fractions 2, 3, 4, and 5 which were identified as methyl sulfide, acetone,[8] butanone, and ethanol, respectively, it served only as confirmation of identity. The identity of the remaining Fractions 1, 6, and 7 were determined only after extensive interpretation and with helpful suggestions from an expert in the field. Beyond limitations of the authors in the field of mass spectrometry, they were confused for some time by the data in that all three traps contained completely unanticipated compounds, and one of the traps represented a mixture of two such compounds. The unlikely postulation of ethyl ether in trap 1 proved correct on

[7] Over 25 distillations showed milk and cream to contain generally the same components, the proportions of which varied rather widely between samples. Skimmilk was not analyzed.

[8] The split in the peak for Fraction 3 is assumed to result from overloading. The mass spectrum showed only acetone in the fraction.

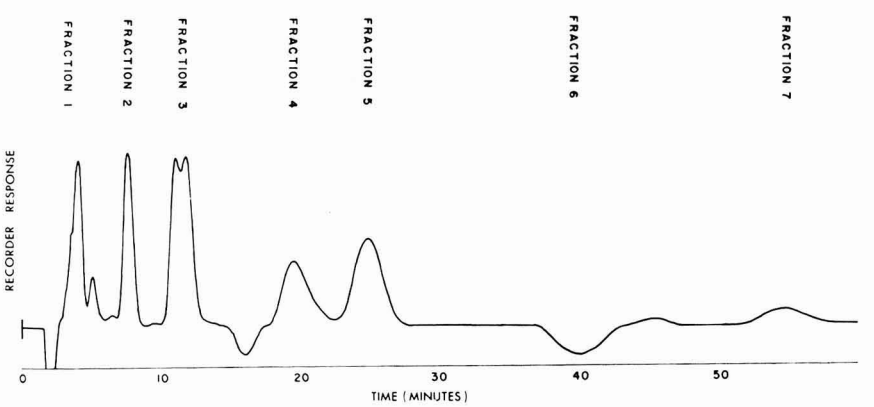

FIG. 2. Gas chromatogram of cream volatiles showing areas trapped. Column temperature 65 C, Argon pressure 8 psi, Helium pressure 12 psi, Column—Carbowax 1540.

comparing the spectrum for the unknown with that of the authentic. It was pointed out that the mass spectrum for trap 6 presented good evidence of chloroform as one component through the characteristic pattern in the region of mass numbers 82 through 87 and the small peaks at mass numbers 41.5, 42.5, and 43.5. When the mass spectrum of chloroform was extrapolated from that of trap 6, the residual pattern was almost completely accounted for by acetonitrile. The main residue was at mass numbers 38 through 41, peak proportions being the same as for acetonitrile. It became evident through expert guidance that trap 7 might also contain a chlorinated hydrocarbon. A search of authentic spectra for such compounds revealed coincidence between data for the unknown and the mass spectrum of ethylene chloride (major peaks in proper proportions at mass numbers 26, 27, 49, 51, 61, through 65, 98, and 100). Although the mass spectral data were considered adequate for identification of the indicated compounds, the evidence was reinforced by gas chromatography. Not only did these compounds have the proper retention times, but chloroform and acetonitrile produced negative peaks as did the contents of Trap 6 when chromatographed under trapping conditions.

The odors of the various components as they were being eluted from the carbowax column were noted. An odor was observed in the area of Trap 6 that was very characteristic of cream flavor. It was described by some as malty, but more appropriately by others as waxy and nutlike. It appeared to have extreme odor potency, because it was easily detectable by odor even when the peak on the chromatogram was small. It could be detected in the crude liquid nitrogen trapping of milk or cream and distinguished from the methyl sulfide. Although chloroform and acetonitrile were responsible for most of the spectrum of Fraction 6, it is postulated that another compound is responsible for this odor and is also eluted at this time in concentration so low as to have escaped detection by the isolation and identification procedures.

Fraction 7 had an ester-like odor. Whether ethylene chloride accounts for the fruity, apple-like odor associated with this peak or overshadows the true flavor components can only be answered by further study.

The identification of chloroform, acetonitrile, and ethylene chloride in milk and cream volatiles awaits confirmation at other laboratories. The detection of these compounds poses somewhat of a mystery. They may arise from the use of solvents in the fat extraction of feeds (seedoil meals), although present knowledge indicates that other solvents are more commonly used. The routine use of chlorinated pesticides in the treatment of dairy cows or their feed may provide a means of transfer or assimilation that results in residues in milk.

This investigation confirmed earlier findings (8) regarding the presence and importance of methyl sulfide in the flavor of fresh milk and cream.

ACKNOWLEDGMENTS

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IDENTIFICATION OF STAPHYLOCOCCI IN NONFAT DRY MILK BY THE FLUORESCENT ANTIBODY TECHNIQUE [1, 2]

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SUMMARY

An investigation was made of the applicability of the fluorescent antibody technique to the detection of staphylococci in nonfat dry milk. Procedures are given for the preparation of milk films and the staining of these films by the indirect fluorescent antibody procedure. Qualitative studies revealed that *Staphylococcus aureus* cells could be specifically identified in the presence of cells of *Streptococcus lactis, Lactobacillus casei, Bacillus subtilis,* and other organisms which might occur normally in milk. Additional studies showed that cells of staphylococci could be quantitatively determined in milk films by this procedure, and that direct microscopic counts of these organisms correlated extremely well with numbers of *S. aureus* cells known to be present. The quantitative detection of staphylococci from nonfat dry milk which had been inoculated with *S. aureus* before spray-drying demonstrated that the heating and desiccation occurring during spray-drying did not affect the results of our tests. Advantages of the fluorescent antibody technique over conventional procedures are presented.

The serious staphylococcal food poisoning outbreaks of 1953 and 1956 (2, 3) which implicated nonfat dry milk as the responsible food prompted investigations into new methods of detecting staphylococci in this product. The ideal technique would be one which would specifically identify staphylococci in small numbers and in the presence of morphologically similar organisms. In addition, it should detect either viable or dead organisms, since most bacteria are killed during the manufacture or storage of nonfat dry milk.

The recent use of fluorescent antibodies as specific staining reagents in a number of other systems (5, 6, 8) was noted and considered as potentially applicable to the above problem. Because this technique uses a serological system and a microscopic system, it should be both highly specific and highly sensitive. Indeed, while the present study was in progress, Carter (4) reported that he could specifically identify coagulase positive staphylococci in smears from cheese and nonfat dry milk. The purpose of our investigation was to see if the fluorescent antibody technique could be applied to the qualitative and quantitative detection of staphylococci in nonfat dry milk. Conventional staining methods of nonfat dry milk films do not distinguish between cells of *Staphylococcus aureus* and thermoduric cocci, but a serological procedure such as the fluorescent antibody technique might provide such a distinction. Also, this antigen-antibody system does not require viable organisms for a positive result, since killed cells will also be detected.

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[3] Present address: Communicable Disease Center, Atlanta, Georgia.

METHODS

Preparation of milk films. Thin slides of any glass except Pyrex were thoroughly cleaned with Bon Ami and allowed to dry in air, after which they were wiped clean and stored in a dust-free container. A few minutes before use they were flamed and allowed to cool. Nonfat dry milk was reconstituted in water according to standard procedures (1) at a concentration of 11 g per 99 ml of water. After the suspension had become homogeneous, it was diluted 1:10 with sterile water of neutral pH. Milk films were prepared according to standard methods and dried at 40-45 C for 5-10 min. They were then immersed in xylene for 2 min, air-dried, immersed in 95% ethanol for 5 min, and dried again.

The films were firmly fixed to glass slides by the following procedure of Olson and Jezeski (7). Each slide was immersed vertically in 2 N NaOH for 5 min, then washed very gently

by moving the slide vertically three or four times, in each of two beakers of water. Horizontal movement was avoided, since it enhanced disintegration of milk films. Slides were then drained and air-dried.

Preparation of immune antisera. Staphylococcus antisera were prepared by injecting rabbits intravenously with heat-killed whole cell preparations adjusted to an O.D. of 2.0 in a Coleman Spectrophotometer (590 mμ λ). An initial i.v. injection of 0.5 ml was followed four days later by 1.0 ml on each of three successive days, then four days rest, and the cycle repeated weekly through the fourth week. Sera were collected ten days after the final injection. Titers of antisera ranged from 1:1280 to 1:5120, by tube agglutination tests, but only those with the highest titers were used for fluorescent antibody work. Fluorescein-labeled sheep antiserum to rabbit globulin was obtained from the Sylvana Company.

Staining with fluorescent antibodies. The indirect staining method of Weller and Coons (9) was employed. Milk films were stained by flooding slides with a small amount of unlabeled staphylococcal antiserum and placing them in a moist chamber for 20-30 min. Excess antiserum was removed, and slides were washed in either phosphate-buffered saline (pH 7.2, 0.05 M phosphate) or distilled water (pH 7), after which they were air-dried. The milk films were then flooded with a small amount of fluorescein-labeled sheep antiserum to rabbit globulin and the slide returned to the moist chamber for 20 min. Excess serum was removed and slides were washed in phosphate-buffered saline (pH 8.0) for 15 min, with occasional gentle vertical agitation. Again, care was exercised to avoid dislodging the milk film. Excess buffer was removed with absorbent tissue, a drop of buffered glycerol (nine parts glycerol; one part phosphate-saline, pH 8) was placed over the film, and a cover slip applied. Slides were then examined in the fluorescence microscope with an oil-immersion objective.

For this work a Leitz Ortholux microscope equipped for fluorescence microscopy was used. The ultraviolet-transmitting filter BG 12, 4 mm thick, was used in conjunction with appropriate eyepiece filters and a Philips CS 150 high-pressure mercury lamp. Photographs were taken on Kodak Tri-X film through a medium yellow eyepiece filter to absorb ultraviolet light.

Controls. The controls used are shown in Table 1. These are grouped as A and B; Group A for specificity experiments in the absence of nonfat dry milk, and Group B for all experiments involving nonfat dry milk. One other type of control might have been used in either group, and this would have employed a fluorescein-labeled normal sheep serum, e.g., as the second staining reagent in control number B5.

Quantitative studies. Commercially prepared nonfat dry milk was reconstituted and inoculated with known numbers of cells of *S. aureus*, as determined in a Petroff-Hauser chamber. Milk films were then prepared and stained as described above, and direct microscopic counts made of fluorescent staphylococci in each field.

Other quantitative experiments were performed with experimentally prepared nonfat dry milk powders obtained from Dr. J. J. Jezeski, of the University of Minnesota, and having the following histories:

Powder No. 1: prepared from skimmilk which had been heated to 93 C for 1 hr, cooled to 4.4 C within 30 min, and held at this temperature overnight. Uninoculated.

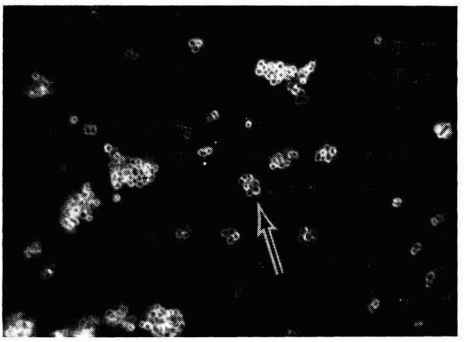
Powder No. 2: prepared from equal parts of milk used for powders No. 1 and 3. Re-

TABLE 1

Controls for the indirect fluorescent antibody technique

Experiment	Control No.	Reagents [a]				
		Milk	*S. aureus*	IAB	NAB	FAB
A	1		×			
	2		×			×
	3		×		×	×
	4		×	×		
B	1	×				
	2	×	×			
	3	×	×	×		
	4	×	×			×
	5	×	×		×	×
	6	×		×		×

[a] Legend: IAB = Unlabeled immune globulin to staphylococci; NAB = Unlabeled normal rabbit globulin; FAB = Fluorescein-labeled sheep anti-rabbit serum.



a. Incandescent illumination b. Ultraviolet illumination

FIG. 1. Fluorescent antibody staining of a mixture of *Streptococcus aureus* and *Streptococcus lactis*.

ported to contain 5×10^7 staphylococci per milliliter of original milk.

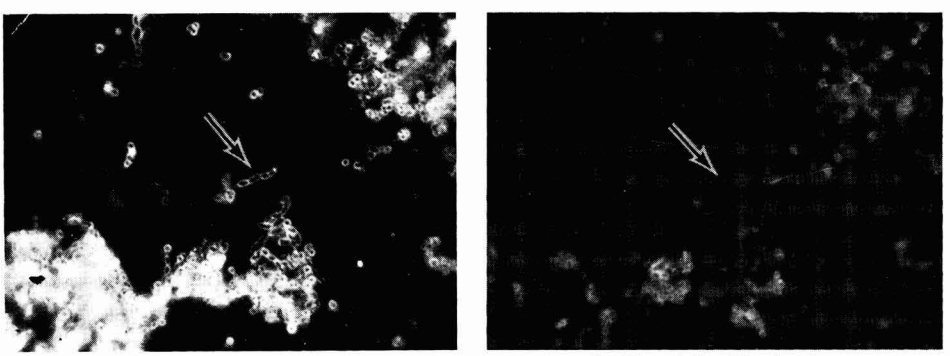
Powder No. 3: prepared from skimmilk which had been heated to 93 C for 1 hr, then cooled to 37 C. It was then inoculated with an enterotoxin-producing strain of *S. aureus* and incubated 18 hr. The staphylococcal population was reported to be 10×10^7 cells per milliliter of milk. Part of it was used to prepare Powder 2, above.

These powders were reconstituted, films prepared on glass slides, and the films stained as described previously. Direct microscopic counts were made of the fluorescent staphylococci in each film.

RESULTS

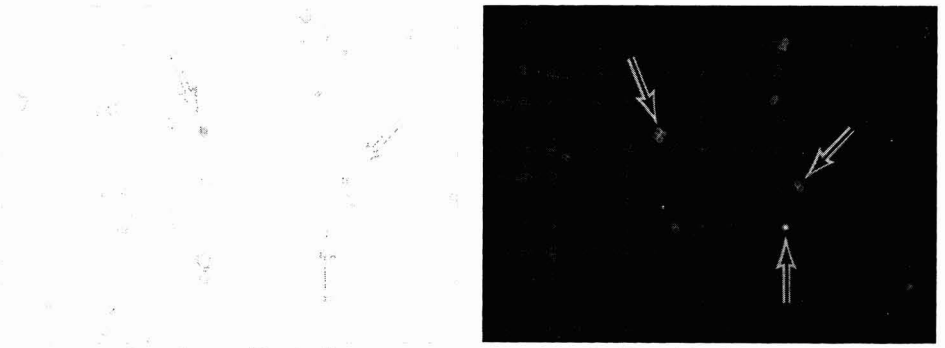
Specificity of staining. A variety of bacterial species which constitute the normal flora of milk and some which might possibly be found in dry milk were used. They were *Bacillus cereus*, *Bacillus coagulans*, *Bacillus subtilis*, *Aerobacter aerogenes*, *Streptococcus lactis*, *Streptococcus faecalis*, *Lactobacillus casei*, and *S. aureus*. Smears were prepared of both pure cultures and mixtures of these organisms, and stained as above.

Cells of each of these species nonspecifically adsorbed a small amount of fluorescent antibody, but cells of *S. aureus* stained far more intensely than did the others. Figures 1a and b and 2a and b show the results on staining mixtures of *S. aureus* and either *L. casei* or *S. lactis*. The arrows in Figures 1a and 1b point to a mixed cluster of *S. aureus* and *S. lactis* cells which are nearly indistinguishable from each other under incandescent illumination (Figure 1a). However, with ultraviolet (UV) illumination, it is apparent that most of these cells are *S. lactis*, since they do not fluoresce (Figure 1b). Figures 2a and 2b illustrate the same point with a mixture of *S. aureus* and *L. casei* cells, the arrows indicating a chain of lactobacilli.



a. Incandescent illumination b. Ultraviolet illumination

FIG. 2. Fluorescent antibody staining of a mixture of *Streptococcus aureus* and *Lactobacillus casei*.



a. Incandescent illumination b. Ultraviolet illumination

FIG. 3. Fluorescent antibody staining of nonfat dry milk containing staphylococci.

Results such as these were typical of all of the mixtures, with the exception of mixtures of *S. aureus* and *B. cereus*. The bacilli of this species, but not of the other species, adsorbed sufficient fluorescent antibody to fluoresce brightly under UV illumination. However, the dissimilarity in morphology between these cells and *S. aureus* cells made differentiation possible.

Preparations of nonfat dry milk which contained *S. aureus* prior to processing were stained as described above, and Figures 3a and 3b demonstrate the results. The diagonally directed arrows indicate *S. aureus* cells which are specifically and easily detectable by their fluorescence, but difficult to identify under incandescent illumination because of the presence of particles of milk constituents. Under UV light these particles usually were not visible, but an exception is denoted by the vertically directed arrows in these figures. These arrows indicate a particle which had adsorbed fluorescent antibody, and thus might give a false positive result. However, observation of several such particles revealed that they always were uniformly fluorescent, whereas cells of *S. aureus* appeared as doughnuts. With this distinction in mind, it was not difficult to differentiate the two, as in Figure 3b.

Although the above experiments demonstrated that staphylococci could be specifically identified in mixtures with other organisms, only one strain of *S. aureus* had been used. To see if the specific antiserum would also react with other staphylococcal strains, a collection of 72 cultures from cheeses, human sources, and bovine sources was made. When these strains were stained with fluorescent antibody, 12 strains did not specifically adsorb the labeled antibody, but 60 strains did react positively. The 12 strains which did not react had no outstanding property in common, such as being coagulase-negative or all of bovine origin. The percentage of negative reactions (16.6%) was of interest because agglutination tests with the same antiserum, unlabeled, failed to react with 23% of 94 staphylococcal strains from the same sources, thus giving the same order of negative reactions.

Quantitative studies. The results of studies involving commercial preparations of nonfat dry milk to which known numbers of *S. aureus* cells were added are shown in Table 2. It is apparent that good results were obtained in all tests except those involving reconstituted milk with only 3.6×10^5 *S. aureus* cells per milliliter. With this cell population, and because of the dilution factor involved in preparing milk films, only one staphylococcus cell per 14 microscopic fields would be expected, on the average. This is too low for accurate quantitative counts.

Quantitative counts on nonfat dry milk powders in which staphylococci were grown prior to processing gave very good results. Examination of milk films prepared from Powder No. 1 revealed that very few bacteria were visible. A few large bacilli were seen, but staphylococci

TABLE 2

Quantitative recoveries of *S. aureus* cells

No. cells/milliliter added to reconstituted nonfat dry milk	No. fluorescent cells/milliliter observed in microscope after staining with fluorescent antibodies
3.6×10^8	2.3×10^8
3.6×10^7	2.8×10^7
3.6×10^6	4.5×10^6
3.6×10^5	Too few to count

were absent. Since this powder was prepared from uninoculated, unincubated milk, this is not surprising. Examinations of milk films prepared from Powders 2 and 3, however, showed large numbers of bacteria and resulted in counts of 6.05×10^7 and 13×10^7 fluorescent staphylococcal cells per milliliter, respectively. Although they were not counted, there was a fairly heavy concentration of large bacilli in these milk films, as compared with films from Powder 1. This might be expected, since bacilli of several types are present in milk, and some sporeforming bacilli might survive the heat treatment and grow during the 18-hr incubation period.

Thus, in milk films reported by Dr. Jezeski to contain 5×10^7 staphylococci cells per milliliter by the direct microscopic count, we found 6.05×10^7 fluorescent staphylococci per milliliter and from milk films reported to contain 10×10^7 cells per milliliter we found 13×10^7 fluorescent cells per milliliter.

DISCUSSION

Preliminary experiments with milk films prepared by standard methods showed that two major difficulties existed in the mechanics of staining the staphylococci with fluorescent antibodies. The first of these was that prolonged exposure of milk films to aqueous and saline solutions usually resulted in disintegration of the milk films. The second problem was that fluorescent staphylococci were nearly impossible to detect because of highly fluorescent background material (milk constituents). Adsorption of fluorescent antibody preparations with both Dowex 2 chloride and acetone-dried mouse liver powders, to remove unconjugated fluorochrome and/or conjugated nonspecific antibodies, failed to decrease this background fluorescence. The procedures which have been presented above under the heading Methods are those which were found to be best for overcoming both of these difficulties. Thus, the brilliant background fluorescence was reduced by a combination of diluting the reconstituted nonfat dry milk 1:10 with sterile water, and by using the indirect staining procedure.

In addition to eliminating excessive background staining, the indirect staining technique has the inherent advantage that only one fluorescein-labeled antibody is required for each type of animal in which specific antisera are being produced. On the other hand, the direct staining procedure requires that each specific antiserum under study be labeled with a fluorescent compound.

All of the controls listed in Table 1 are not necessary for routine use of this procedure as a diagnostic tool. Controls numbered B2 through B5 are essential ones, provided a positive control is also included in each day's tests. This might be prepared from a batch of nonfat dry milk which was either experimentally or naturally seeded with *S. aureus* cells.

The photographs illustrating the specificity of the technique show that little difficulty should be encountered in identifying staphylococci in milk films, even in the presence of morphologically similar organisms such as *L. casei* or *S. lactis*. In addition, some evidence has been presented that a large majority (83.4%) of the strains of *S. aureus* which were tested could be detected by the indirect fluorescent antibody technique. This was approximately the same percentage as obtained by agglutination tests with the unlabeled serum.

The results presented herein do not show that only coagulase-positive staphylococci react, since such results were not found to be true. To the contrary, and in contrast to the results of Carter (4), we found that both coagulase-negative and -positive strains were stained. It should be pointed out, however, that Carter used different antigens and that we made no attempt to produce antisera specific for coagulase-positive strains. Furthermore, our results have no immediate bearing on the detection of enterotoxin or enterotoxigenic staphylococci in nonfat dry milk. However, if a more specific antiserum can be produced, i.e., against such strains of staphylococci specifically, it may be that it will be useful as a diagnostic reagent in the fluorescent antibody technique.

The data from quantitative experiments show that good correlation was obtained between numbers of cells of staphylococci known to be in nonfat dry milk and those actually counted as fluorescent in the UV microscope. This correlation is emphasized when one considers the extremely high microscopic factor (5×10^6) introduced by diluting the reconstituted nonfat dry milk. Only the lowest concentration of cells of *S. aureus* was not counted quantitatively, but this does not mean that staphylococci could not be detected at this concentration, or at even lower concentrations. Thomason, Moody, and Goldman (8) have reported that the bacterium *Malleomyces mallei* could be detected with fluorescent antibody when only four cells were found on a smear covering about 1.5 sq cm. Thus, it would be possible theoretically to detect a single cell of *S. aureus* on a milk film, provided the entire area of the milk film were scanned microscopically.

The most valuable quantitative and diagnostic results were obtained from experiments with nonfat dry milk powders in which staphylococci were grown prior to processing. These results not only showed that *S. aureus* cells could be quantitatively counted, but that the various treatments given skimmilk before and during spray-drying did not seriously lessen the staining of staphylococcal cells with fluorescent antibodies.

The fluorescent antibody technique has some distinct advantages over conventional cultural methods of detecting organisms: (a) it is rapid; (b) it is as specific as the serological system employed; and (c) it will detect either viable or dead cells. Its major advantage over conventional microscopic methods is that the fluorescent antibody technique employs a specific staining reagent (labeled antiserum), thus eliminating the painstaking and tedious examination of every stained organism. One glance at a microscopic field is sufficient to tell if fluorescent staphylococci are present, whereas one must often spend considerable time trying to differentiate conventionally stained staphylococci from morphologically similar organisms or from milk components. The major disadvantages of the fluorescent antibody technique, as applied to our purposes, are that (a) more expensive equipment is required for UV microscopy, and (b) specific antisera must be prepared and maintained, thus necessitating a small-animal colony. Both of these disadvantages might be minimized, however, by establishing regional or cooperative laboratories to which all of the milk drying plants within a given area might send samples for testing.

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ACID-SOLUBLE NUCLEOTIDES IN COW'S AND GOAT'S MILK[1]

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SUMMARY

Acid-soluble nucleotides in cow's and goat's milk at different stages of lactation were examined by ion-exchange column chromatography. In goat milk CMP5′, DPN, AMP5′, AMP-X, GMP5′, UMP5′, orotic acid, GDP-mannose, UDP-acetylglucosamine, UDP-acetylgalactosamine, UDP-glucose, UDP-galactose, GDP, UDP, UDP-uronic acid, and other unidentified nucleotides were found throughout the lactation period. Total content of acid-soluble nucleotides attained maximum at the colostral stage (330 μM per 100 ml) and decreased with the advance of lactation. The percentage of UDP-acetylhexosamine and UDP-hexose in acid-soluble nucleotides are not only highest in colostrum (68%) but also in the mid-stage of lactation (48%). Active P^{32} incorporation into these nucleotides was observed following the injection of P^{32}. Milk of a goat with lactation induced by the administration of hormones showed the same chromatographic pattern as that of the milk at normal lactation.

Considerable species difference of the acid-soluble nucleotides of milk was observed between cow's and goat's milk. With the exception of the colostral stage, orotic acid constituted a major part of acid-soluble nucleotides of cow milk. Cow's milk also contained CMP5′, uric acid, AMP5′, and an unidentified AMP derivative. There was no difference in the patterns of chromatograms between Holstein and Jersey milk.

A recent study of phosphorus compounds of the lacteal secretion during early lactation has shown that the amount of ester P[1] is highest in colostrum and that uridine nucleotides, guanosine nucleotides, and adenosine nucleotides are found in goat colostrum and milk. These substances were also detected in cow colostrum, but disappeared with advancing lactation (12).

Several investigators have reported that UDP-glucose, a coenzyme in lactose synthesis, is particularly abundant in the mammary gland (6, 14, 20), and that no great difference exists between the patterns of acid-soluble nucleotides of the mammary glands of the cow and goat (14).

There have been few investigations on the composition of acid-soluble nucleotides in cow's and goat's milk. Also, the relationship between the changing content of acid-soluble nucleotides and mammary gland function has not been clarified. This paper describes marked differences in the acid-soluble nucleotide composition of cow's and goat's milk, as well as nucleotide variation with the stage of lactation and P^{32} incorporation into these substances.

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[1] In this paper, the following abbreviations are used: P, phosphate; AMP, adenosine monophosphate; CMP, cytidine monophosphate; GMP, guanosine monophosphate; UMP, uridine monophosphate; ADP, adenosine diphosphate; GDP, guanosine diphosphate; UDP, uridine diphosphate; ATP, adenosine triphosphate; GTP, guanosine triphosphate; UTP, uridine triphosphate; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; G-1-P, glucose-1-phosphate; G-6-P, glucose-6-phosphate; DNA, desoxyribonucleic acid; RNA, ribonucleic acid.

METHODS AND PROCEDURE

Experimental samples were obtained from Holstein cows and Saannen goats of normal lactation kept at the National Institute of Agricultural Sciences in Chiba, Japan. Milk of a goat with lactation induced by the administration of estrogen and progesterone was also analyzed.

Forty to one hundred milliliters of milk was used for the experiment. Immediately after milking, the milk was cooled to 2 C and 2.5 volumes of ice-cold 10% trichloroacetic acid was added. The mixture was stirred with a

glass rod and protein centrifuged off in the cold. The protein residue was homogenized and re-extracted with 2.5 volume of 10% trichloroacetic acid. The acid-soluble supernatant solutions were combined. The acid was then removed from the extract by shaking vigorously with five volumes of ice-cold ether until neutral, aerated to remove as much as possible of the remaining ether. The acid-soluble phosphate of the solution was adsorbed in an anion exchange resin column in 5 C, which was washed with 200 to 300 ml of water.

Ion exchange column chromatography. For separating nucleotides, a gradient elution method with a formate system and Dowex I, ×-8, 200-400 mesh (formate) in a 1.6- by 25-cm column was used (11). A 500-ml mixing flask was employed. The five elution ranges were obtained by changing the series in 1 N formic acid, 4 N formic acid, 0.2 M ammonium formate in 4 N formic acid, 0.4 M ammonium formate in 4 N formic acid, and 0.8 M ammonium formate in 4 N formic acid, respectively. Ten milliliters of fractions were obtained at the rate of 0.8 ml per minute with an automatic fraction collector. The optical density of the fractions was determined at 260 mμ with a Hitachi Model EPU spectrophotometer, using distilled water as the blank.

Identification of nucleotides. To identify nucleotides, fractions of the column chromatograms were pooled and the eluents were removed. Samples were vacuum-dried at low temperature on KOH. In fractions containing much ammonium formate, NH_4^+ was exchanged for H^+, using columns of Dowex 50, ×-8, 200-400 mesh (H^+ form).

The condensed samples were chromatographed with known standard substances in one dimension on paper with two or more of the following solvent systems (17, 22).

1. *iso*-amylalcohol + *iso*-propylalcohol + 75% lactic acid + 5% trichloroacetic acid 10:30:1:20)
2. *iso*-butyric acid + 0.5 N ammonia (20:12)
3. *n*-butyric acid + 0.5 N ammonia (10:6)
4. 95% ethanol + 1 M ammonium acetate (pH 3.8)
5. 95% ethanol + 1 M ammonium acetate (pH 7.5)

Rf values of the individual acid-soluble nucleotides were determined according to Bandurski and Axelrod (2). All paper chromatogram spots estimated by the method of Markham and Smith (15) were cut out and eluted with 0.01 N HCl. Ultraviolet absorption spectra of the eluates were determined at different pH. Phosphorus content of the acid-soluble nucleotides was determined by the method of Horecker (10). The content of ribose was detected by orcinol reaction (I). The purine bases and pyrimidine nucleotides of each peak, obtained on hydrolysis in 1 N HCl at 100 C for an hour, were chromatographed with authentic samples in the following solvent systems:

6. *iso*-propanol + HCl + water (170:41:39)
7. *n*-butanol (saturated with ammonia and water)

The carbohydrate-moieties of sugar containing nucleotides were identified after hydrolysis of the nucleotides in 0.01 N HCl at 100 C for 30 min. After removing anion and cation with Dowex 50 (H^+ form) and Amberlite 1R 4B (OH^- form), the samples were chromatographed with the solvent systems.

8. *n*-butanol (saturated with water) + acetic acid + water (4:1:5)
9. *n*-butanol + pyridine + water (3:2:1.5)
10. Ethylacetate + pyridine + water (10:5:6)

The sugars were determined with aniline hydrogen phthalate, naphthoresorcinol, ninhydrin, and the Elson and Morgan reagent (18, 19). DPN was confirmed by the increase of ultraviolet absorption at 340 mμ in the glucose dehydrogenase system.

For the estimation of nucleotide contents, the following extinction coefficients were used (at pH 2): adenosine nucleotide 14,200, guanosine nucleotide 11,800, uridine nucleotide 9,900, cytidine nucleotide 6,800, orotic acid 4,200, DPN 18,500.

The incorporation of P^{32} into the acid-soluble phosphorus compounds of milk. To study the metabolism of acid-soluble phosphorus compounds in milk, an isotope experiment was performed. Three mc of P^{32} labelled orthophosphate supplied by the Radiochemical Center, Amersham, England, in isotonic sodium chloride was injected into the jugular vein of a Saannen goat in the mid-stage of lactation. Before injection, milk was removed from the udder. Two hours later the goat was milked, using five units of oxytocin injected to facilitate complete collection of the milk. A 60-ml milk sample was used for column chromatography of acid-soluble phosphorus compounds. Methods of extraction, column chromatography, and identification were the same as those described previously. Radioactivity of the acid-soluble phosphorus compounds was estimated by a Geiger-Müller counter.

TABLE 1

Composition of acid-soluble nucleotides of goat milk at different stages of lactation

Fraction	Main components	Percentage of total extinction at 260 mμ				
		Stage of lactation (days from parturition)				
		2	10	30	121 [a]	Artificially [b] induced lactation
A	CMP5′ DPN	2.6	1.7	3.2	4.3	2.1
B	AMP5′	7.6	9.4	12.3	9.3	21.2
C	AMPX	1.0	0.4	1.2	0.6	0.5
D	GMP5′	0.6	1.1	0.5	0.4	0.5
E	Cytidine derivative	....	0.6	....	0.3	0.3
F	UMP5′	7.3	3.8	4.4	4.2	4.9
G,G′	Orotic acid, ADP-X	1.7	7.0	14.2	5.2	4.9
H	GDP-mannose	1.2	9.0	11.0	7.0	6.0
H′	ADP-X, GDP-sugar	0.3	0.4	....	3.0	2.2
I	UDP-acetylglucosamine UDP-acetylgalactosamine	41.1	26.6	17.5	13.8	23.9
J	UDP-glucose UDP-galactose	28.4	23.9	25.5	34.1	20.4
J′	GDP-X	1.2	2.6	....	2.0	1.7
K	GDP, UDP-sugar	1.9	5.0	5.7	7.5	4.9
L	UDP, UDP-uronic acid	4.9	8.0	3.6	8.0	6.5
M	Guanosine derivative	0.2	0.5	0.9	0.3	0.5
Total extinction at 260 mμ		3,182.39	1,846.89	1,712.65	2,271.02	1,841.60

[a] Acid-soluble nucleotides obtained from residual milk by oxytocin injection. This milk had fairly rich ester P content.

[b] After administration of estrogen and progesterone, lactation was initiated by milking. This sample was obtained on the 70th day from the initiation of milking.

RESULTS AND DISCUSSION

The acid-soluble nucleotides of goat colostrum and milk were found to be composed of 13 or 14 peaks. The main components of each fraction were estimated as shown in Table 1. The chromatographic patterns of the acid-soluble nucleotides of goat colostrum and milk are shown in Figures 1, 2, 3, and 4, whereas those of cow's milk are shown in Figures 5 and 6. Table 2 shows the main components of cow's milk. The identification results of nucleotides of cow's and goat's milk were as follows:

Fraction A: In cow's milk, only CMP5′

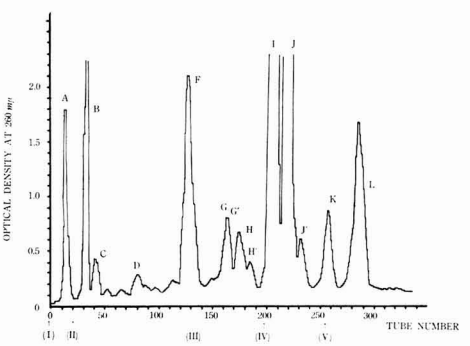

FIG. 1. Chromatography of the acid-soluble nucleotide fraction of goat colostrum, second day of lactation. The optical density at 260 mμ of the eluted fractions is plotted for each chromatographic fraction. The readings of the tubes which were too high to be plotted were B 4.4, I 12.0, and J 11.0. From Figure 1 to Figure 6, the arrows show the changing points of the following eluent solvents: (I) 1 N formic acid, (II) 4 N formic acid, (III) 4 N formic acid + 0.2 M ammonium formate, (IV) 4 N formic acid + 0.4 M ammonium formate, (V) 4 N formic acid + 0.8 M ammonium formate.

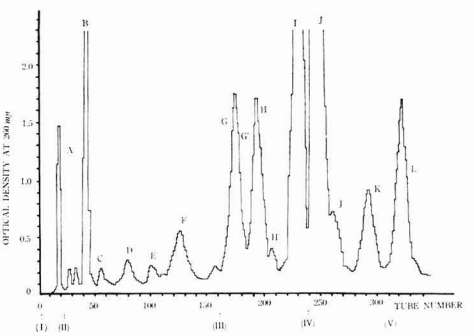

FIG. 2. Chromatography of the acid-soluble nucleotide fraction from 70 ml of goat milk, tenth day of lactation. The readings of the tubes which were too high to be plotted were B 3.2, I 6.5, and J 6.8.

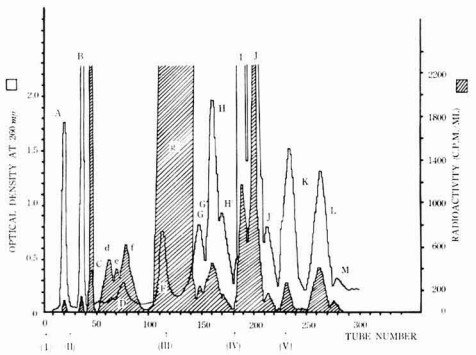

FIG. 3. Chromatography of the acid-soluble phosphorus compounds of goat milk; 2 hr after intravenous administration of three mc P^{32}, milk was obtained by oxytocin injection. The acid-soluble phosphates of 60 ml were chromatographed on a 1.5- by 25-cm column. Ultraviolet absorption at 260 mμ is represented by empty and radioactivity by cross-hatched areas. The readings of the optical density which were too high to be plotted were B 4.4, I 12.0, and J 11.0. Fractions of d, e, and f are hexose monophosphates. Fraction g is inorganic phosphate.

was found. In all goat milk except that taken before parturition, DPN and CMP5′ were obtained. The identification of DPN was confirmed by an increase of light absorption at 340 mμ upon addition to glucose dehydrogenase system, as well as Rf values of several paper-chromatograms. The DPN percentage of the optical density of this fraction varied from 17-50%.

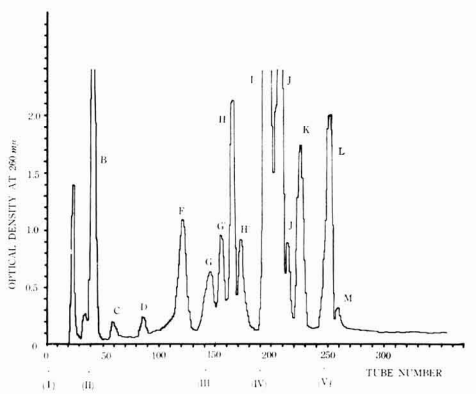

FIG. 4. Chromatography of the acid-soluble nucleotides of milk of a goat whose lactation was induced by the administration of hexestrol and progesterone for 90 days. The sample in this case was obtained on the 70th day after the initiation of milking. The readings of the optical density which were too high to be plotted were B 10.0, H 2.7, I 8.5, and J 8.2.

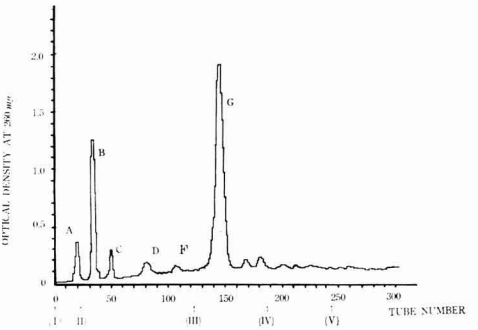

FIG. 5. Chromatography of the acid-soluble nucleotide fraction from 60 ml of cow's milk, 33rd day of lactation. The optical density at 260 mμ of the eluted fractions is plotted for each chromatographic fraction.

Fraction B: Cow's milk of 105th and 121st day contained only uric acid. AMP5′ was the main component of this fraction and uric acid was found only in trace amounts in goat milk.

Fraction C: Both cow and goat milk contained an adenosine nucleotide. The ratio of adenine to ribose to total phosphorus was 1:1:1. The Rf value on paper chromatograms of solvent system I was 0.38, while that of AMP5′ was 0.41. In goat milk, it was found that this adenosine nucleotide had the highest specific activity of P^{32} among nucleotides.

Fraction D: This fraction contained GMP5′ in goat milk.

Fraction E: An unidentified cytidine derivative was found.

Fraction F: This fraction consisted of UMP5′ only in goat milk.

Fraction G,G′: In cow milk, orotic acid was the only constituent of this fraction and it had neither phosphorus nor ribose. This fraction

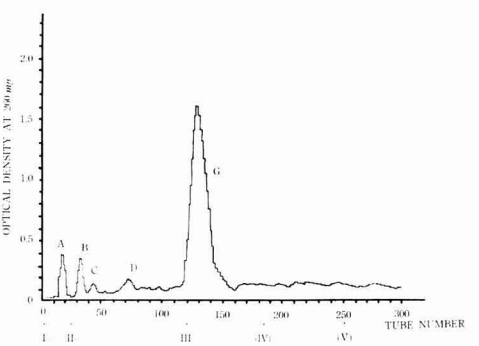

FIG. 6. Chromatography of the acid-soluble nucleotide fraction from 60 ml of cow milk, 105th day of lactation.

TABLE 2

Composition of acid-soluble nucleotides of cow's milk

Fraction	Main components	Percentage of total extinction at 260 mμ			
		Stage of lactation (days from parturition)			
		33	105	121[a]	206[b]
A	CMP5′	3.0	3.9	1.4	3.7
B	Uric acid, AMP5′	25.5	6.6	12.0	15.6
C	AMP-X	3.9	0.2	0.4	1.8
D	GMP5′	3.6	1.7	....	....
E	Cytidine derivative	....	....	0.6	0.3
G	Orotic acid	63.6	87.6	85.6	78.6
Total extinction at 260 mμ		270.69	351.54	425.79	182.65

[a] Alcohol test positive milk with normal acidity.
[b] Jersey cow milk.

of goat milk sometimes separated itself into two peaks, G and G′, as shown in Figure 4, according to the change in formic acid concentration. Fraction G contained orotic acid and G′ consisted of ADP-X, besides orotic acid.

Fraction H: GDP-mannose (Rf value of solvent system 3 was 0.22) was the main component of this fraction.

Fraction H′: This fraction consisted of a GDP-sugar and an unidentified adenosine derivative.

Fraction I: The bulk of this large fraction contained a mixture of UDP-acetylglucosamine and UDP-acetylgalactosamine. The solution containing these substances, after removing ammonium formate and formic acid, was concentrated in vacuo. After hydrolyzing with 0.01 N HCl for 30 min at 100 C, the interfering substances were removed with anion and cation resins, and they were tested by paper-chromatography. They gave a positive reaction with Elson-Morgan reagent without acetylacetone. It was also found that these substances changed to arabinose and lyxose, respectively, after ninhydrin degradation at 100 C for 30 min of the hydrolyzed and deacetylated substances.

Fraction J: This fraction consisted of a mixture of UDP-glucose and UDP-galactose. When the acid-soluble phosphates precipitated from trichloroacetic acid extract with 25% barium acetate and ethanol at pH 8.2 were column-chromatographed, this fraction almost disappeared. On the other hand, UMP5′, which corresponds to the amount of UDP-glucose and UDP-galactose, increased, although UDP-acetylhexosamine and other sugar-containing nucleotides did not show any remarkable change (unpublished data). Paladini and Leloir (17) observed that UDP-glucose is alkali-labile.

Fraction J′: Besides UDP-glucose and UDP-galactose, an unidentified GDP-x was found.

Fraction K: GDP and an unidentified UDP-sugar (aniline-phthalic acid reagent positive) were found.

Fraction L: This fraction contained UDP and UDP-uronic acid. Uronic acid was detected by Dishe's carbazole reaction. The concentrated elution from paper chromatograms of this fraction showed positive pink color reaction when heated with H_2SO_4 for 20 min in a boiling water bath and mixed with 0.1% carbazole.

Fraction M: This fraction contained an unidentified guanosine and an adenosine derivative.

In goat milk, total acid-soluble nucleotides, UDP-acetylhexosamine, and UDP-hexose contents are highest in colostrum, being 330, 132, and 92 μM per 100 ml, respectively. With the advance of lactation, the content of total acid-soluble nucleotides decreased, whereas the content of adenosine nucleotides and guanosine nucleotides increased (Table 1). The lacteal secretion of 11 days before parturition contained only trace amounts of nucleotides. Total extinction at 260 mμ per 100 ml was 59.16. The acid-soluble nucleotides of milk of the goat whose lactation was induced by the administration of estrogen and progesterone were also detected. Three samples from goats that produced at about one liter of milk per day were examined. In every case, the chromatographic patterns of nucleotides were the same as those of normal lactation. One example is shown in Figure 4.

On the other hand, chromatographic patterns of acid-soluble nucleotides of normal cow milk are considerably different from those of goat milk (Figures 5 and 6). Orotic acid is the main component of the acid-soluble nucleotides of cow milk. Milk of the 33rd, 105th, and 121st days of parturition contain 40, 73, and

86 μM of orotic acid per 100 ml, respectively. There are no remarkable differences in the chromatographic patterns of nucleotides between Holstein milk and Jersey milk. Fraction B of the 121st day milk contained uric acid only. Minor amounts of AMP5′, an unidentified AMP derivative, GMP5′, and relatively large amounts of CMP5′ were also found in the acid-soluble fraction of cow milk.

These data confirm our earlier results that uridine diphosphate nucleotides and guanosine diphosphate nucleotides are detected only in cow colostrum. In milk taken during a later stage of lactation, only inorganic P and hexose phosphates and no diphosphate nucleotide were detected on the paper-chromatogram (12). This agrees with the data of Deutsch and Mattsson (7), who have detected orotic acid and monophosphorylated nucleotides in whole and skimmed cow milk. Hallanger et al. (9) also found orotic acid in cow milk, goat milk, and ewe milk by microbiological assay. Recently, Denamur et al. (4-6) reported that ewe milk contained large quantities of acid-soluble nucleotides, including UDP-acetylhexosamine, GDP-fucose, and UDP-hexose. They found that the percentage content of UDP-hexose to the total acid-soluble nucleotides was highest in colostrum immediately after parturition, and decreased gradually with advancing lactation.

In our data for goat milk, the variation of UDP compounds was rather different from that of ewe milk. With goat milk the percentage content of UDP-acetylhexosamine compared with total acid-soluble nucleotides was highest in colostrum and decreased gradually with the advance of lactation. On the other hand, the percentage content of UDP-hexose to total acid-soluble nucleotides rather increased up to the middle stage of lactation.

There is noteworthy species variation in the composition of acid-soluble nucleotides in milk even among ruminants, cow milk showing a unique composition pattern of acid-soluble nucleotides in comparison with goat and ewe milk. UDP-hexose, UDP-acetylhexosamine, or GDP-hexose is not specific to the milk of goat or sheep, but is also found in human milk or mare milk (unpublished data).

Then, where do the acid-soluble nucleotides come from and what do they imply?

Colostrum and milk usually contain leucocytes and degenerated secretory cells (3). These cells contain DNA and RNA (16). One might assume decomposition of these nucleic acids by ribonuclease or deoxyribonuclease during the process of milk secretion and accumulation in the udder. Our recent study indicated that although lacteal secretions of late pregnancy were fairly high in nucleic acid P, the amount of ester P including nucleotides was small (12). Milk from cows suffering from mastitis generally has a larger number of leucocytes and contains more nucleic acid than normal milk. Milk from the cow with severe mastitis did not show any increase of ester P content (12). It is probable, therefore, that the major part of acid-soluble nucleotides does not come from the decomposition of nucleic acid.

Blood serum contains a trace amount of hexose phosphate (8), but there has been no information with regard to blood serum nucleotides. On the other hand, large amounts of acid-soluble nucleotides were found in the mammary gland (6, 14, 20). In particular, the UDP-glucose content of the mammary gland is three times that of the liver (20). High UDP-glucose and UDP-galactose contents of goat milk and ewe milk are recognized. ATP, UTP, and GTP found in the mammary gland are present in very small quantities, if not undetectable, in milk. The results of the isotope experiment with a lactating goat demonstrate the active incorporation of P^{32} into the acid-soluble nucleotides, inorganic phosphate, and hexose phosphates (Figure 3). The P^{32}

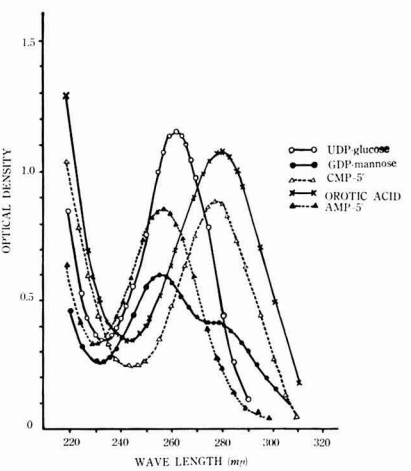

FIG. 7. Ultraviolet absorption spectra of acid-soluble nucleotides of milk at pH 2. After removing eluents, each fraction obtained from ion-exchange column chromatography was chromatographed on paper. The spots of nucleotides were cut out and eluted with 0.01 N HCl. The elution was washed with ether to remove interfering substances and the spectra were estimated. At pH 2, the maximum absorption spectra of each nucleotide are as follows: UDP-glucose 262 mμ; GDP-mannose 256 mμ; CMP5′ 278 mμ; AMP5′ 258 mμ; orotic acid 280 mμ. These values agree well with those of the authentic samples.

incorporation into the acid-soluble nucleotides is 4.7 times that of hexose phosphates; Fraction I of UDP-glucose and UDP-galactose, Fraction J of UDP-acetylglucosamine and UDP-acetylgalactosamine, and Fraction C of an adenosine nucleotide showed especially high P^{32} uptake. An adenosine nucleotide and a small amount of inorganic phosphate, probably derived from decomposition of the former, were detected on the paper chromatograms of Fraction C. Although its content is small, this is a noteworthy nucleotide in milk because of high specific activity.

Therefore, it seems that the acid-soluble nucleotides of milk are secreted selectively from epithelial cells of the mammary gland and are active substances in the mammary gland metabolism. The role of UDP-acetylhexosamine or GDP-hexose in milk synthesis has not been clarified as that of UDP-glucose in lactose synthesis was, but it is of interest that several hexosamines are recognized in the glycoproteins of milk fat globule membranes (21). Orotic acid is an intermediate in uridine nucleotide synthesis and is known as an essential growth factor of a lactobacillus bulgaricus (9). Orotic acid metabolism in the mammary gland remains to be investigated.

Further experiments are continuing on the acid-soluble nucleotides of other milks.

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COMPOSITION OF MAMMARY LYMPH IN LACTATING GOATS[1, 2]

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SUMMARY

Eleven supramammary lymph ducts in eight healthy, lactating, nonpregnant goats were cannulated under anesthesia. Lymph flowed externally from freely moving, unanesthetized animals for an average period of 14 days. Jugular blood and mammary lymph from the same goat were collected simultaneously and were analyzed for protein, nonprotein nitrogen, glucose, lactose, sodium, potassium, calcium, magnesium, chloride, bicarbonate, and pH. Results showed no change in lymph composition with variations in rate of milk secretion and, except for protein concentration, no change in composition during different rates of lymph flow. Higher lymph flows were associated with decreases in lymph protein concentrations. NPN and glucose levels in lymph were not significantly different from plasma levels. Lactose was not apparent in either fluid. The electrolyte composition of lymph was similar to that of plasma when allowance was made for Donnan membrane equilibrium adjustments. Composition of mammary lymph with regard to the substances investigated appears to result from diffusion of plasma from capillaries and with no back diffusion from mammary alveoli or ducts.

Investigations of lymph composition from certain organs show that the activity of the cells in these organs contributes to the formation of its lymph. In the liver, protein is added to lymph (16), and in the kidney, renal lymph reflects renal tubular reabsorption (8, 10). It would be of interest to know if the cells of the mammary gland, in the course of secreting milk, also contribute to the formation of mammary lymph.

The gross structure of the lymphatic system in mammary glands of man and other mammals is well described, and information about the composition and flow of mammary lymph is increasing. Samples of bovine lymph and serum collected at the time of slaughter have been analyzed for composition by Heyndrickx and Peeters in Belgium. They used lactating cows and analyzed for electrolytes (6), sugars, ketones, and organic acids (7), and lipids and proteins (5). By cannulating supramammary lymph ducts, Linzell (11), in England, analyzed composition of mammary lymph in four unanesthetized, lactating goats, contrasting this with composition of serum from other goats in the same herd. More recently, Lascelles and Morris (9), in Australia, also using a cannulation technique, have determined composition of mammary lymph and plasma in both dry and lactating, unanesthetized Merino sheep. Results from these three laboratories, widely separated as to location and each using a different ruminant, show agreement on most of the substances analyzed in common, but disagreement on relative concentrations of sodium, potassium, and glucose. The following experiments on lactating goats are reported in order to add more information of the composition of mammary lymph and to describe further the relationship, if any, between production of mammary lymph and secretion of milk.

Method. Eleven chronic cannulations of a supramammary lymph duct were done in eight healthy, lactating, nonpregnant goats. In three of the goats a duct was cannulated first on one side and later on the other. The experiments were conducted over a period of 1.5 yr and represent a wide range of stages of lactation.

With the goat anesthetized with sodium pentobarbitol, one of the supramammary lymph ducts lying along the external pudendal artery between the supramammary lymph node and the inguinal ring was exposed. Polyethylene tubing, varying in internal diameter from .030 to .047 inches (no. 60 to no. 190), according to the size of the duct, was inserted approximately

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3 cm into the duct and tied in place. The tube was brought out through the suspensory ligament, so that it was directed in a line with the lymph duct. It was brought through the skin on either the caudal or cranial surface of the udder close to the base of the gland and the tip hung six to eight inches below the point of exit from the skin. From the moment of cannulation, lymph dripped freely by gravity from the tube and continued for many days. Clotting of lymph was a problem only if a good flow was not maintained during the first few hours after surgery. Following cannulation the goat was returned to her 5 ft[2] boxstall, and within 24 hr she was eating, drinking, and milking normally. Except for wearing a broad rigid collar, to prevent nibbling at the cannula, she was permitted to move freely in her stall during the entire period of cannular lymph flow, even during collection of samples.

Lymph was collected for composition analysis by hanging a 1-oz plastic bottle on the end of the cannula until it was filled. The bottle was partially open at the top and contained a few granules of dry heparin. Filling the bottle required from 20 to 60 min, depending on the rate of lymph flow. For analyses of CO_2 content and pH, lymph was collected with the goat standing in a stock. A 4-ft piece of polyethylene tubing, with an internal diameter of .125 inches (no. 350), was coupled tightly to the cannula. It was held in place by attachment to a harness worn by the goat. When lymph had filled the large tube and was dripping from it, it was clamped off 1 ft from the distal end. The sample was drawn into an airtight syringe from the proximal 3 ft of the tube which had had no contact with air.

Plasma samples were obtained from blood drawn into heparinized syringes from a jugular vein at the beginning of each lymph collection period. For analyses of plasma pH and CO_2 content, airtight syringes were used, and these were centrifuged in the manner described by Davenport (3).

Lymph and serum were collected three to five times during the first week after cannulation and two to three times during the second week. Both fluids were analyzed in duplicate from duplicate samples. Concentrations of the following substances were measured. Nonprotein nitrogen, and plasma and lymph protein concentrations in half of the samples, were measured by micro-Kjeldahl procedures (4). The remainder of the protein concentrations were measured with an Abbé 3-L Refractometer,[3] a quantitative relationship having been established in this laboratory between micro-Kjeldahl analyses and refractive indices for protein concentration in goat serum and lymph. Glucose and lactose were determined qualitatively by paper chromatography (15), an ethyl acetate-pyridine-water solvent being used for running the serum and lymph samples on to the paper and alkaline silver nitrate in acetone used to develop the chromatogram. Sodium and potassium from the first six experiments were measured with a Beckman DU Flame Photometer,[4] while a Patwin Flame Photometer[5] was used for the last five experiments. Calcium was determined by the method of Clark and Collip (1), chloride with an Aminco-Cotlove Chloride Titrator[6] (2), and magnesium by the titan yellow method (13). Total carbon dioxide and bicarbonate were measured with a Van Slyke manometric apparatus (14), pH was measured with a Beckman Model G pH meter[4] and a Beckman Constant Temperature Blood Type glass electrode[4] equilibrated at 40 C in a constant temperature bath.

Results. The duration of lymph flow averaged 14 days for all experiments and ranged from three to 23 days. Throughout each flow period, the milk secretion and general health of the goat did not appear to be impaired, with the exception of one goat in which both plasma and lymph protein concentration fell to very low values. Nine experiments ended by having the cannula come out in some unknown manner during the night. In the other two experiments, lymph simply ceased to flow through the tube. Regardless of how the lymph flow stopped, and even though the lymph duct remained tied off, there was never any swelling or externally observable change in the appearance of the udder or in the milk production.

Lymph flows were measured in four goats. Although they varied considerably with many factors, a basal flow could be obtained after each animal had been standing quietly for at least one-half hour. Under these conditions the rate was 15 ± 5 ml per hour on the third postoperative day. If the goat moved about, the flow could increase to 150 ml per hour. Concentrations of substances measured in the lymph were not affected by these wide variations in the rate of lymph flow, with the exception of

[2] A preliminary report of this investigation appeared in Federation Proc., 19: 240. 1960.

[3] Bausch and Lomb, Rochester, N. Y.

[4] Beckman Instruments, Inc., Pasadena, Calif.

[5] Patwin Instruments, Waterbury, Conn.

[6] American Instrument Co., Silver Spring, Md.

protein concentration, which decreased slightly at very high flows.

There was a gradual daily increase in basal rate of lymph flow following cannulation. In spite of this, and in spite of the fact that lymph draining from the cannula was lost from the goat, all of the substances determined, with the exception of protein, showed no significant change in concentration throughout the entire post-cannulation period. Protein, however, in both plasma and lymph decreased gradually with time. Clotting time was not measured, but it appeared to lengthen with the fall in lymph protein concentration.

The cannulated goats varied in milk production from 30 to 2,700 ml per day. Although there may have been a direct relation between lymph flow and rates of milk secretion, there was none between the composition of serum and mammary lymph and the amount of milk being secreted daily.

Values obtained during the first week have been averaged and are compiled in Table 1. The difference in composition between plasma and lymph is evident. Protein concentration in lymph is approximately half of that in plasma. The other substances showed no significant variation between plasma and lymph. However, potassium and chloride were always higher in lymph than in plasma, and calcium and magnesium were always slightly lower in lymph, while nonprotein nitrogen, sodium, bicarbonate, and pH showed no consistent difference. According to the chromatogram, glucose appeared to be present in lymph in about the same quantity as in plasma, and lactose could not be detected in either lymph or plasma.

Discussion. Some differences exist between the electrolyte values which Heyndrickx (6) reported in his studies on lymph from slaughtered cows and those reported here. In eight lymph samples he found sodium to be slightly lower than in serum, potassium to be much higher, magnesium to be higher, rather than lower, and bicarbonate to be lower than in serum. He found lymph glucose to be considerably less than plasma glucose, and he also found small amounts of lactose in both plasma and lymph (7). It may be that mammary lymph from the bovine is different from that of the caprine, but collection of lymph from slaughterhouse cows, which may have had greatly distended udders, as well as immediate post-mortem changes, could explain these differences. Linzell (11), on the other hand, using only four unanesthetized goats and a can-

TABLE 1

Average values of substances in jugular plasma and supramammary lymph of lactating goats, collected during first week after lymph cannulation

	No. cannulations	Jugular plasma	Mammary lymph
Protein *(g/100 ml)*	9	6.6 ± .5[a] (5.8–7.5)	3.3 ± .6[a] (2.6–4.6)
NPN *(mg/100 ml)*	7	28 ± 4 (20–30)	24 ± 6 (16–33)
Glucose	6	+	+
Lactose	6	0	0
Sodium *(meq/liter)*	9	146 ± 3 (143–151)	146 ± 4 (142–152)
Potassium *(meq/liter)*	9	4.5 ± .3 (4.0–5.2)	4.9 ± .4 (4.0–6.0)
Calcium *(meq/liter)*	11	4.6 ± .2 (4.1–5.1)	4.1 ± .4 (3.1–4.8)
Magnesium *(meq/liter)*	2	1.9 ± .2 (1.7–2.1)	1.4 ± .1 (1.2–1.5)
Chloride *(meq/liter)*	11	107 ± 4 (97–115)	111 ± 5 (100–117)
Bicarbonate *(meq/liter)*	4	25 ± 1 (24–26)	26 ± 1 (25–27)
pH	4	7.45 ± .03 (7.42–7.50)	7.47 ± .02 (7.45–7.50)

[a] Standard deviation.

Each cannulation consists of 3-5 plasma, or lymph, collections for composition analyses.

nulation method similar to the one described here, found values for mammary lymph composition which agree well with those reported here. The results of measurements made by Lascelles and Morris (9) on lymph from mammary glands of sheep is in agreement with this author's results for all substances except potassium and sodium. They found these electrolytes to be lower in lymph than in plasma in both dry and lactating sheep. However, with no statistical treatment of their data, it is difficult to assess the significance of their composition differences.

In general, mammary lymph composition in terms of cation-anion equivalence is similar to plasma and quite unlike milk. This is shown in Figure 1, where electrolyte concentration values for these fluids have been arranged in a Gamble-type chart. The primary difference between mammary lymph and plasma is in the protein fraction, the concentration being considerably less in the former. The slight variations in concentration of other substances, such as decrease in calcium and magnesium and increase in chloride, appear to be the result of diffusion forces probably in accordance with the Donnan equilibrium. On the other hand, the difference between the electrolyte composition of mammary lymph and milk is striking. The chemical structure of milk, values for which were taken from Macy (12), is more like that of an intracellular fluid. There does not appear to be any back diffusion of those minerals, which are present in milk in such relatively large quantities, from milk into lymph. As a result, it can be stated that, except for possibly supplying organic materials such as glucose (11) and fatty acids (5, 9) to be used by the cells in the production of milk, mammary lymph, whether from an active or inactive gland, appears to be a filtrate of plasma.

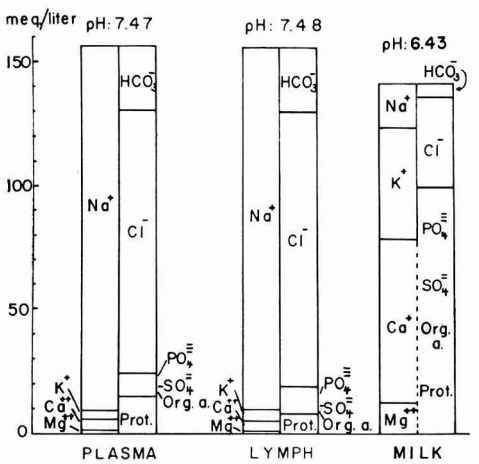

FIG. 1. Chemical equivalence of cations and anions in goat plasma, lymph, and milk. Base equivalence of protein was calculated by using the Van Slyke factor 2.43. Values for substances in goat milk were taken from Macy (12). The dotted line separating calcium and magnesium from phosphate, sulfate, organic acid, and protein anions is intended to indicate that to some extent these substances exist in milk as undissociated compounds.

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NUTRITIONAL EVALUATION OF PASTURES WITH DAIRY CATTLE [1]

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SUMMARY

Three digestion trials were conducted during the summer months at three different periods of permanent pasture (oats, rye grass, clovers, and volunteer grasses) growth from May through August, 1959. A combination of internal (lignin) and external (Cr_2O_3) indicators was used to determine digestibility and intake of grazed herbage as the only source of forage in the total ration of milking cows. Forage samples were collected regularly throughout the experimental period. Milk weights, pasture scores (quantity and quality), and ambient temperatures were recorded daily.

Milk production adjusted for expected decline in lactation was significantly higher ($P < .05$) in Trial I than in Trials II and III. The average forage digestion coefficients of 68.27% for dry matter, 68.41% for crude protein, 63.31% for crude fiber, and 77.66% for NFE were significantly higher ($P < .01$) in Trial I than those of 51.59, 39.15, 55.04, and 62.01% in Trial III, and 41.22, 31.18, 49.06, and 51.13% in Trial II, respectively. The average forage digestion coefficients for dry matter and nitrogen-free extract in Trial III were significantly higher ($P < .01$) than those in Trial II. The average herbage dry matter intake adjusted for body weight of 27.62 lb in Trial I was significantly higher ($P < .01$) than the average intakes of 13.36 and 14.19 lb in Trials II and III, respectively. Cows in Trial I gained 25.5 lb per cow for the 14-day trial period, which was significantly different ($P < .01$) from the losses of 32.1 and 20.6 lb per cow in Trials II and III, respectively.

Regression analyses indicated that pasture quality score was the only significant variable in predicting milk production among the variables studied. Thus, a simple method of pasture evaluation appears to be of benefit in determining when to commence supplemental summer forage feeding to lactating cows grazing permanent pasture as the sole source of forage.

The introduction of indicator methods for determining herbage intake and digestibility has been one of the important advances in nutrition research in the past few years. This has facilitated research on herbage evaluation with grazing animals. Pasture furnishes approximately 50% of the feed for dairy cattle in the Southern and Southwestern states, compared to approximately 25% in the Northwestern and North Atlantic states (7). Consequently, there is a great need for the evaluation of pastures and their contribution to the total ration of dairy cattle.

This study was undertaken with lactating cows grazing permanent pastures (a) to evaluate permanent pastures in terms of chemical composition, digestibility, animal performances, and visual inspection, and (b) to analyze these measures of pasture quality to determine the reliability or accuracy of each measure, or both.

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[1] The data are from a thesis presented by the senior author to the graduate faculty of Louisiana State University in partial fulfillment of the requirements for the Ph.D. degree.

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EXPERIMENTAL PROCEDURE

Pastures. Nine permanent pastures with the same type of herbage, varying in size from four to 30 acres and grazed by the Iberia Livestock Experiment Station dairy herd, were available for this study. A five-year pasture

rotation system is carried out at this station, which consists of first-year summer seeding of Alyce clover for hay, followed by oats, rye grass, and clovers in the fall, with second, third, and fourth year reseeding clovers, volunteer grasses, and sod-seeded oats or rye grass.

A pasture scoring system, considering quantity and quality of herbage and based on the score card of Hodgson and Shepherd (8), was used. The pasture quantity scores (surplus = 21-30 points, adequate = 11-20 points, or deficient = 0-10 points) and the pasture quality scores (excellent = 31-40 points, good = 21-30 points, fair = 11-20 points, or poor = 0-10 points) were obtained daily on the pasture currently occupied by the milking herd by the same person throughout the study. The cows were followed during grazing and a hand-plucked sample as representative as possible to that the cows were actually grazing was collected.

Animals. The milking herd consisted of Jerseys, Holsteins, and Sindhi-crossbreds. These animals, including the experimental animals, received a concentrate ration and pasture as the sole source of forage during the 204-day experimental period (April 1–October 21, 1959). The concentrate ration (16% dairy feed) was fed at the rate of 1 lb for each 4 lb of milk produced daily by the Holsteins and 1 lb for each 3 lb of milk produced daily by the Jerseys. Rotation of the herd from field to field varied from periods of one day to 2 wk. In many instances, the cows had access to two, three, or even four fields at the same time. Estimates of quantity of forage and physical condition of the ground were used as main criteria for rotations. Milk weights and ambient temperatures were recorded daily.

Ten cows from the milking herd with lactations past their milk production peak were chosen for calculating milk production data. The expected lactation curves were calculated by using the persistency figure, 0.9144, of Corley (4) for each cow starting at her peak production and calculating the decline for each 30-day period. Differences between the actual milk production and the expected milk production were used as the measures of milk production, to avoid a correlation between milk production and forage quality which could occur because of a normal decline in forage quality as the pasture period advanced in the summer and a normal decline in lactation.

Correlation coefficients were computed using Y's (actual minus expected milk production first day, actual minus expected milk production next day, actual minus expected milk production two days later, and actual minus expected milk production three days later) and all the X's (pasture quantity score, pasture quality score, mean daily temperature—average of maximum and minimum, per cent lignin in herbage, per cent crude protein in herbage, per cent ether extract in herbage, per cent nitrogen-free extract in herbage, per cent fiber in herbage, and per cent ash in herbage), to determine the proper Y to use in accounting for lag in milk production. Data for any days in which an X value was missing were discarded. Multiple regression analyses were computed using $Y1$, actual minus expected milk production first day, and different combinations of the independent variables.

Digestion trials. Three digestion trials (May 2-16, June 23-July 7, and August 17-31) were conducted during the experimental period on the same field, as it was the largest unit and permitted completion of the trial without rotation of the herd. This pasture was in the fourth year of the five-year rotation system and appeared to be about average to those available at the time the trials were conducted. These trials were conducted at three stages in which it was felt that there was a change in quality of herbage from the previous period. Each trial consisted of a seven-day preliminary period and a seven-day fecal collection period. Eight milking cows and two dry cows were used as the experimental groups in digestion Trials I and II, and seven milking cows and two dry cows in digestion Trial III. The dry cows were not fed any grain. Chromium oxide was fed to animals at the rate of 20 g per day (10 g at each milking time) as an external indicator to measure fecal output, and lignin was used as an internal indicator to determine digestibility. Fecal samples were collected from the experimental animals at 6 AM and 4 PM for the seven days of each collection period. A seven-day total collection of feces was performed on three milking cows and two dry cows, to check recovery of chromium oxide. A slight modification of the collection harness and bag of Gorski and co-workers (6) was used with grazing cows. Data in the paper are confined to the cows used in the three digestion trials and the ten cows used for measuring the deviation in milk production. Therefore, all other cows in the herd were incidental. However, digestion trial cows and test cows were handled as a part of the entire milking herd.

The proximate analyses of feed and fecal samples were determined according to the procedures recommended by the Association of Official Agricultural Chemists (2). The lignin

content was determined by the method outlined by Achacoso (1). Chromium oxide in feces was determined by a method outlined by Austin and Bonner (3). Total ration digestion coefficients were calculated by an indirect method essentially similar to the method of McCullough (10). To determine per cent digestibility of the forage, digestion coefficients for the concentrate ration were calculated according to the formulas of Schneider et al. (17) for the organic nutrients. The digestibility of available ash was considered to be 50%, based on the report of the National Research Council (16). From the above information, it was possible to correct for the portion of the fecal material voided due to the concentrate, because it is known how much concentrate each cow had consumed daily. The amount of fecal dry matter voided was determined by the chromium oxide content. The amount of lignin in the feces due to the concentrate feed was also subtracted from the total amount of lignin found in the feces. From the lignin analysis of herbage consumed on the dry matter basis, and the total amount of lignin left in the feces after correction for the amount contributed by the concentrate, it was possible to calculate herbage dry matter consumed. Then, by knowing herbage dry matter consumed and its proximate analysis, along with the amount of dry matter, crude protein, crude fiber, nitrogen-free extract, and ether-extract in the feces due to herbage consumed (after subtracting that contributed by the concentrate), digestion coefficients were calculated.

Statistical analyses. The analyses of variance and covariance were conducted according to the methods of Snedecor (18), accounting for unequal subsample numbers where applicable. Duncan's multiple range test (5), as modified by Kramer (9), for unequal numbers per treatment was employed to test significance between the digestion trial group means.

RESULTS AND DISCUSSION

Forage data. The herbage in Trial 1 (May 2 to 16) consisted mainly of oats (*Avena sativa*) and rye grass (*Lolium multiflorum*) in the boot stage, a small amount of hop clover (*Trifolium dubium*), small amounts of California burclover (*Medicago hispida*) and spotted burclover (*Medicago arabica*), a good growth of S-1 clover (*Trifolium repens*), with Bermuda grass (*Cynodon dactylon*) and Dallis grass (*Paspalum dilatatum*) just starting out.

The herbage in Trial II (June 23 to July 7) was inferior to that of Trial I, because the clovers had played out and a short dry spell occurred in the latter part of June. The species were mainly Bermuda grass and Dallis grass, about five to seven inches high. There was a small amount of S-1 clover left in a few spots and some Vasey grass (*Paspalum urvellei*) along the ditch banks.

The herbage in Trial III (August 17 to 31) was of somewhat better appearance than in Trial II, because of July and August rains. The dominant species were Bermuda grass, Dallis grass, a small amount of Vasey grass, and crab grass (*Digitaria sanguinalis*). Many of the grasses were seeded at the time.

The herbage consumed in Trial I was higher in crude protein and lower in crude fiber and lignin than that in Trials II and III, owing to its higher content of clovers in proportion to grasses (Table 1). The proximal content of herbage consumed in Trials II and III did not differ to any extent.

Milk production. Actual and expected milk production of the ten cows during the three digestion trials is given in Table 2. Milk production adjusted for expected decline in lactation was significantly higher ($P < .05$) in Trial I than in Trials II and III. The higher ad-

TABLE 1

Lignin and proximate analyses of herbage consumed by cows in the digestion trial periods (dry basis)

Herbage constituents	Trial I (May 2-16)	Trial II (June 23-July 7)	Trial III (August 17-31)
	(%)		
Lignin [a]	3.75	5.58	4.94
Crude protein	13.96	10.89	10.43
Crude fiber	23.63	26.71	27.19
Nitrogen-free extract	51.00	50.27	50.75
Ether extract	2.20	2.79	2.44
Ash	9.21	9.34	9.19

[a] Lignin is not included in the 100% make-up of proximate analysis.

TABLE 2

Actual and expected milk production of ten cows during the three digestion trials

Cow no.	Stage of lactation [a]	Trial I (May 2-16)		Trial II (June 23-July 7)		Trial III (August 17-31)	
		Average daily milk production per cow					
		Actual	Expected	Actual	Expected	Actual	Expected
	(day)	*(lb)*					
J-310 [b]	78-282	20.8	28.2	15.9	24.1	12.0	20.3
J-339	39-243	30.0	29.9	23.0	25.6	21.5	21.7
B-2695	32-236	28.9	41.4	27.3	35.5	23.4	30.0
H-201	69-273	26.4	26.5	19.4	22.6	17.3	19.1
H-717	101-305	29.2	29.4	20.5	25.2	18.3	21.4
H-3466	111-315	32.0	32.5	24.0	27.8	15.5	23.6
H-3474	39-243	40.6	41.9	27.5	35.9	22.5	30.4
S-66	135-339	30.4	27.6	25.5	23.5	20.9	20.1
SX-58	66-270	33.9	41.8	29.8	35.9	26.2	30.2
SX-250	109-313	33.7	29.9	29.1	25.5	23.2	21.6
Avg	78-282	31.6	32.9	24.2	28.2	20.1	23.9
Average milk production adjusted for expected decline in lactation			28.7 [c]		24.3		22.9

[a] Stage of lactation of cows in the 204-day experiment (April 1-October 21).
[b] H signifies Holstein, J or B signifies Jersey, and S or SX signifies Sindhi-crossbreds.
[c] Significantly different at ($P < .05$).

justed milk production in Trial I appeared to be due to the higher-quality forage available.

Digestion trials. Total forage mean digestion coefficients for the three digestion trials are presented in Table 3. The average forage digestion coefficients of 68.27 for dry matter, 68.41 for crude protein, 63.31 for crude fiber, and 77.66% for NFE were significantly higher ($P < .01$) in Trial I than those of 51.59, 39.15, 55.04, and 62.01% in Trial III, and 41.22, 31.18, 49.06, and 51.13% in Trial II, respectively. The values for dry matter and NFE in Trial III were significantly higher ($P < .01$) than those in Trial II. Digestion coefficients for ether-extract were variable and of doubtful value. These results indicate that succulent herbage with a high content of clovers was more highly digested by milking cows. Dry and coarse herbage consumed during the hot part of the summer under the condition of this study was low in digestibility.

The average dry matter intake adjusted for body weight, of 27.62 lb in Trial I, calculated from the combined lignin and chromium oxide indicators, was significantly higher ($P < .01$) than the average intakes of 13.36 and 14.19 lb for the animals in Trials II and III, respectively (Table 4). There appeared to be a relationship between dry matter digestibility and dry matter consumption, which has also been reported by other workers (11-14). The cows in Trial I gained 25.5 lb per cow for the 14-day period, which was significantly different ($P < .01$) than the losses of 32.1 and 20.6 lb per cow for Trials II and III, respectively (Table 4). These figures were in line with all other

TABLE 3

Forage digestion coefficients

Trial [a]	Dry matter	Crude protein	Crude fiber	NFE	Ether extract
	(%)				
I [b]	68.27 [c]	68.41 [c]	63.31 [c]	77.66 [c]	52.55 [c]
II	41.22 [e]	31.18 [d]	49.06 [d]	51.13 [e]	19.88 [d]
III	51.59 [d]	39.15 [d]	55.04 [d]	62.01 [d]	56.02 [c]

[a] Trial I was conducted May 2-16, Trial II June 23-July 7, and Trial III August 17-31.
[b] Ten animals (eight milking cows and two dry cows) were used in Trials I and II, whereas only nine animals (seven milking cows and two dry cows) were used in Trial III.
[c, d, e] Means with different superscripts are significantly different at $P < .01$.

TABLE 4

Herbage dry matter intake and body weight changes by trials

	Trial		
	I	II	III
	(lb)		
Avg dry matter intake adjusted for body weight	27.62 [a]	13.36 [b]	14.19 [b]
Avg body weight change	25.5 [a]	−32.5 [b]	−20.6 [b]

[a, b] Means with different superscripts are significantly different at $P < .01$.

results obtained, emphasizing the greater nutritive value of herbage in the early part of the growing season as reflected in body weights.

Total fecal collections on five cows showed an average chromium oxide recovery of 99.5%, with a range of 86.8 to 109.1%. The chromium oxide as an external indicator was relatively accurate in predicting fecal output.

Correlation data. Correlation coefficients between all X's and Y's are shown in Table 5. Actual minus expected milk production first day was as highly correlated with the X's as any of the other Y's. This was contrary to the lag one might expect; however, changes in herbage quality and summer temperatures were gradual and abrupt changes in milk production from day to day due to these independent variables were negligible. Significant correlations existed among some of the independent variables (Table 5). Pasture quality score was highly correlated ($r = 0.60$) with pasture quantity score, indicating that pasture quality score accounted for 36.0% of the variability of pasture quantity score, and vice versa. Pasture quality score was inversely correlated ($r = -0.49$) with per cent lignin in herbage, indicating that herbage scoring high for quality was lower in lignin. Pasture quality score was highly correlated ($r = 0.61$) with per cent crude protein in herbage, indicating that the quality score was a good measure of the protein content of herbage. This correlation was expected, because the quality score was influenced by the amount of clovers present in the herbage and the presence of clovers increased the amount of crude protein.

Multiple regression data. Percentage ether extract in herbage and percentage ash in herbage were eliminated before running the multiple regression analyses. These two independent variables were not correlated with any of the Y's and this would have a very minor effect if included (Table 5). For the data to fit the IBM multiple regression program designed for

TABLE 5

Simple correlation coefficients between all independent variables and the four measures of milk production

	X1 [a]	X2	X3	X4	X5	X6	X7	X8	X9
Y1 [b]	0.19 [c]	0.31 [c]	−0.12 [c]	−0.17 [c]	0.21 [c]	0.03	−0.11 [c]	−0.12 [c]	−0.05
Y2	0.18 [c]	0.31 [c]	−0.13 [c]	−0.16 [c]	0.21 [c]	0.03	−0.10 [c]	−0.13 [c]	−0.05
Y3	0.17 [c]	0.29 [c]	−0.13 [c]	−0.17 [c]	0.19 [c]	0.04	−0.10 [c]	−0.11 [c]	−0.05
Y4	0.16 [c]	0.27 [c]	−0.12 [c]	−0.15 [c]	0.21 [c]	0.04	−0.11 [c]	−0.12 [c]	−0.01
X1	1.00	0.60 [c]	−0.04	−0.22 [c]	0.25 [c]	0.25 [c]	−0.24 [c]	−0.05	−0.14 [c]
X2		1.00	−0.24 [c]	−0.49 [c]	0.61 [c]	0.15 [c]	−0.30 [c]	−0.42 [c]	−0.05
X3			1.00	0.23 [c]	−0.41 [c]	0.34 [c]	0.17 [c]	0.25 [c]	−0.01
X4				1.00	−0.33 [c]	0.01	0.17 [c]	0.25 [c]	−0.11 [c]
X5					1.00	−0.02	−0.62 [c]	−0.59 [c]	0.22 [c]
X6						1.00	−0.31 [c]	0.05	0.24 [c]
X7							1.00	−0.17 [c]	−0.28 [c]
X8								1.00	−0.35 [c]
X9									1.00

[a] X1 = Pasture quantity score, X2 = Pasture quality score, X3 = Mean daily temperature (average of maximum and minimum), X4 = Per cent lignin in herbage, X5 = Per cent crude protein in herbage, X6 = Per cent ether extract in herbage, X7 = Per cent nitrogen-free extract in herbage, X8 = Per cent crude fiber in herbage, and X9 = Per cent ash in herbage.

[b] Y1 = Actual minus expected milk production first day, Y2 = actual minus expected milk production next day, Y3 = actual minus expected milk production two days later, and Y4 = actual minus expected milk production three days later.

[c] Significantly different at $P < .01$.

TABLE 6

Data from multiple regression analysis I

	X1 [a]	X2	X3	X4	X5	X7
b	0.05	0.33	−0.05	−0.08	0.03	−0.01
s_b	0.06	0.06	0.04	0.11	0.06	0.06
t	0.78	5.94 [b]	−1.39	−0.74	0.45	−0.01
b′	0.03	0.26	−0.04	−0.02	0.02	−0.01
x	18.7	19.1	79.3	5.20	12.81	49.76

Y1 = −3.67; n = 1,387 [c]; d. f. = 1,380; R = 0.31 [b]; a = −6.85.

[a] X1 = Pasture quantity score, X2 = pasture quality score, X3 = mean daily temperature (average of maximum and minimum), X4 = per cent lignin in herbage, X5 = per cent crude protein in herbage, X7 = per cent nitrogen-free extract in herbage, and Y1 = actual minus expected milk production first day.

[b] Significantly different from zero at $P < .01$.

[c] The appropriate Y values were used on an individual cow basis each time a complete set of X values appeared. All values for a day were disregarded unless complete in its entirety.

six independent variables, it was necessary to eliminate another variable. Percentage lignin in herbage and percentage crude fiber in herbage were considered as related measures. Both were measuring substances with varying degrees of resistance to the digestive process. However, the correlation ($r = 0.25$) between the two measures was not as high as expected (Table 5). It was decided to compute two multiple regression analyses, using percentage lignin in herbage in one analysis with the five other independent variables remaining and percentage crude fiber in herbage in the other.

Results of multiple regression analyses I and II are shown in Tables 6 and 7, respectively. The partial regression coefficients for pasture quality score were highly significant in both analyses. Pasture quality score appeared to be the only measure of value in predicting milk production. The standard partial regression coefficients add validity to the above statement. Pasture quality score in Analysis I (Table 6) was 10, 6, 12, 13, and 870 times more valuable than pasture quantity score, mean daily temperature (average of maximum and minimum), percentage lignin in herbage, percentage crude protein in herbage, and percentage nitrogen-free extract in herbage, respectively, in predicting milk production.

The multiple correlation coefficients (R = 0.31 and R = 0.32) give an indication of the prediction powers of the regression equations (Tables 6 and 7). Actually, only about 10% of the variance of the dependent variable was due to the regression on the independent variables in both analyses. Inspection of the simple correlation coefficients in Table 5 shows that the correlation ($r = 0.31$) between pasture quality score and the dependent variable is of the same magnitude as the multiple correlation coefficients (R = 0.31 and R = 0.32) in Tables 6 and 7. However, this does not give the complete picture, because the other independent variables are also accounting for some variability in the dependent variable. By inspection of the correlations existing between pas-

TABLE 7

Data from multiple regression analysis II

	X1 [a]	X2	X3	X8	X5	X7
b	0.03	0.35	−0.04	0.18	0.21	0.15
s_b	0.06	0.05	0.04	0.12	0.13	0.11
t	0.59	6.72 [b]	−1.06	1.58	1.59	1.36
b′	0.02	0.27	−0.03	0.11	0.15	0.09
x	18.7	19.1	79.3	25.14	12.31	49.76

Y1 = −3.67; n = 1,387 [c]; d.f. = 1,380; R = 0.32 [b]; a = −22.85.

[a] X1 = Pasture quantity score, X2 = pasture quality score, X3 = mean daily temperature (average of maximum and minimum), X8 = per cent crude fiber in herbage, X5 = per cent crude protein in herbage, X7 = per cent nitrogen-free extract in herbage, and Y1 = actual minus expected milk production first day.

[b] Significantly different from zero at $P < .01$.

[c] The appropriate Y values were used on an individual cow basis each time a complete set of X values appeared. All values for a day were disregarded unless complete in its entirety.

ture quality score and the other individual X's, it can be seen that pasture quality score accounted for a large portion of their contributions.

General. The decline in milk production experienced in Louisiana during the summer months appears to be a nutritional problem due to the low-quality herbage available at that time. In standardization of a feeding program to obtain year-round maximum production, the feeding of high-quality supplemental forage must be considered when pasture quality declines in the summer. A simple method of pasture evaluation, such as pasture quality scoring, could be used for determining when to start supplemental summer forage feeding of milking cows. This pasture quality scoring warrants further consideration as an index of the nutritional value of herbage in permanent pastures.

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EFFECTS OF EXPANDING OR PELLETING UPON FEED DIGESTIBILITY AND HEIFER GROWTH [1]

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SUMMARY

The effects of processing feed by expanding or pelleting were evaluated. Ration digestibilities and growth of 12 eight-month-old Guernsey heifers when fed rations so processed were compared with the response to a control ration containing nonexpanded grain (50% yellow corn and 50% raw soybeans) and long hay. The digestibilities of nutrients in the complete rations were determined by the chromic oxide method. Digestion coefficients were highest in the expanded and lowest in the pelleted ration. Mineral matter was retained to the greatest extent in the pelleted ration. The effects on growth were studied over an 8-wk period using a randomized block design. Ad libitum daily total dry matter consumption in pounds was: 9.17 of the expanded, 8.66 of the control, and 6.51 of the pelleted ration. Daily weight gain in pounds was: 1.68, 1.47, and 1.19, respectively. Increase in heart girth was greatest in the group fed the expanded grain. Chronic bloating and a significantly lower rumen pH were associated with the feeding of the completely pelleted ration.

It has been shown that the nutritive value of starch is improved by cooking (2, 11). Flaking of maize, a process by which the starch granules are burst and partly dextrinized, produces a higher amylase potency in the rumen of sheep, when consumed, than unflaked maize (10). Corn strach is more readily attacked by rumen microorganisms after heating and hydration (11). Cooking and expanding of cereal grains has enjoyed rising popularity for human breakfast food and small-animal feeds in recent years. Expansion is a continuous process of high-pressure steam cooking and extrusion of the hot mass into the normal atmosphere, where the sudden external pressure drop causes escape of the steam, rupture of the cellular structure of the ground grains, expansion and decrease in density of the original material. Expanded grains have been fed to swine, but no reports about such work with ruminants were found.

Pelleting hay and grains for ruminants has received considerable attention in recent years, especially from a management point of view. Reports of the nutritive value of pelleted rations have, however, indicated possible disadvantages under certain circumstances (5, 6, 9).

The objectives of this study were to compare the effects of processing grains by the expansion method with a nonexpanded grain plus long hay control ration and with a completely pelleted ration. The criteria of evaluation were comparative digestibilities of nutrients and growth of heifers under ad libitum feeding conditions. Preliminary results of this work have been reported (3).

EXPERIMENTAL PROCEDURE

Twelve Guernsey heifers, six to eight months of age and weighing between 249 and 316 lb, were used. Six heifers belonged to the University of Delaware dairy herd and six were borrowed from a nearby farm. They were first grouped according to weight into four fairly uniform trios. Heifers within each trio were assigned at random to the three experimental treatments: expanded grain plus long hay, control grain plus long hay, and completely pelleted ration, making four heifers per treatment. The heifers were stanchioned and bedded on sawdust. In previous experiments it was found that straw or peanut hull bedding was occasionally eaten by the animals on trial. This was prevented by bedding on sawdust or rubber mats.

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The statistical design for analysis of the growth study was a randomized block with single classification and equal numbers. The heifers were individually fed twice and watered three times daily. Intake and refusals of water, grain, hay, and pellets were recorded. Samples of the feeds for chemical analyses were taken during the trial.

Hay from a pure stand of alfalfa, third-cutting (September 9, 1960) and heat-dried, was used for this experiment. One-third of it was ground through a 5/8-inch screen and mixed with ground grain (50% yellow corn and 50% raw soybeans) in a ratio of 2 :1 by weight. This mixture was pelleted through a 5/8-inch die without steam or other pelleting aids. The resulting pellets weighed about 28 lb per cubic foot. The same grain ration as used for the pellets served also as the control treatment. An identical grain ration was subjected to the process of expansion. A temperature of about 280 F and steam injection at 70 psi were used with a residence time of only 15–30 sec. This treatment may be equivalent to 4 hr in a pressure cooker. Subsequent extrusion of the feed through small apertures brought about rupture of the grain cells and expansion in bulk of the material to about 50% of its original density. After the extruded feed had been chopped and dried, it had the appearance of small, crisp biscuits.

All rations were fed ad libitum with an attempted restriction of a hay-to-grain ratio of 2:1. Salt bricks were available to the heifers. The animals were weighed before the PM feeding at weekly intervals. Height at withers and heart girth also were determined.

The heifers were started on an 8-wk growth trial after a 1-wk preparatory period. At the end of the sixth, seventh, and eighth week of the trial and shortly before the PM feeding rumen liquor in liter quantities was drawn by stomach tube from the rumen of each heifer. This was subsampled and the pH immediately determined in duplicate, using a portable calomel electrode pH meter.

After finishing the growth studies, one heifer from each group was selected at random and used for the determination of digestion coefficients of hay alone.[4] The remaining three heifers in each group were kept on their previous rations for the determination of digestibility. A ten-day period for the establishment of constant, 100% intake levels preceded the four-day fecal collection period. Chromic oxide was used as an indicator of daily fecal excretions in place of total collections and was administered in 10-g gelatin capsules twice daily before feeding at 6 AM and 4 PM. At the same hours, 50-g fecal collections were made from rectal grab samples and immediately frozen for later analyses of the combined four-day samples.

All chemical analyses were run in duplicate. Chromic oxide was determined according to the New Zealand method (4). Other feed and fecal constituents were determined by proximate analysis (1). The results were evaluated by analyses of variance (12) and the Multiple Range Test (8).

RESULTS AND DISCUSSION

The average chemical composition of the feeds is given in Table 1. Part of a difference in composition between the expanded and the control grain may be due to the expanding process, especially with respect to fat. Unrealistically low values resulted with the usual ether extraction method. Fat content of expanded grain recorded in Table 1 was, therefore, determined by the hydrolytic fat determination method, which is recommended for baked goods (1). Other differences in composition of the two grain mixtures may reflect differences in composition of the original grains.

Digestion coefficients of complete rations composed of hay with one of the two types of grain mixtures or of the completely pelleted ration are presented in Table 2. The digestibility of all organic nutrients was higher in the ration supplemented with the expanded grain than in the control ration. Differences were statistically highly significant for crude fiber. All organic nutrients under study were digested to a significantly lesser degree in the pelleted than in the other rations. Normal cud chewing and rumination was not observed in the pellet fed group. A significantly higher retention of mineral matter (ash) in the feed occurred in the heifers fed the pelleted ration. A summary of the analyses of variance of the digestion coefficients is contained in Table 3.

Averages of the animal data obtained from the 8-wk growth trial are presented in Table 4. Their analyses of variance are summarized in Table 5. All rations were fed ad libitum with the attempted restriction of a hay-to-grain ratio of 2:1. Hay was refused to a greater extent in the group fed expanded grain than in the two other groups, while a significantly greater percentage of the ground control grain was left over at each feeding in the mangers. The apparent preference for expanded grain was

[4] The loss of a heifer from pneumonia made it necessary to introduce one new heifer into the group on hay alone, rather than a randomly selected animal from the group receiving expanded grain.

TABLE 1

Chemical composition of feeds

Feeds	Dry matter	Dry matter constituents: Crude protein	Fat	N-free extract	Crude fiber	Ash
	(%)					
Expanded grain (50% corn + 50% raw soybeans)	91.5	26.8	10.5	55.3	3.3	4.1
Nonexpanded control grain (50% corn + 50% raw soybeans ground)	87.1	26.9	12.1	52.4	5.0	3.7
Alfalfa hay (3rd-cutting, heat-dried)	89.0	18.3	2.8	43.9	31.9	3.1
Alfalfa hay, corn and raw soybeans, ratio 4:1:1, ground and pelleted	86.1	20.0	5.7	44.1	23.9	6.3

TABLE 2

Average digestion coefficients of rations[a] (hay-to-grain ratio 2:1)

Rations	Dry matter	Crude protein	Fat	N-free extract	Crude fiber	Ash
Hay + expanded grain	69.0[b]	75.8[b]	62.9[b]	80.0[b]	49.5[b]	9.5[b]
Hay + control grain	65.0[b]	73.6[b]	62.4[b]	77.2[b]	41.5[c]	9.9[b]
Completely pelleted ration (ground hay + ground grain)	53.6[c] **	65.7[c] **	50.4[c] **	68.3[c] **	22.6[d] **	32.6[d] **
Hay, alone	56.5	73.8	31.7	70.2	38.0	−28.8

* and ** Mean differences statistically significant at $P < .05$ and $P < .01$, respectively.

[a] Each figure is the average of three heifers.

[b, c, d] Means within group with a different superscript are significantly different at $P < .05$ by the Multiple Range Test (8).

TABLE 3

Analyses of variance of digestion coefficients

Nutrients	Mean squares: Treatment	Error	LSD[a] at $P < .05$
Dry matter	191.34	5.78	6.47
Crude protein	84.12	7.67	7.46
Fat	149.49	20.06	11.88
N-free extract	112.84	5.20	6.15
Crude fiber	572.86	8.44	7.83
Ash	522.60	46.00	18.27

[a] LSD = Least significant difference (8).

TABLE 4

Average growth trial results [a]

Group	Consumption per day				Total d.m. per lb wt gain	Gain of body wt per day	Increase in ht at withers per trial	Increase in heart girth per trial
	Water	Hay	Grain	Total				
	(lb)						(inches)	
Hay + expanded grain	35.4	5.12	4.05	9.17 [b]	5.51	1.68	1.46	4.37 [b]
Hay + control grain	44.4	5.33	3.33	8.66 [b]	5.98	1.47	1.79	2.75 [c]
Completely pelleted ration (ground hay + ground grain)	31.5	.....	.....	6.51 [c] *	5.46	1.19	1.12	1.87 [c] *

* Mean differences statistically significant at $P < .05$.

[a] Each figure is the average of three heifers.

[b, c] Means within group with a different superscript are significantly different at $P < .05$ by the Multiple Range Test (8).

of interest, since information about the palatability of expanded grain for heifers was not available.

Total feed and dry matter consumption was highest in the group fed the expanded ration and lowest in the group of heifers on the pelleted ration. Rates of daily weight gain over the 56-day growth period were greatest for the expanded ration and lowest for the pelleted ration. The differences among the three groups approached statistical significance. However, pneumonia occurred in each heifer in the middle of the trial and probably reduced all of the growth rates to some degree. One heifer died on the 29th day. Growth data of that heifer and its trio mates in the other two groups were, therefore, removed from statistical analyses. The heifers on expanded grain also showed slight diarrhea at the beginning of the trial, while all animals on pelleted feed had a chronic bloating problem.

The efficiency of feed conversion (pounds of feed dry matter per pound of gain) was higher in the pelleted and expanded feed groups than in the control group, but not significantly different. The heifers receiving the control grain consumed more water than those in the other groups. They also showed the greatest increase in height, while those in the pellet group showed the least. Increase in heart girth was significantly greater in the expanded grain-fed heifers, while the heifers on the completely pelleted feed appeared stunted in growth and showed a rough hair coat.

Factors that were correlated with mean rates of weight gain in individual heifers seemed of interest, especially because of the observed differences in water consumption among groups. The following correlation coefficients were found:

Daily weight gain:
- Daily water consumption, $r = .62^*$
- Daily dry mater consumption, $r = .81^*$

Daily dry mater consumption:
- Digestible dry matter content of ration, $r = .75^*$
- Daily water consumption, $r = .72^*$

Daily water consumption:
- Digestible dry matter content of ration, $r = .28$

(* r is significant at $P < .05$)

A multiple correlation and regression equation between daily weight gain in pounds ($\hat{Y}$), digestible dry matter content of ration in per cent (X_1) and daily dry matter consumption in pounds (X_2) was found to be:

TABLE 5

Analyses of variance of growth trial results

Item	Mean square		LSD [a] at $P < .05$
	Treatment	Error	
Water consumption	27.36	10.08	18.81
Total d.m. consumption	5.96	0.68	2.14
Total d.m. per lb wt gain	0.24	0.60	2.08
Gain of body wt	0.18	0.05	0.63
Increase in ht at withers	0.33	0.42	1.75
Increase in heart girth	4.82	0.58	2.05

[a] LSD = Least significant difference (8).

$$\hat{Y} = 0.1129 - 0.002863X_1 + 0.1898X_2 \pm 0.192, \quad r = .82$$

Thus, the weight production potentials of the rations could have been estimated from the daily dry matter consumption and the per cent digestible dry matter content of the ration.

Chronic bloating of a nonserious, low-pressure type was constantly observed in the pellet-fed group. The bloating may have been partly responsible for the decreased rate of feed consumption. Since the pelleted ration consisted of three major components: alfalfa hay, corn, and raw soybeans, possibly one of them may have caused the bloating. Therefore, new pellets were made consisting of either the alfalfa hay only, or hay and corn, or hay and raw soybeans. These were fed to three groups of heifers after termination of the growth trial. The different composition of the pellets caused no significant difference in hours that bloating first appeared after placing the heifers on the pellets. The ration prior to this change, however, did have an effect.

Heifers that had been on a pelleted ration previously, appeared bloated within 28 hr, while heifers previously on other rations containing long hay needed an average of 152 hr before bloat manifested itself.

The possibility that an altered type of rumen fermentation was responsible for the bloating and lowered digestibility of the pelleted ration is indicated by results of determining the pH of rumen liquor in the heifers during the last 3 wk of the growth trial. For the three groups: expanded grain ration, control ration, and completely pelleted ration, the mean pH values were: 7.3, 7.0, and 6.3, respectively. The depression of the latter was statistically significant by the Multiple Range Test (8). Lowered rumen pH after feeding pellets has been reported by other workers (6, 9). The pH value of the group fed the expanded grain is within the normal range as given by Dukes (7).

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COMPARISON OF LARD, TALLOW, BUTTER, AND HYDROGENATED COTTONSEED OIL IN STARTERS AND OF PELLETED VS. NONPELLETED COASTAL BERMUDAGRASS HAY FOR CALVES[1]

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SUMMARY

A total of 99 calves was used in three experiments to study; (a) the effect of adding various fats to starters, (b) the influence of calcium level in high fat starters, and (c) the affect of grinding and pelleting vs. grinding Coastal Bermudagrass hay on calf performance. In Experiment 1, 56 baby calves were fed one of seven starters and pelleted or coarsely ground Coastal Bermudagrass hay ad libitum in an 8-wk growth trial. The addition of 10% butter, tallow, lard, or hydrogenated cottonseed oil to starters did not significantly ($P = 0.05$) affect weight gains, days of diarrhea, or hay consumption. Level of calcium in starters containing hydrogenated cottonseed oil had little influence on calf performance. Pelleting the hay approximately doubled its consumption and reduced the starter eaten, but did not affect the total amount of feed consumed.

In cafeteria trials (Experiment 2) the control starter was decidedly more palatable than those containing added fat. The following order of decreasing palatability was indicated for the others; butter, lard, hydrogenated cottonseed oil, and tallow. Calves exhibited a decided preference for the pelleted hay.

In Experiment 3, pelleting the hay increased hay consumption and gains of older calves with either a low or a medium level of concentrates.

Earlier experiments from this laboratory (7) demonstrated that high levels of brown grease and to a lesser extent hydrogenated cottonseed oil in calf starters reduced growth, feed consumption, and palatability. Other studies (5) showed that adding tallow to starters tended to improve the growth rate of calves. Subsequently, it has been shown that the addition of corn oil to diets of sheep depressed the utilization of calcium (11, 12). Calculations of the calcium intakes of calves in the original study from this laboratory (7) indicated that the level approximated NRC requirements (10). Thus, the adverse effects of the high levels of added fats possibly could have been influenced by a depression in calcium utilization.

Gardner et al. (4) have shown that pelleting alfalfa increased hay consumption, and tended to increase growth in young dairy calves while decreasing the amount of concentrates consumed. No published results of similar studies have been found in which Coastal Bermudagrass hay was used. Such studies appeared to be needed, as Coastal Bermuda is rapidly becoming the dominant forage in the southeastern United States and is very different from alfalfa. Although in raising dairy calves from two to five months of age, it is usually considered necessary to feed liberal amounts of concentrates, the amount required is influenced by the quality and amount of forage consumed. Since grinding and pelleting Coastal Bermudagrass hay will increase feed consumption and weight gains of yearling beef steers above that obtained with grinding only (3), it seemed desirable to determine whether a similar benefit would be exhibited in dairy calves.

The objectives of the studies herein reported were to determine: (a) the effect of the addition of various types of fats to starters on calf performance; (b) whether the calcium level influenced the relative performance of calves fed starters containing high levels of fat; (c) the effect of grinding and pelleting Coastal Bermudagrass hay, compared with grinding only, on the performance of calves, and whether any interaction existed between the form of

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the hay and the addition of fats to starters; and (d) the effect of pelleting Coastal Bermudagrass hay for intermediate-aged dairy calves at low and medium levels of concentrate feeding.

EXPERIMENTAL PROCEDURE

Three experiments were conducted with 99 dairy calves; (a) growth experiment with young calves, (b) a cafeteria study with young calves, and (c) a growth study with older calves.

Experiment 1. Seven starters and two hays were fed to 42 Holstein and 14 Jersey baby calves in a 7 × 2 factorial design. The control starter had the following composition: 29.0 lb soybean oil meal (44%), 66.0 lb ground yellow corn, 2.1 lb defluorinated rock phosphate, 1.0 lb trace-mineralized salt (6), 200 g vitamin A supplement (20,000 USP units/g), 200 g vitamin D_3 supplement (1,500 IU/g in soybean oil meal), and 174 g chlortetracycline concentrate (10 g/lb). Starters 2, 3, 4, and 5 contained 10% added fat. To make the ratio between energy and other nutrients more nearly equal, 10% more of each of the vitamin, mineral, and antibiotic supplements and crude protein were added to these four starters. The change in protein was accomplished by increasing the soybean oil meal to 40 lb and reducing the corn to 45 lb. The experimental starters were numbered as follows on the basis of the kind of fat each contained: No. 2, bleachable fancy tallow; No. 3, hydrogenated cottonseed oil; No. 4, lard; and No. 5, butter. Starters No. 6 and 7 were identical with Nos. 1 and 3, respectively, except the defluorinated rock phosphate was reduced to 1.3 lb.

Proximate analyses determined by AOAC procedures (2) are presented in Table 1. Characteristics of the various fats were determined by a commercial laboratory, using AOCS procedures (1).

The calculated calcium contents of the starters in per cent were respectively: 0.80, 0.93, 0.93, 0.93, 0.93, 0.53, and 0.55. The hay contained 0.44% calcium. The tallow, hydrogenated cottonseed oil, lard, and butter had the following characteristics, respectively: free fatty acids (%), 7.26, 0.02, 0.17, and 0.31; iodine no. 38, 58, 54, and 30; saponification no., 194, 192, 200, and 220; titer test for solidification point of the fatty acids (degree C), 42.2, 46.4, 39.0, and 35.2; and moisture (%), 0.36, 0.02, 0.03, and 17.65. Other analyses which characterized the hydrogenated cottonseed oil were as follows (%): total fatty acids, 95.7; unsaponifiable, 0.4; direct saturated fatty acids, 13.9; oleic acid, 77.0; linoleic acid, 0.8; linolenic acid, 0.01; and trans bonds, 39.6. The butter scored 92.0.

The hays were (a) coarsely ground and (b) ground and pelleted. Both were from third-cutting Coastal Bermudagrass which was harvested and artificially dehydrated in mid-July, 1958. It had had 3 wk to grow since the second cutting. The field was fertilized with 1,000 lb of 0-10-20 and 100 lb of N per acre in the spring, plus an additional 100 lb of N after each of the first two cuttings. Part of the chemical analyses of the hay is presented in Table 1. The lignin and carotene contents were 7.0% and 75.4 mg/lb of hay, respectively. The carotene analysis, however, was made July 21, 1958, and is not necessarily indicative of the value available when the hays were fed during the fall and winter. The ground hay was run through a hammer mill with a 9/16-inch screen; whereas, the pelleted hay was ground using a 5/8-inch screen and made into pellets with a 1/2-inch die.

The calves were fed and cared for as described previously (6). Beginning on the sixth day, the experimental starters and hays were fed ad libitum to the calves, individually, for 8 wk. Each calf was given 50,000 IU of vitamin A by

TABLE 1

Chemical analyses of the feeds used

Starter no.	Crude protein	Crude fiber	Ether extract	Ash
	(%)[a]			
1	22.2	3.5	3.7	6.4
2	24.3	3.5	13.9	6.9
3	24.1	3.3	14.0	6.6
4	24.6	3.3	13.8	6.7
5	25.8	3.5	12.1	7.1
6	23.1	3.1	4.2	5.4
7	25.3	3.4	14.6	6.0
Hay	16.8	27.2	2.3	6.6
Concentrate (Experiment 3)	16.8	11.5	2.5	6.8

[a] Dry matter basis.

capsule twice per week until 5 wk of age, and once per week thereafter.

Experiment 2. The relative palatabilities of Starters 1 through 5 and of the two physical forms of the hay were determined in cafeteria studies involving 23 calves for 9 wk, beginning at six days of age. Two types of cafeteria trials were conducted. In one, five calves were each given a choice of all five of the starters for 9 wk. Since it has been shown that offering more than two choices may lead to appreciable bias in cafeteria studies (8), four other trials were conducted in which only two starters per trial were offered to each of three calves. The relative palatability of the pelleted and the ground hay was studied in a comparable trial with six calves. Other details of the procedure were similar to those described previously (6).

Experiment 3. The two physical forms of the hay were fed with two levels of concentrates to 20 calves in an 8-wk growth trial beginning when they were between 70 and 76 days of age. Two replications of Holstein steers, two of Holstein heifers, and one of Jersey steers were utilized in a 2 × 2 factorial design. The two levels of concentrates were 5 and 3 lb per calf per day of a mixture composed of: 73.5 lb ground snapped corn, 23.5 lb soybean oil meal, 45 g vitamin A supplement (20,000 USP units/g), 146 g chlortetracycline concentrate (10 g/lb), 45 g vitamin D_3 supplement (1,500 IU/g), 1 lb trace-mineralized salt (6), and 1.5 lb defluorinated rock phosphate. The amounts of concentrates were 3.9 and 2.3 lb per calf per day, when the cobs and shucks were calculated as roughage.

RESULTS AND DISCUSSION

Experiment 1. The means of weight gains, starter consumption, hay consumption, and days of diarrhea are presented in Table 2. There were no significant differences ($P = 0.05$) in weight gains, or days of diarrhea among calves fed the various starters. Calves fed the starter containing butter (No. 5) had the highest average

TABLE 2

Effect of starter and form of hay on weight gains, starter and hay consumption, and diarrhea (Experiment 1)

Starter no.	Hay form	Weight gains	Feed consumption			Diarrhea
			Starter	Hay	Total	
		(lb/day)				*(total days)*
1	Chopped	1.05	1.68	0.22	1.90	0.5
	Pelleted	1.10	1.63	0.29	1.92	1.8
	Avg	1.08	1.66	0.26	1.91	1.2
2	Chopped	1.11	1.81	0.14	1.95	1.5
	Pelleted	0.99	1.13	0.71	1.85	2.0
	Avg	1.05	1.47	0.42	1.90	1.8
3	Chopped	0.88	1.43	0.16	1.59	0.5
	Pelleted	0.96	1.32	0.55	1.87	1.0
	Avg	0.92	1.38	0.36	1.73	0.8
4	Chopped	0.88	1.30	0.27	1.57	1.2
	Pelleted	1.09	1.61	0.57	2.18	0.5
	Avg	0.98	1.46	0.42	1.88	0.8
5	Chopped	1.28	2.06	0.20	2.26	0.0
	Pelleted	1.00	1.54	0.34	1.88	0.5
	Avg	1.14	1.80	0.27	2.07	0.2
6	Chopped	1.18	1.92	0.29	2.21	1.0
	Pelleted	1.03	1.25	0.55	1.80	0.8
	Avg	1.10	1.58	0.42	2.00	0.9
7	Chopped	0.96	1.44	0.16	1.60	0.0
	Pelleted	0.99	1.49	0.32	1.81	0.2
	Avg	0.98	1.46	0.24	1.70	0.1
Avg	Chopped	1.05	1.66	0.21	1.87	0.7
	Pelleted	1.02	1.42	0.48	1.90	1.0
$S_{\bar{X}}$	(starters)[a]	0.08	0.11	0.11		
$S_{\bar{X}}$	(hay form)	0.04	0.06	0.06		

[a] $S_{\bar{X}}$ = standard error.

starter consumption and those fed the ones containing hydrogenated cottonseed oil (no. 3 and 7) the lowest. The calves fed these starters also had the highest and the lowest gains, respectively. The calves receiving the control starters with either the high (No. 1) or the lower level (No. 6) of calcium had the second-highest starter consumption and weight gains, whereas those fed starters containing lard (no. 4) or tallow (no. 2) were next to lowest. The level of calcium apparently had little effect on the performance of the calves. The average calcium intakes (weekly basis) and the percentages of requirements (10) of calves fed the various starters are presented in Table 3. Calves fed Starters no. 1 through 5 received more calcium than is considered to be required (10), during most of the period. However, calves fed the two low calcium starters (no. 6 and 7) received slightly less than recommended levels throughout most of the experiment. Since this is less than was fed in the previous study (7), the results suggest that the adverse effects of high fat levels on growth in that study probably were not due to a deficiency of calcium caused by the high levels of fat decreasing the efficiency of calcium utilization.

Those calves fed the pelleted Coastal Bermudagrass consumed about twice as much hay as those fed the ground material, but significantly less starter, so that the total feed intakes were approximately the same. The difference in hay consumption was highly significant statistically ($P = 0.01$), but there were no significant differences in weight gains or days of diarrhea.

The addition of high levels of the fats did not decrease weight gains or starter consumption significantly ($P = 0.05$), as it did in the previous test (7). However, the starter consumption and weight gains of calves fed hydrogenated cottonseed oil (the only fat used in both studies) were lower, on an average, than those of any other group. Thus, it appears that the type of fat may have a bearing on whether its addition to dry calf starters will adversely affect calf performance.

Several differences were considered in an attempt to determine why the use of hydrogenated cottonseed oil did not have as serious detrimental effects in this study as in the previous one (7). The basal starter used in this study was a simple high-density feed, whereas the one used previously was complex and bulky. Other studies have shown that there were no important differences in starter consumption, growth, or diarrhea between calves fed the dense and the bulky starter (9). How-

TABLE 3

Calcium intake of calves fed the various starters by weeks (Experiment 1)

Experimental week		Starter no.						
		1	2	3	4	5	6	7
1	g/day [a]	2.9	3.2	3.4	3.2	3.6	3.1	3.1
	(%) [b]	(51)	(52)	(57)	(52)	(58)	(52)	(52)
2	g/day	5.8	6.1	6.0	5.9	5.9	5.1	5.4
	(%)	(97)	(97)	(97)	(92)	(94)	(81)	(87)
3	g/day	7.0	7.3	7.7	6.9	8.0	6.0	6.7
	(%)	(111)	(109)	(118)	(104)	(121)	(91)	(102)
4	g/day	8.5	8.2	9.0	8.8	10.1	6.5	7.1
	(%)	(125)	(117)	(130)	(128)	(136)	(93)	(100)
5	g/day	9.4	9.7	9.2	9.9	11.1	6.9	7.0
	(%)	(127)	(123)	(120)	(127)	(134)	(88)	(89)
6	g/day	10.4	11.3	9.9	11.6	12.0	8.1	6.7
	(%)	(124)	(124)	(122)	(136)	(130)	(91)	(78)
7	g/day	12.8	13.3	12.5	12.6	14.7	9.6	7.7
	(%)	(136)	(134)	(134)	(130)	(144)	(96)	(80)
8	g/day	15.0	15.6	13.4	16.0	17.5	11.0	8.4
	(%)	(142)	(140)	(131)	(157)	(148)	(97)	(82)
Avg	g/day	9.0	9.3	8.9	9.4	10.4	7.0	6.5
	(%)	(114)	(112)	(114)	(115)	(121)	(86)	(84)

[a] Grams calcium/calf/day.
[b] Average per cent of calcium requirement (10).

TABLE 4

Weight gains, feed consumption, and feed efficiency of older calves fed two forms of hay with two levels of concentrates (Experiment 3)

Hay	Grain fed [a]	Wt gain	Feed consumption		Feed/lb of gain	Starting wt
			Hay	Total		
		(lb/day)			*(lb/lb)*	*(lb)*
Pelleted	5(3.9)	2.34	4.30	9.30	3.97	166
Pelleted	3(2.3)	2.29	6.05	9.05	3.95	173
Ground	5(3.9)	2.06	2.80	7.80	3.79	182
Ground	3(2.3)	1.97	4.46	7.46	3.79	182
Pelleted *(avg)*	4(3.1)	2.32	5.18	9.18	3.96	170
Ground *(avg)*	4(3.1)	2.02	3.63	7.63	3.79	182
Medium grain *(avg)*	5(3.9)	2.20	3.55	8.55	3.88	173
Low grain *(avg)*	3(2.3)	2.13	5.26	8.26	3.87	178
$S_{\bar{X}}$		0.08	0.21	0.21	0.13	

[a] Snapped corn calculated as grain values in ( ) based on cobs and shucks calculated as roughage.

[b] $S_{\bar{X}}$—standard error.

ever, hay consumption was higher for calves fed the dense starter. Since considerably more pelleted than ground hay was consumed, it is interesting that there was a much more pronounced effect of starters on growth among calves fed the ground hay. The range among average gains of calves fed the various starters and pelleted hay was only 0.14 lb per day, compared to 0.40 lb among those fed the ground hay. Thus, the higher hay consumption probably was at least partially responsible for a diminution or elimination of the depressing effect of the hydrogenated cottonseed oil in this study, as contrasted to the previous one (7). However, the element of chance should not be ignored, as the level of difference was within the range of normal experimental errors.

Experiment 2. When Starters 1 through 5 were offered simultaneously to five calves in a cafeteria experiment, the following percentages were consumed respectively: 53, 3, 9, 15, and 20. Thus, they ate significantly more control starter than any other.

In the four trials in which only two starters were offered, the following ratios of starters were consumed: Trial A, 83% of No. 1 (Control) and 17% of No. 5 (butter); Trial B, 63% of No. 5 (butter) and 37% of No. 2 (tallow); Trial C, 60% of No. 4 (lard) and 40% of No. 3 (hydrogenated cottonseed oil); and Trial D, 59% of No. 4 (lard) and 41% of No. 2 (tallow).

From these two types of trials, it is apparent that calves preferred the control starter to those containing any of the added fats, and that the differences among the various types of added fats were not as large as those between added fat and no added fat. However, it did appear that the type of fat had some influence on the relative palatability of the starters. The following order of decreasing relative palatability was indicated; buter, lard, hydrogenated cottonseed oil, and tallow.

The pelleted hay was much more palatable than the ground hay. Sixty-five per cent of the total consumption was pellets.

Experiment 3. The weight gain, feed consumption, and feed efficiency data are presented in Table 4. Calves fed the pelleted Coastal Bermudagrass gained significantly ($P = 0.05$) faster than those fed the ground hay. The differences due to level of concentrates and the interaction were not statistically significant. Calves fed the pelleted Coastal Bermudagrass consumed highly significantly ($P = 0.01$) more feed than those fed the ground Coastal. However, the feed efficiency of those fed the pellets was slightly lower (Table 4). There was no significant difference in feed consumption or feed efficiency between the groups fed the high and low levels of concentrates.

While the calves fed the ground Coastal Bermudagrass with only 2.3 lb of concentrates had the lowest average gain, it was a highly satisfactory gain for calves of this age, sex, and breed composition. Also, this would often be a lower cost ration. Thus, it would probably be the most satisfactory for raising dairy calves. In making practical recommendations, however, a number of items should be considered. The hay was unusually high-quality Coastal Bermudagrass; it was ground, which probably increased the feed consumption and weight gains of the calves (4); and the concentrate mixture was well fortified with minerals, vitamins, and antibiotics. All of these factors probably contributed to the excellent performance of these calves.

All experiments. Several conclusions seem justified when all of the experiments are considered together with the previous research (3, 4, 5, 7, 9, 11, 12). It appears that the effects of adding high levels of fats to calf starters can vary from a serious reduction in growth and performance of the calves to slightly beneficial effects, the exact results being greatly influenced by several factors. The type of fat may be important, with such fats as brown grease and hydrogenated cottonseed oil probably being more undesirable than butter, lard, and tallow. Apparently, the palatability of calf starters is reduced by high levels of all types, and this may be an important reason for the frequently adverse effects on performance. The amount of hay eaten seems to have some importance in determining the effect of the fat on calf performance. The type of starter also may have an influence, although part of this appears to be the indirect effect of causing the calf to eat relatively more or less hay. In general, however, the addition of fats to calf starters does not seem to offer enough promise to justify its recommendation on a practical basis.

Grinding and pelleting compared with only grinding Coastal Bermudagrass hay appears to improve its palatability and to increase the intake when it is offered ad libitum. When the level of starter or concentrates is limited, it appears that pelleting the hay increases the growth rate but not the efficiency of the gains. However, when the starter is not limited, the increase in hay intake due to pelleting is offset by a reduction in starter consumption, so that the growth rate is not increased.

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RELATIVE DEAMINATION RATES OF AMINO ACIDS BY RUMEN MICROORGANISMS [1]

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SUMMARY

Studies were made of the relative deamination rates of amino acids in vitro by rumen microorganisms. Two media were investigated—cheesecloth-strained rumen liquor and washed suspensions of rumen bacteria. Individual amino acids may be divided into three groups with regard to their relative rate of deamination. Serine, cysteine, aspartic acid, threonine and arginine were attacked most completely, followed by glutamic acid, phenylalanine, lysine, and cystine forming an intermediate group and a third group in which deamination was much less pronounced was tryptophane, δ-amino valeric acid, methionine, alanine, valine, isoleucine, ornithine, histidine, glycine, proline, and hydroxyproline.

Dissimilation rates are more rapid and complete in rumen liquor than in washed cell suspensions. A mixture of three or four amino acids were not dissimilated to a greater degree than the catabolic summation of each individual amino acid in the mixture. The D and L forms of serine and tryptophan were catabolized at the same relative rate whereas the D enantiomorphs of aspartic acid, lysine, threonine, and phenylalanine were not catabolized. Modifications of the washed cell medium failed to demonstrate the factors responsible for the marked decrease in deaminating power.

Discrepancies concerning resistance of amino acids to deamination have appeared in the literature from studies in vitro involving rumen liquor and washed cell suspensions (2, 5, 6). In addition, there is a serious lack of knowledge regarding the inability of amino acid studies in vitro to duplicate reaction rates noted in vivo.

This investigation had three purposes. First, to compare and quantitate catabolic reactions of amino acids in two media, strained rumen liquid and washed cells; secondly, to compare the dissimilation rates of optical isomers of amino acids, and third, to add materials to washed cell suspensions in an effort to increase the dissimilation rates of individual amino acids.

MATERIALS AND METHODS

Animal management. Mature cows with rumen fistulas closed by acrylic resin plastic cannulae were fed 6 to 8 lb of alfalfa hay and 10 to 14 lb of a 16% protein-concentrate mixture. These rations were fed for at least 3 wk prior to sampling. The animals had free access to water.

Incubation mediums. Cheesecloth-strained rumen fluid served as one medium in vitro, whereas a second medium consisted of a washed suspension of rumen bacteria. The latter suspensions were prepared by differential centrifugation. The cheesecloth-strained rumen liquid was centrifuged 5 min at 250 × G to remove the large feed particles and protozoa. The resulting supernatant was then subjected to a force of 27,600 × G for 10 min. The supernatant was discarded and the bacterial residue was twice washed in 25 ml of 0.1 M phosphate buffer at pH 6.5, previously boiled and cooled, to which was added 0.02% (w/v) $Na_2S \cdot 9\ H_2O$. Three parts of the bacterial suspension (dry weight 4 to 7 mg/ml) were then added to ten parts of the buffer containing the amino acid or amino acids.

The concentration of each amino acid in each medium was 10 μM/ml. Whenever tryptophan was studied, the amino acid was solubilized in 5 ml of 1 N NaOH prior to the addition of the rumen fluid or phosphate buffers; aspartic and glutamic acids were neutralized to pH 6.9 with sodium carbonate prior to incorporation in the incubation medium.

Anaerobic conditions were obtained by gassing

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5 min with carbon dioxide freed from oxygen by bubbling the gas through a chromous acid solution (4). The rumen fluid incubations were conducted for 8 or 24 hr at 39 C, whereas the washed suspensions were incubated at 39 C for 24 hr. One-half-milliliter subsamples for chromatography were taken before and after each incubation. Fermentation was terminated by addition of sulfuric acid after taking these subsamples. Endogenous products were determined by simultaneous incubation of the medium with substrate omitted.

Analytical methods. Ammonia was determined by aeration or steam distillation from an alkaline sample into a 2% solution of boric acid and titrated with standard acid. All the ammonia values reported were corrected for the endogenous values. The percentages of amino acid dissimilated were determined as 100 times the total ammonia, minus endogenous ammonia divided by theoretical substrate ammonia. Amino acid disappearance was studied using smaples preserved with four parts of absolute ethanol. Each amino acid was chromatographed unidimensionally on Whatman No. 1 filter paper and the diminution of ninhydrin coloring visually compared to standard solutions and to the per cent dissimilation computed from ammonia production. Butanol, acetic acid, water (4:1:1); phenol, water (80:20), and other well-known developing solvents were utilized.

RESULTS

The relative magnitudes of 24-hr dissimilation in vitro of individual amino acids (10 μM/ml) added to either cheesecloth-strained rumen liquor or washed suspensions of rumen bacteria are found in Table 1.

Serine, cysteine, aspartic acid, threonine, arginine, phenylalanine, glutamic acid, lysine, and cystine were readily dissimilated when added to either medium (47 to 100% in rumen liquor; 21 to 99% in washed cells). Tryptophane, histidine, methionine, ornithine, valine, alanine, leucine, isoleucine, δ-amino valeric acid, glycine, hydroxyproline, and proline were dissimilated at lesser rates (8 to 37% in rumen liquor; 1 to 22% in washed cells). A visual comparison of amino acid disappearance on paper chromatograms closely paralleled the increase noted in ammonia values.

The amino acids catabolized in rumen fluid were likewise dissimilated by washed cell suspensions and in approximately the same sequence of magnitude. The dissimilation rates, however, were more rapid and complete in rumen liquor than in washed cell suspensions.

TABLE 1

Percentages of individual amino acids dissimilated in two mediums

Amino acid	% Dissimilation [a, b]	
	Rumen fluid	Washed cells
L-serine	100	99
L-cysteine	96	85
L-aspartic acid	95	86
L-threonine	83	69
L-arginine	80	57
L-phenylalanine	75	21
L-glutamic acid	64	57
L-lysine	57	37
L-cystine	47	34
DL-tryptophane	37	15
L-histidine	33	14
L-methionine	32	18
L-ornithine	29	14
L-valine	29	11
L-alanine	28	14
L-leucine	24	11
L-isoleucine	22	10
Delta-amino valeric acid	21	22
Glycine	10	2
L-hydroxyproline	9	2
L-proline	8	1

[a] Mean of three values.
[b] Maximum range ± 10%.

The one exception was δ-amino valeric acid, which was not dissimilated at a distinguishably faster rate in rumen fluid.

When three or four amino acids were incubated together with washed suspensions of rumen bacteria, the only phenomenon which differed from amino acids incubated alone was the marked increase in the dissimilation of proline and alanine incubated together.

The unreplicated results of a second phase of this study, dealing with the catabolism of the optical isomers of six amino acids are summarized in Table 2. Again, the ammonia production corresponded well with the amount of amino acid dissimilated. These results indicate that both isomers of certain amino acids, serine and tryptophan, are dissimilated equally

TABLE 2

The per cent dissimilation of optical isomers of six amino acids

Amino acid	Medium	% Dissimilation		
		L	DL	D
Serine	Rumen liquor	100	100	
Serine	Washed cell	99	99	
Aspartic acid	Rumen liquor	95	53	
Threonine	Rumen liquor	83	42	
Phenylalanine	Rumen liquor	75	44	
Lysine	Rumen liquor	57	41	
Tryptophan	Washed cell	19	17	15

well, whereas only the L-isomers of other amino acids, aspartic acid, lysine, threonine, and phenylalanine, are readily dissimilated and the D-enantiomorphs are either catabolized slowly or not at all.

Six amino acids were dissimilated, using rumen liquor as the incubating medium, to elucidate the relative rate of ammonia production from individual amino acids. An 8-hr incubation was long enough to yield ammonia production over endogenous level which was adequate to resolve relative differences and was short enough to prevent substrate depletion from affecting the unreplicated results found in Table 3. In 8 hr, arginine contributed approximately five times as much total ammonia as did any one of the other five amino acids studied.

TABLE 3

Ammonia production from six amino acids in 8-hr rumen fluid incubations

Amino acid	Ammonia N (*mg/100 ml*)		
	Theoretical yield	Actual yield	% Dissimilation
L-arginine	56.04	24.14	43
L-aspartic acid	14.01	5.16	37
L-serine	14.01	4.52	32
L-cysteine	14.01	4.39	31
L-lysine	28.02	2.56	18
DL-tryptophan	14.01	1.82	13

Several alterations in the washed cell technique failed to significantly promote ammonia production over the control values (Table 4). The enrichment culture in Column 1 of Table 4 refers to bacteria from a 48-hr incubation period (30 μM/ml of an amino acid) resuspended in the same amino acid medium. Additions of pyridoxamine, pyridoxal phosphate, methylene blue, catalase, or magnesium ion and an all-potassium cationic buffer system were also tested (Table 4). Three international units (IU) of potassium penicillin G per milliliter had no effect on amino acid dissimilation, whereas a penicillin concentration of 30 IU per milliliter markedly inhibited amino acid dissimilation, as shown in Table 5. Each value in Tables 4 and 5 represents a single determination.

TABLE 5

Effect of two levels of penicillin, 3 IU and 30 IU/ml, on amino acid dissimilation by washed suspensions of rumen microorganisms

Substrate	Control	3 IU	30 IU
None	0.88	1.04	0.18
L-arginine	25.46	27.64	0.46
L-aspartic acid	10.82	10.16	0.16
L-lysine	9.06	9.36	0.04
L-glutamic acid	7.42	8.06	0.12
L-ornithine	3.88	3.72	0.18
L-phenylalanine	3.66	2.26	0.00
DL-tryptophan	3.12	3.18	0.00
δ-amino valeric acid	3.10	2.56	0.00

DISCUSSION

Since ammonia is formed in the latter stages of protein catabolism, and is the prime nitrogen source in protein anabolism, it is desirable to understand the conditions under which ammonia is released from amino acids. This study was designed for just such a purpose.

Because the rate of attack upon single amino acids is considerably more rapid and extensive in vivo and in rumen liquor than when washed

TABLE 4

Ammonia production from amino acids incubated with washed suspension of rumen microorganisms

Substrate	Experiment [a]							
	0	1	2	3	4	5	6	7
	(*mg ammoniacal N/100 ml*)							
Control	1.64	0.63	1.61	1.72	0.84	0.98	2.12	1.22
L-arginine	25.82	1.62	23.14	25.54	24.62	23.35	28.54	30.16
L-lysine	9.60	0.23	7.88	8.86	8.54	8.38	4.46	8.74
δ-amino valeric acid	3.02	0.06	2.76	2.84	3.08	2.96	1.48	3.36
L-aspartic acid	12.10	1.12	12.68	11.98	10.22	10.78	10.64	9.84
DL-tryptophan	3.06	0.29	3.50	3.68	3.36	3.42	1.48	3.64
L-glutamic acid	7.60	0.52	6.90	7.20	7.46	7.90	7.80	8.10
L-phenylalanine	3.40	0.20	2.86	2.94	3.18	3.46	3.84	3.72
L-ornithine	4.06	0.40	3.48	4.12	3.72	4.28	2.22	3.46

[a] 0—No addition; 1—48-hr enriched cultures resuspended; 2—2.5×10^{-5}M pyridoxamine; 3—2.5×10^{-6}M pyridoxal phosphate; 4—3×10^{-4}M magnesium ion; 5—.097 M potassium buffer; 6—0.1% methylene blue; 7—50 units of catalase.

cells are used, it appears that the method of preparation of the suspension somehow inactivated the enzymes responsible for the deamination. A second possibility for decreased deamination could have been a selection of the nondeaminating population in the washed cell preparation or a lysis or death of the deaminating bacteria (7). The first supposition appeared more likely and was the one investigated. No single additive to the washed cell suspension significantly promoted ammonia production over the control values, which are low as compared to rumen liquor and ammonia production in vivo. Lewis (5) obtained increases in the activity of washed suspensions of rumen bacteria by modifications of the environment. However, the stimulations were slight and these conditions also must have differed significantly from those present in rumen liquor. The factor or factors responsible for the marked decrease in the deaminating power of washed cell suspension still is unanswered and requires more extensive investigation. Increasing the number of amino acids to three or four in the suspensions failed to produce greater ammonia levels, as might be expected if a Stickland type of reaction predominated.

The studies in vitro demonstrated that amino acids may be divided into three groups with regard to their relative rate of deamination. These findings agree qualitatively with Sirotnak et al. (6) and Lewis (5) with a few exceptions, notably arginine, lysine, and tryptophan. Both of these authors reported that washed cell suspensions deaminate aspartic acid at a much greater rate than arginine, whereas rumen fluid incubations demonstrated that arginine contributed approximately five times as much total nitrogen in 8 hr as did aspartic acid. It must be remembered, though, that cheesecloth-strained rumen liquor studies were not limited to rumen bacteria but also included rumen protozoa.

Since Barrentine et al. (1) and Emery, Smith, and Huffman (3) obtained practical control of bloat with penicillin, a trial was designed to study the effects of penicillin on amino acid dissimilations. Three IU of penicillin per milliliter, the approximate ruminal level in the study by Emery, Smith, and Huffman, had no apparent effect on amino acid catabolism, whereas 30 IU markedly inhibited amino acid dissimilation.

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DURATION OF SECRETION OF BACTERIOSTATIC DRUGS IN MILK. I. PENICILLIN, FOLLOWING ORAL AND PARENTERAL ADMINISTRATION [1]

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SUMMARY

The duration and concentration of penicillin in milk were determined following intramuscular and oral administration of procaine penicillin G in sesame oil with 2% aluminum monostearate and intramuscular, intravenous, and intrauterine administration of potassium penicillin G in aqueous suspension.

Following intramuscular injection at the rate of 3,000 units per lb of body weight, the maximum interval that penicillin was detected in the milk of the test animals was 96 hr for cows that received penicillin in oil, and 72 hr for those that received aqueous penicillin. Milk from only 25% of the cows in both groups contained detectable penicillin at the maximum intervals. Intravenous injection of 2,000 units of aqueous penicillin per pound of body weight yielded detectable penicillin in the milk of 25% of the test animals at 20 hr, and in 8.3% at 44 hr post-injection. Penicillin was found in the milk of 16.7% of the test animals 27 to 31 hr after intrauterine infusion of 1,000,000 units of aqueous penicillin, and at subsequent intervals no penicillin was detected in milk from any of the cows. A single dose of 10,000,000 units of penicillin in oil fed with concentrate resulted in detectable penicillin in the milk a maximum of 86 hr post-feeding, at which time only 8.3% of the samples were positive.

Correlations between the duration of secretion of milk containing penicillin and milk production, fat content of the milk, and body weight were nonsignificant ($P > .05$) for all methods of administration.

Penicillin is used in the treatment of many diseases of bacterial origin in dairy cattle. This antibiotic is administered to dairy cattle by many routes, including intramuscular and intravenous injections, intrauterine infusions, and orally. A survey conducted during 1959 (8) showed that 3.5% of market milk samples tested contained penicillin. Dermatitis reactions in persons have been associated with the presence of penicillin in milk (7). Thus, the minute quantities of penicillin in milk represent a potential health hazard. Therefore, the Food and Drug Administration is enforcing a law that prohibits interstate shipment of market milk containing this drug. As indicated by reviews (2, 4, 10), information on the duration of secretion of penicillin in milk following intramammary infusions is voluminous. Conversely, the information regarding the duration of secretion of penicillin in milk following administration of this antibiotic to dairy cows by other routes was considered inadequate. For the foregoing reasons, a study was begun to determine the concentration and duration of penicillin secretion in milk following its administration to dairy cows by intramuscular, intravenous, intrauterine, and oral routes, and to evaluate the effect of certain independent variables on differences in responses of individual cows.

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EXPERIMENTAL PROCEDURE

Intramuscular injections. Twelve lactating cows were injected intramuscularly with aqueous penicillin (potassium penicillin G in aqueous suspension) at the rate of 3,000 units per pound of body weight in a single does. An additional 12 lactating cows were injected by the same route and dose with penicillin in oil

(procaine penicillin G in sesame oil with 2% aluminum monostearate). These injections were made immediately after the morning milking.

Intravenous injections. Aqueous penicillin was injected, in a single dose, into the jugular of 12 cows at approximately 8 AM at a level of 2,000 units per pound of body weight.

Intrauterine infusions. Twelve lactating cows received intrauterine infusions of 1,000,000 units of aqueous penicillin. In two of the cows, the penicillin was infused during estrus, whereas the other ten cows were infused approximately ten days post-partum. All intrauterine infusions were made between 8 and 12 AM.

Oral administration. Penicillin in oil was fed to 12 cows at the rate of 10,000,000 units per cow in a single dose. The penicillin, mixed with 1 lb of concentrate, was fed during the afternoon milking.

Collection of samples and penicillin assay. All cows were machine-milked between 4:30 and 6:30 AM and 2:30 and 4:30 PM daily. After milking each cow, the milking machine was rinsed thoroughly, to minimize contamination of milk from the next cow. An aliquot of the total milk taken from the four quarters was collected at the two daily milkings for the penicillin assay. Collection of the milk samples continued until two consecutive samples were found to contain no detectable penicillin by the overnight cylinder plate assay (9), using *Sarcina lutea* as the test organism. The minimum concentration of penicillin detected by this assay was approximately 0.0063 unit per milliliter of milk.

Total milk produced at each milking was weighed and recorded. Also, the milk produced by each cow during two consecutive milkings was assayed for milk fat content by the Babcock method.

RESULTS

Concentrations of penicillin in milk, at 10- and 14-hr intervals, following administration of the drug to dairy cows intramuscularly, are given in Tables 1 and 2. The penicillin content

TABLE 1

Concentration and duration of detectable levels of penicillin in milk following intramuscular injection of a single dose of potassium penicillin G in aqueous suspension at the level of 3,000 units per pound of body weight

Post-treatment time	Concentration of penicillin		Positive samples
	Mean	Range	
(hr)	*(units/ml)*		*(%)*
10	0.121	0.049–0.222	100.0
24	0.027	0.016–0.054	100.0
34	0.009	0.000–0.018	83.3
48	0.004	0.000–0.011	58.3
58	0.001	0.000–0.006	16.7
72	0.001	0.000–0.006	25.0
82	0.000		0.0
96	0.000		0.0

TABLE 2

Concentration and duration of detectable levels of penicillin in milk following intramuscular injection of a single dose of procaine penicillin G in sesame oil with 2% aluminum monostearate at the level of 3,000 units per pound of body weight

Post-treatment time	Concentration of penicillin		Positive samples
	Mean	Range	
(hr)	*(units/ml)*		*(%)*
10	0.018	0.012–0.026	100.0
24	0.036	0.006–0.223	100.0
34	0.016	0.011–0.031	100.0
48	0.013	0.000–0.024	91.7
58	0.013	0.000–0.024	91.7
72	0.012	0.006–0.019	100.0
82	0.004	0.000–0.014	41.7
96	0.002	0.000–0.008	25.0
106	0.000		0.0
120	0.000		0.0

of milk from cows injected intramuscularly with aqueous penicillin was highest at the 10-hr post-injection milking. In contrast, the greatest mean concentration of penicillin in milk from cows injected intramuscularly with penicillin in oil was detected at the 24-hr post-injection milking. After the peak concentration was reached, the mean level of penicillin in milk from cows that received the injection of penicillin in oil decreased at a slower rate than that in milk from cows receiving the injection of aqueous penicillin. The maximum post-injection interval at which penicillin was detectable in milk from any of the cows was 72 and 96 hr for the aqueous penicillin and penicillin in oil, respectively.

The duration of penicillin in milk for the aqueous penicillin intramuscular injection (Table 1) is greater than that reported by other investigators (13-15), who injected between 1,800 and 5,800 units per pound of body weight. Also, the maximum concentration of penicillin in milk (0.222 unit/ml) following intramuscular injections with 3,000 units of the aqueous penicillin per pound of body weight was greater than the maximum concentration reported by Wright and Harold (15), but considerably less than the maximum concentration (0.52 unit/ml) reported by Vaid et al. (14), using the same penicillin salt. However, the cows used by Wright and Harold were milked at 6- and 18-hr intervals, whereas those used by Vaid et al. were milked at 12-hr intervals, and those used in the current study were milked at 10- and 14-hr intervals. In the study by Vaid et al., and in this study, an aliquot of the total milk was used for the assay, whereas Wright and Harold used a composite of fore milk and strippings. These variations in experimental procedures may account for the differences in the maximum period that penicillin was detected in the milk and the differences in the maximum concentrations detected. In all studies involving intramuscular injections of aqueous penicillin, the maximum concentration of the penicillin residue appeared in the first post-injection milking.

The data, Table 2, for the intramuscular injections of penicillin in oil are within the range of concentrations reported by others (3, 11, 14, 15), in which cows were injected with 2,000 to 6,000 units per pound of body weight. In contrast, Albright et al. (1) and Snyder et al. (13) failed to detect penicillin in milk during the first 48 hr post-injection of a similar preparation at the rate of 3,870 to 5,854 units per pound of body weight. The lowest level of detectable penicillin in the test used by these investigators (1, 13) was 0.05 unit per milliliter. In the current investigation and in two others (10, 15) in which a more sensitive method of assay (9) was used, only 11.5% of all milk samples collected during the first 24 hr contained more than 0.05 unit of penicillin per milliliter. Thus, the failure of Albright et al. (1) and Snyder et al. (13) to detect penicillin in milk with a relatively insensitive method was to be expected. This difference in sensitivity of methods focuses attention on an important point, namely, that minute traces of penicillin probably persisted in the milk for periods greater than those given in Table 1, or reported by others.

The peak mean concentrations of penicillin in milk following intravenous injections with aqueous penicillin occurred at the 6-hr post-injection milking, Table 3. The 44-hr interval was the last milking at which penicillin was detected in milk of any of the cows. This is 20 hr longer than the duration reported by Edwards and Haskins (4), who employed a less sensitive test.

Milk from some cows that received intrauterine infusions of 1,000,000 units of aqueous

TABLE 3

Concentration and duration of detectable levels of penicillin in milk following intravenous injection of a single dose of potassium penicillin G in aqueous suspension at the level of 2,000 units per pound of body weight

Post-treatment time	Concentration of penicillin		Positive samples
	Mean	Range	
(hr)	*(units/ml)*		*(%)*
6	0.092	0.064–0.137	100.0
20	0.002	0.000–0.008	25.0
30	0.001	0.000–0.006	8.3
44	0.001	0.000–0.006	8.3
54	0.000		0.0
68	0.000		0.0

penicillin contained a detectable level of penicillin during the first three post-infusion milkings (27 to 31 hr), but penicillin was not detectable in the milk at subsequent milkings, Table 4. As with intramuscular and intravenous injections of aqueous penicillin, the highest mean concentration of the drug was detected in milk at the first post-infusion milking.

Following oral administration of 10,000,000 units of penicillin in oil (Table 5), the maximum duration of a detectable level of penicillin in milk was 86 hr. The peak mean level of penicillin in milk occurred at the 14-hr post-feeding interval. Skaggs and Miller (12) reported detectable levels of penicillin in milk following oral administration of approximately 173,000 and 270,000 units of procaine penicillin. On the other hand, Wright and Harold (15) did not detect penicillin in milk of cows fed 1,000,000 units daily or 2,500,000 units twice daily of buffered potassium penicillin. It would seem that the type, rather than the amount, of penicillin preparation fed may have been involved in the differences in findings of the three studies. Cows fed the 10,000,000 units in a single feeding showed several symptoms of physiological disturbances including: (a) severe scouring, (b) loss of appetite, (c) reduction of milk yield, and (d) increase in milk fat percentage of the milk produced. Emery, Smith, and Huffman (6) also noted a drop in milk production and loss of appetite as a result of feeding 400 mg of penicillin per cow daily. The physiological disturbances when 10,000,000 units were fed may have altered the absorption and metabolism of the drug.

The variation among individual cows in duration of penicillin secretion in milk following treatment with this drug by various routes is given in Tables 1 to 5. It will be noted that all cows that received intramuscular or intravenous injections of penicillin produced milk with a detectable level of the drug at one or more post-injection milkings. Yet, at subsequent intervals the percentage of the cows that produced milk containing penicillin varied. For this reason, a statistical study was made to determine the correlation between the post-treatment duration of production of milk con-

TABLE 4

Concentration and duration of detectable levels of penicillin in milk following intrauterine infusion of 1,000,000 units of potassium penicillin G in aqueous suspension

Post-treatment time	Concentration of penicillin		Positive samples [a]
	Mean	Range	
(hr)	*(units/ml)*		*(%)*
3–7	0.012	0.000–0.031	75.0
17–21	0.004	0.000–0.011	41.7
27–31	0.001	0.000–0.007	16.7
41–45	0.000		0.0
51–55	0.000		0.0

[a] No detectable penicillin was found in the milk from two of the 12 cows at any time.

TABLE 5

Concentration and duration of detectable levels of penicillin in milk following oral administration of 10,000,000 units of procaine penicillin G in sesame oil with 2% aluminum monostearate

Post-treatment time	Concentration of penicillin		Positive samples [a]
	Mean	Range	
(hr)	*(units/ml)*		*(%)*
14	0.010	0.000–0.055	75.0
24	0.006	0.000–0.030	58.3
38	0.003	0.000–0.010	41.7
48	0.006	0.000–0.043	33.3
62	0.001	0.000–0.006	16.7
72	0.002	0.000–0.009	25.0
86	0.001	0.000–0.006	8.3
96	0.000		0.0
110	0.000		0.0

[a] All test cows had at least one positive sample.

TABLE 6

Correlations between duration of secretion of milk containing penicillin and other variables following administration by various routes [a]

Other variables	Vehicle and routes of administration				
	Intramuscular		Intravenous	Intrauterine	Orally
	Oil	Aqueous	Aqueous	Aqueous	Oil
	(r)				
Milk production	−0.052	−0.062	0.405	−0.026	−0.025
Milk fat percentage	0.121	−0.284	−0.233	0.375	−0.493
Body weight	−0.181	0.081	−0.039	0.367	−0.179

[a] d.f. = 11 for all tests.

taining detectable levels of penicillin and average daily milk production, fat percentage in milk, and body weight. All correlations between duration of penicillin in milk and the other variables were low (Table 6). These correlation data indicate that neither the amount of milk produced, the fat percentage in milk, nor body weight of the cows contributed significantly ($P > .05$) to the variation in duration of secretion of penicillin in milk.

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REPRODUCTIVE CAPACITY OF DAIRY BULLS. VIII. DIRECT AND INDIRECT MEASUREMENT OF TESTICULAR SPERM PRODUCTION [1, 2, 3]

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SUMMARY

It was desired to determine testicular daily sperm production (DSP) and compare it with daily sperm output (DSO). Two independent calculations of DSP for each testis from eight 2-yr-old and four mature Holstein bulls were based on counts of gonadal sperm reserves and on quantitative testicular histology. Limitations of the proposed methods and formulas were discussed. The duration of spermatogenesis in bulls was calculated to be 60 days. The average DSP values calculated from gonadal sperm reserves and histological data were 9.98 and 12.76 billion per bull. The differences between DSP values for the 24 testes by the two methods of calculation were highly significant, but the two values were correlated (+0.81). However, testis weight accounted for most of the relationship, since the correlation was not significant when calculated on the basis of DSP per gram of testis (+0.28). Since the validity and repeatability of these methods has not been established, the two DSP values were averaged and ranged from 7.7 to 13.2 billion for eight bulls of similar age and body weight.

The DSO during collection of six ejaculates weekly with intensive sexual preparation (6×) also was obtained for the 12 bulls. About five months elapsed between collection of DSO data and postslaughter determination of DSP. The DSO on 6× averaged 4.81 ± 0.35 billion, or only 42% of the mean DSP of 11.49 ± 0.64 billion. However, the highly significant correlation between DSP and DSO on 6× (+0.87) suggests that DSP can be estimated in individual living bulls by collecting semen at a suitable high ejaculation frequency.

While there are many reports on the sperm output and semen characteristics of bulls collected at various ejaculation frequencies, quantitative data on production of spermatozoa by the testes are lacking. Recent reports (2, 8, 12, 13, 18) have suggested that testicular daily sperm production could be inferred from daily sperm output by collecting semen at a sufficiently high ejaculation frequency. To determine the relationship between sperm production and the number of sperm which can be collected, however, direct measurement of testicular production is essential. Kennelly (14) has calculated testicular sperm production for boars.

The objectives of the present study were to develop methods of determining testicular sperm production in bulls and to assess the relationship between daily sperm production and daily sperm output. Two methods of calculating testicular sperm production are proposed. The first is based on counts of gonadal sperm reserves (1, 7, 9); the other involves the more tedious techniques of quantitative histological evaluation of the testes (5). Since both methods required sacrifice of the animal, the testicular sperm production for 12 bulls was compared with their daily sperm output during collection at a frequency of six ejaculates per week, using intensive sexual preparation. A preliminary report of this work was presented in 1960 (4).

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GENERAL PROCEDURE

Two groups of bulls were used. Group A consisted of eight 29-month-old Holstein bulls which were ejaculated six times per week (6×) for 5 wk, unilaterally vasectomized, and then either sexually rested or ejaculated two or eight times per week for 20 wk just prior to slaughter. Group B included four intact, mature Holstein bulls. One was sexually rested and the others were ejaculated either six, seven, or eight times weekly preceding slaughter. However, data for daily sperm output (DSO) on 6× had been obtained for all four Group B bulls within five months prior to slaughter.

In each case, 6× collection was continued for a minimum of five consecutive weeks and the first week's data were excluded as an adjustment period. During collection at 6×, two successive ejaculates were collected by artificial vagina each Monday, Wednesday, and Friday. Intensive sexual preparation, consisting of one false mount, 2 min of restraint, and two additional false mounts, was used prior to each ejaculation. Thus, with the exception of Bull 137, who was collected at 6× for 26 wk immediately before slaughter, the DSO data which are compared with the calculated testicular daily sperm production (DSP) values were obtained about five months prior to slaughter. The semen characteristics, gonadal and extragonadal sperm reserves, and quantitative testicular histology of these bulls have been described (1, 5, 8, 9).

At the time of slaughter, testicular weight and sperm reserves were determined (7) and tissue samples for histological evaluation were taken from three loci of each testis (5). The percentage by volume of seminiferous tubules in the testis and uncorrected major and minor diameters of Stage I seminiferous tubules were determined as previously described (5). The mean number of spermatids in Stage I seminiferous tubules for each testis was based on counts of ten randomly selected 7-μ tubule cross sections in each of three loci, or a total of 30 Stage I tubules per testis. The actual counts were corrected for variation in nuclear diameter (5). To compensate for tissue shrinkage resulting from histological processing a correction term was used. Amann (5) found that in four testicular samples processed in a manner identical to that used in the present work, linear shrinkage averaged 19.5% and volumetric shrinkage 47.8%. Thus, the volume of each testis recorded at slaughter was multiplied by a factor of 0.522, so that it would be comparable with the volumes of the Stage I seminiferous tubule cylinders used in the spermatid counts. A third correction was necessary to account for the nonproductive areas of the testis, the tunica albuginea and mediastinum. Determinations on five bull testes showed that the tunica albuginea comprised 8.7 ± 0.2% (±S.E.) of the testicular weight and that the mediastinum occupied about 1% of the testis volume (5). Since testicular weight and volume are similar (density 1.04 g/cm^3) (5), a factor of 0.903 was used to correct for these nonproductive areas.

CALCULATION OF TESTICULAR SPERM PRODUCTION

Duration of spermatogenesis. An accurate estimate of the duration of spermatogenesis is essential for any calculation of sperm production. For bulls, this was obtained from recent studies on the relative frequency of the stages of the cycle of the seminiferous epithelium (5), the pattern of spermatogenesis (6), and the time interval between injection of P^{32} and appearance of radioactive sperm in the proximal caput epididymis or ejaculated semen (11, 15-17, 19, 20). With rams, Ortavant (18) demonstrated that P^{32} was incorporated into the DNA of developing germ cells only in the preleptotene phase of primary spermatocytes or in the more primitive spermatogonia. Thus, the first radioactive sperm to appear in the caput epididymis would be those which had been in the preleptotene phase at the time of P^{32} injection. In bulls, spermatogenesis requires about 4.5 cycles of the seminiferous epithelium (5, 6). Preleptotene primary spermatocytes are found in Stage I of the third cycle prior to release of the resulting spermatozoa (see Figure 4, Reference 6). Therefore, the time interval between P^{32} administration and release of radioactive spermatozoa into the bovine seminiferous tubule lumen is 3.0 cycles of the seminiferous epithelium or 3.0/4.5 of the duration of spermatogenesis.

From the data of Koefoed-Johnsen (15, 16) and Dawson (11) it was calculated that about 39 days elapsed between injection of P^{32} into bulls and release of radioactive sperm from the seminiferous tubules. This value is based upon extrapolation from the subsequent arrival of these radioactive sperm in ejaculated semen. Although frequently cited incorrectly, careful examination of the report by Ortavant (19) reveals that he found an interval of 40 to 42 days between the injection of P^{32} into bulls and the appearance of radioactive spermatozoa in the lumen of the seminiferous tubules. This is not the duration of spermatogenesis. More

recently, Orgebin and co-workers (17, 20) reported that it required 41 days between the injection of P^{32} in bulls and the arrival of radioactive sperm in the proximal caput epididymis. Thus, it must require about 40 days for daughter spermatozoa from a preleptotene primary spermatocyte to be released into the seminiferous tubule lumen. Using this value, the duration of spermatogenesis in bulls can be calculated in the following manner:

$$\text{Duration of one cycle of the seminiferous epithelium} = \frac{40}{3} = 13.3 \text{ days}$$

$$\text{Duration of spermatogenesis} = (13.3)(4.5) = 60 \text{ days}$$

If 41 days rather than 40 is used, the duration of spermatogenesis would be 61.5 days.

Determination of sperm production from gonadal sperm reserves. It is logical to assume that the gonadal sperm reserve, which is the total number of sperm and late spermatids counted in an aqueous testicular suspension, represents some function of daily testicular sperm production. Morphological examination of spermatids in fixed seminiferous tubule cross sections suggests that only the older generation of spermatids in Stages VI, VII, and VIII of the cycle of the seminiferous epithelium would be counted in these testes suspensions. These stages represent 24.6% of the cycle of the seminiferous epithelium (5), which has a duration of 13.3 days. Thus, testis sperm counts represent 3.27 days' sperm production, and theoretical daily sperm production from sperm count data (TDSPC) can be calculated as follows:

$$\text{TDSPC} = \frac{\text{testis sperm count}}{3.27}$$

The validity of values calculated by this method is limited primarily by the assumption that the gonadal sperm reserves represent spermatids and spermatozoa present in Stages VI, VII, and VIII of the cycle of the seminiferous epithelium.

Determination of sperm production from histological data. Estimation of theoretical daily sperm production from quantitative histological data (TDSPH) requires the use of a formula such as that shown below.

This formula is based on the assumption that DSP can be expressed as a function of the number of spermatids per unit volume reaching a certain fixed point in spermatogenesis (Part *c* of formula), when the number of unit volumes in the testis (*ab/ef*) is known. In this application, a unit volume is the ellipsoidal cylinder of a seminiferous tubule cross section 7 μ high and with major and minor diameters of approximately 225 and 205 μ. Although spermatids are not released from a tubule until Stage VIII of the cycle of the seminiferous epithelium, Stage I was chosen as the optimum point to count spermatids. This is the last point in spermiogenesis when spermatids are essentially round and can be counted readily. In the subsequent seven stages prior to their liberation as spermatozoa, spermatids become elongated and grouped in bundles. Selection of Stage I spermatids rather than primary spermatocytes or spermatogonia is desirable, since there probably is little loss of potential spermatozoa after this point (6). Thus, it should not be necessary to correct for testicular degeneration of germ cells.

Introduction of the required time factor is simplified by assuming that 100% of the seminiferous tubule cross sections in a testis are in Stage I rather than the actual value of 30.3% (5). This assumption is valid, since (*abc/def*) is equal to (*abc/ef*) (30.3%)/(*d*) (30.3%), which is the correct expression. Thus, TDSPH is the total number of Stage I spermatids in the testis (*abc/ef*) divided by the duration of one cycle of the seminiferous epithelium (*d*). Although it requires 4.5 cycles of the seminiferous epithelium to form spermatozoa, because there are four or five generations of germ cells in a seminiferous tubule cross section and spermatozoa are released at each Stage VIII, it is proper to divide by the duration of one cycle, i.e., 13.3 days. In a preliminary report of this formula (4), the value for 4.5 cycles was erroneously given.

As suggested above, testis volume must be corrected for histological shrinkage and for the volume occupied by the tunica albuginea and mediastinum. Determinations of the volume percentage of seminiferous tubules in the testes

$$\text{TDSPH} = \frac{\begin{pmatrix}(a)\\ \text{Corrected testis volume}\end{pmatrix}\begin{pmatrix}(b)\\ \text{Per cent seminiferous tubules in the testis}\end{pmatrix}\begin{pmatrix}(c)\\ \text{Corrected number of spermatids per Stage I cross section}\end{pmatrix}}{\begin{pmatrix}\text{Duration of one cycle of the seminiferous epithelium}\\ (d)\end{pmatrix}\begin{pmatrix}\text{Area of Stage I tubule cross section}\\ (e)\end{pmatrix}\begin{pmatrix}\text{Thickness of histological section}\\ (f)\end{pmatrix}}$$

excluded these latter areas. Although testis volumes were recorded at the time of slaughter, testis weight was considered a more accurate measure. Thus, the corrected testis volume (*a*) for use in the TDSPH formula was calculated as shown below.

$$\text{Corrected testis volume} = \frac{\left(\begin{array}{c}\text{Testis}\\ \text{weight}\end{array}\right)\left(\begin{array}{c}\text{Shrinkage}\\ \text{correction}\end{array}\right)\left(\begin{array}{c}100\%-\%\\ \text{tunic and}\\ \text{mediastinum}\end{array}\right)}{(\text{Testis density})}$$

$$= \frac{(\text{Testis weight})\,(0.522)\,(0.903)}{(1.04)}$$

$$= (0.452)\ (\text{Testis weight})$$

The percentage of seminiferous tubules in the testis (*b*), excluding the tunica albuginea and mediastinum, and the corrected number of spermatids per Stage I tubule cross section (*c*) were previously reported (5). The duration of one cycle of the seminiferous epithelium (*d*) was calculated to be 13.3 days. The area of a Stage I seminiferous tubule cross section (*e*) was calculated from the major and minor diameters for 30 tubules per testis, although for simplicity Table 1 shows only an average tubule diameter for each testis. All histological sections were cut at a thickness of 7 μ (*f*). Individual testis averages for percentage of seminiferous tubules, corrected number of spermatids per Stage I seminiferous tubule cross section, and area of Stage I tubule cross sections were used (Table 1).

The accuracy of TDSPH values is limited by several factors. Probably of greatest importance is the degree of tissue shrinkage resulting from histological processing. Since data for each testis used in this study were not available, the correction for tissue shrink-

TABLE 1

Testicular characteristics at slaughter

Bull	Age at slaughter	Testis weight		Gonadal sperm reserve		Volume of tubules in testis		Avg tubular diameter [a]		Avg no. spermatids per Stage I tubule cross section [b]	
		Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
	(months)	*(g)*	*(g)*	*($\times 10^9$)*	*($\times 10^9$)*	*(%)*	*(%)*	*(μ)*	*(μ)*		
					Group A bulls						
160	36	303 [c]	356	18.6	17.7	76	80	215	220	231	224
162	36	260	279 [c]	14.8	15.0	72	74	220	224	224	245
163	36	316 [c]	321	19.1	19.6	80	78	229	232	205	213
165	36	251 [c]	255	9.4	9.7	76	78	206	207	170	168
168	36	319 [c]	346	14.2	16.5	77	79	214	218	166	188
169	36	286	289 [c]	14.1	13.7	75	77	204	206	193	184
170	36	251	250 [c]	12.8	12.7	78	77	206	205	198	201
173	33	285	290 [c]	17.5	18.1	76	76	211	215	179	181
Avg	36	284	298	15.1	15.4	76	77	213	216	196	200
					Group B bulls						
Regal	104	343	327	15.2	15.3	76	76	220	221	184	196
135	77	302	313	15.8	15.1	74	75	223	224	209	207
137	65	423	405	20.4	21.3	72	70	233	237	244	254
Wayne	104	457	478	22.3	22.9	66	73	225	224	225	216
Avg	88	381	381	18.4	18.6	72	74	225	226	216	218

[a] Average of the major and minor tubular diameters uncorrected for histological shrinkage.
[b] Average corrected number of spermatids per 7-μ cross section, excluding those free in the seminiferous tubule lumen.
[c] Testis on the vasectomized side.

age was based on that occurring in four other testes processed in an identical manner (5). There appears to be some variation in the degree of shrinkage among testicular samples even when processed in an identical manner. Thus, in future applications of this formula it would appear desirable to determine the percentage of tissue shrinkage for each testis. The technical problems of such measurements are increased by differential shrinkage between seminiferous tubules and interstitial tissue and the influence of size of the tissue block. Errors involving the percentage of the testis occupied by the tunica albuginea and mediastinum, the correction term for spermatid counts, degeneration of spermatids during the last cycle of the seminiferous epithelium, and in the basic histological data probably are relatively minor. The duration of spermatogenesis should be quite accurate, but needs confirmation.

RESULTS

The characteristics of the 12 Holsteins and the values used in calculating DSP from gonadal sperm reserves and histological data are presented (Table 1). There was considerable variation in testes weight and gonadal sperm reserves among bulls. However, the correlation between testis weight and testis sperm count was highly significant (+0.82). In Group A bulls, the volume of seminiferous tubules in the testis was quite consistent near a value of 77%. Testes of mature Group B bulls generally contained a lower percentage of spermatogenic tissue, even though the seminiferous tubule diameters for these bulls were greater than for the younger bulls. The mean number of spermatids per 7 μ Stage I seminiferous tubule cross section varied considerably and for the 24 testes ranged from 166 to 254. These values have been discussed in detail (5).

Using either the TDSPC or TDSPH method, the difference in sperm production between the left and right testis of a bull was small (Table 2). Within both groups of bulls the DSP varied considerably, but the DSP for mature Group B bulls was higher ($P < 0.01$) than that for the younger bulls when calculated by either method. The average values for all 12 bulls were 9.98 and 12.76 billion sperm per day for TDSPC and TDSPH. The differences between sperm production values for the 24 testes by the two methods of calculation were highly significant, but the two values were highly significantly correlated (+0.81). However, the correlation was influenced greatly by differences in testis weight. When the TDSPC and TDSPH methods were compared on the basis of DSP per gram of testis the correlation was insignificant (+0.28). Thus, it appears that errors of measurement are relatively large. Future refinements of the quantitative tech-

TABLE 2

Comparison of daily sperm production as calculated from testis sperm count data (TDSPC) and from histological data (TDSPH) with daily sperm output on six ejaculates per week using three false mounts per ejaculate

(Sperm in billions)

	TDSPC				TDSPH				
Bull	Left testis	Right testis	Total	% ejac. on 6×	Left testis	Right testis	Total	% ejac. on 6×	DSO on 6×
				Group A bulls					
160	5.70	5.40	11.09	54.5	7.10	8.14	15.25	39.6	6.04
162	4.51	4.57	9.09	41.0	5.33	6.25	11.57	32.2	3.73
163	5.85	6.00	11.85	45.1	6.09	6.22	12.31	43.4	5.34
165	2.88	2.96	5.84	56.1	4.73	4.76	9.50	34.5	3.28
168	4.35	5.05	9.40	60.0	5.49	6.66	12.14	46.4	5.64
169	4.32	4.19	8.51	57.5	6.23	5.98	12.21	40.1	4.90
170	3.91	3.89	7.81	37.5	5.63	5.68	11.31	25.9	2.93
173	5.36	5.54	10.90	43.7	5.43	5.36	10.78	44.2	4.77
Avg			9.31	49.4			11.88	38.3	4.58
				Group B bulls					
Regal	4.64	4.66	9.31	40.0	6.11	6.17	12.28	30.3	3.73
135	4.84	4.61	9.45	47.0	5.76	5.96	11.72	37.9	4.44
137	6.23	6.51	12.74	47.5	8.46	7.99	16.44	36.8	6.05
Wayne	6.81	7.00	13.81	49.6	8.32	9.34	17.66	38.8	6.84
Avg			11.33	46.0			14.52	36.0	5.26

niques may reduce these errors. Further research on the two methods should be concerned with their validity and degree of repeatability. At present, it is impossible to determine which, if either, method accurately measures DSP. Therefore, the mean value for the two methods was used to estimate DSP.

Based on the mean value for each testis, the 12 bulls produced 11.49 ± 0.64 billion sperm per day. The DSO of these bulls on 6× per week (Table 2) was 4.81 ± 0.35 billion and represented $48.3 \pm 2.1\%$ of the TDSPC and $37.5 \pm 1.7\%$ of the TDSPH. This suggests that less than one-half of the sperm produced were collected on 6× with intensive sexual preparation. These values may be slightly low because of the 5 months between 6× collection and slaughter. However, even at ejaculation frequencies as high as ten per day without sexual preparation or two per day with intensive sexual preparation (13), less than 50% of the sperm produced were collected from bulls Wayne and 137.

Estimating DSP in living bulls would be advantageous for many experiments. The correlations between DSO on 6× and both TDSPC and TDSPH were highly significant (Table 3). This suggests that DSO on 6× with intensive sexual preparation can be used to estimate DSP. Although testes weight is in the formula for TDSPH, the correlations between paired testes weight and DSP probably are meaningful. However, since age affects testis characteristics and function (5), it appears that DSO is a more reliable approach. Presumably, DSO on 14× with intensive sexual preparation would provide even a better estimate of DSP than that obtained with 6× collection (8).

Using the average of the TDSPC and TDSPH values, the resulting estimate of DSP was highly significantly correlated to DSO on 6× (+0.87). Thus, a rough estimate of DSP can be obtained by linear regression. The appropriate equation based on these 12 Holstein bulls is shown below. Both DSP and DSO on 6× with intensive sexual preparation are expressed in billions of sperm.

$$DSP = (1.58)(DSO) + 3.80$$

Insufficient data are available for calculating the regression between DSP and DSO at a frequency of 14× per week. If paired testes weight is known, DSP can be estimated by the following equation in which testes weight is expressed in grams and DSP in billions of sperm per day ($r = +0.92$).

$$DSP = (0.0159)(\text{paired testes weight}) + 1.19$$

DISCUSSION

Although several workers (14, 18) have determined the gonadal sperm reserves of domestic animals, they have not used the resulting values to estimate DSP. The absence of this interpretation of gonadal sperm reserves probably resulted from insufficient data on the duration of spermatogenesis and on the pattern of spermatocytogenesis in bulls, boars, and rams. As this information becomes available (5, 6, 14-20), the formula for TDSPC should be applicable to species other than the bull. The only species modifications requiring attention are (a) the duration of one cycle of the seminiferous epithelium and (b) the percentage of a cycle of the seminiferous epithelium represented by the spermatids and spermatozoa counted. This latter value may differ with various liberation and counting techniques (1, 7, 14, 18), as well as the species.

The only formula for DSP, similar to that for TDSPH given above, was proposed by Kennelly (14). His formula, shown below, was based on an estimate of the total volume of primary spermatocytes in the boar testis, with a correction for the subsequent degeneration of potential spermatozoa.

$$\text{DSP per testis} = \frac{\left(\begin{array}{c}\text{Total number of sper-}\\ \text{matocytes in the}\\ \text{testis}\end{array}\right)\left(\begin{array}{c}\text{Per cent of spermato-}\\ \text{cytes which complete}\\ \text{spermatogenesis}\end{array}\right)\left(\begin{array}{c}\text{Number of spermato-}\\ \text{zoa derived from one}\\ \text{spermatocyte}\end{array}\right)}{\left(\begin{array}{c}\text{Per cent of cycle of seminiferous}\\ \text{epithelium required for}\\ \text{spermatocyte evolution}\end{array}\right)\left(\begin{array}{c}\text{Duration of one cycle}\\ \text{of the seminiferous}\\ \text{epithelium}\end{array}\right)}$$

He derived the total number of spermatocytes per testis as follows:

$$\begin{array}{c}\text{No. sper-}\\ \text{matocytes}\\ \text{per testis}\end{array} = \frac{\begin{array}{c}\text{(Testis vol.) (vol. \% of}\\ \text{spermatocytes in testis)}\end{array}}{\text{(Mean vol. of a spermatocyte)}}$$

Kennelly did not have data on the duration of spermatogenesis, the cycle of the seminiferous epithelium, and the extent of testicular germ

TABLE 3

Correlation coefficients for 12 bulls

	TDSPC [a]	TDSPH [b]	DSO on 6×	Body weight
Paired testes weight	0.84 [c]	0.91 [c]	0.82 [c]	0.53
Body weight	0.24	0.35	0.04	
DSO on 6×	0.85 [c]	0.82 [c]		
TDSPH	0.83 [c]			

[a] Testicular daily sperm production per bull based on sperm count data.
[b] Testicular daily sperm production per bull based on histological data.
[c] Significant at the 1% level of probability.

cell degeneration in boars. Therefore, a number of assumptions based on research for other species were made. The inadvisability of such extrapolations has been emphasized (5, 14, 20). Kennelly's formula has one distinct advantage over that for TDSPH, however, in that it is based on the relative volume of spermatocytes in the testis. Hence, it is probably unaffected by the problem of histological tissue shrinkage.

The calculated DSP for the 12 bulls in the present study of 11.5 billion sperm per day indicates that the rate of testicular sperm production is much higher than previously suspected. However, Kennelly (14) estimated that six young Berkshire boars produced an average of 19.6 billion sperm per day. This value represented a production of 27.6 million sperm per gram of testis each day, as contrasted to 17.7 million for the 12 bulls. This difference between boars and bulls is not surprising if, as suggested by Ortavant (19), a stem A-spermatogonium in boars produces 96 spermatids as contrasted to 64 spermatids in bulls and rams (6, 18). In addition, although not precisely determined, the duration of spermatogenesis appears to be shorter in boars than in rams or bulls (20).

Cupps and Laben (10) compared counts of spermatids in Stage II seminiferous tubule cross sections from the testes of 13 abnormal and normal bulls with the concentration of sperm in their ejaculated semen. Although the semen samples had been collected at weekly intervals and ranged in mean sperm concentration per bull from 10 to 2,060 $\times 10^6$/ml, a correlation of +0.82 was reported. For the 12 bulls in the present study, the correlations between the mean number of spermatids per Stage I seminiferous tubule cross section and the mean sperm concentration on 6× (−0.08) or the total number of sperm per ejaculate on 6× (+0.35) were not significant. This disagreement may be associated with the experimental animals used. At least one-half of the 13 animals studied by Cupps and Laben (10) apparently had abnormal testicular histology, whereas the testes of the animals in the present study were normal (5). Since the correlation between TDSPH and total number of sperm per ejaculate (+0.82) was highly significant, the variations in testicular weight and seminiferous tubule diameter also are important considerations when trying to equate testicular histology and semen characteristics. While sperm concentration and ejaculate volume are not entirely independent, it would appear that the total number of sperm per ejaculate should provide a better estimate of testicular function than sperm concentration. As recently demonstrated (8, 13) with unilaterally vasectomized bulls, unless a high frequency of ejaculation is used even a simulated deficiency of 50% in sperm production can pass undetected when evaluated by semen characteristics. Thus, we believe that, except for drastic abnormalities, testes function of living bulls can be evaluated best by collection of two ejaculates daily for at least 3 wk (8, 9).

Kennelly (14) concluded that 30% of the sperm production of boars was resorbed in the excurrent ducts, since the six boars with a DSP of 19.6 billion ejaculated only an average of 14.0 billion sperm per day while on daily collection. Although lower than the value of 58% resorption for the bulls on 6× in the present study, both values suggest that the resorption of sperm in the excurrent ducts is an important physiological process. These differences cannot be accounted for by sperm losses during the collection process. As suggested by Kennelly (14), and discussed in detail by Amann and Almquist (9), the rate of sperm resorption apparently is inversely related to the output of sperm by collection and masturbation and partially dependent upon the degree to which the storage capacity of the epididymis is filled. During sexual rest, the rate of sperm resorption in the epididymis approaches 100% (9).

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DILUENTS FOR BOVINE SEMEN. XII. FERTILITY AND MOTILITY OF SPERMATOZOA IN SKIMMILK WITH VARIOUS LEVELS OF GLYCEROL AND METHODS OF GLYCEROLIZATION[1, 2]

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SUMMARY

Laboratory studies showed that the beneficial effect of glycerol on the maintenance of sperm motility in skimmilk diluent during 5 C storage was related both to level and method of glycerol addition. Optimum livability was obtained with 5% glycerol and when the glycerol-containing fraction was added stepwise to the partially diluted semen at 5 C. Direct addition of semen to completely formulated skimmilk–13% glycerol diluent at room temperature significantly reduced sperm livability as compared to that in partially diluted semen glycerolated in a stepwise fashion at 5 C. Even when glycerol level in the complete diluent was lowered from 13 to 5%, and the semen added at room temperature, livability during 14 days of storage was slightly better in the skimmilk–13% glycerol control samples glycerolated stepwise at 5 C. Dilution at room temperature into completely formulated skimmilk-glycerol diluents containing more than 5% glycerol also resulted in abnormal sperm motility (circular or backward movement).

In skimmilk-glycerol diluents containing 5, 10, and 20% glycerol, livability was superior ($P < 0.01$) when the glycerol fraction was added to the partially diluted semen at 5 C in three steps rather than all at once at 5 C or dropwise during the cooling process. Irrespective of glycerolization method, there was a highly significant increase in mean livability with each decrease in glycerol level. However, based on a split-sample field trial involving 9,606 first inseminations, fertility on any of the four days of use was not significantly different in skimmilk-diluted semen containing 5% rather than 10% glycerol.

Heated milk containing 10% glycerol is being used successfully by many artificial breeding organizations as a liquid semen diluent. A recent report from this laboratory (4) concerned the motility of bovine spermatozoa in skimmilk diluents at glycerol levels ranging from 10 to 25%. Glycerol levels of 10 and 13% maintained motility significantly greater than no glycerol. Fertility trials showed that the addition of 10% glycerol to milk diluents significantly reduced the decline in fertility with increasing age of semen; however, no significant difference in fertility was found between 10 and 15% glycerol.

There is relatively little information on the effectiveness of other glycerol levels, especially levels under 10%, for maintaining fertility and motility of bovine spermatozoa. In addition, present glycerolization procedures are involved and time-consuming. They require the preparation and handling of two diluent fractions: (a) a nonglycerol fraction into which the semen is diluted at room temperature to one-half the desired final concentration, and (b) a glycerol fraction of equal volume, containing twice the desired final concentration of glycerol, which is added gradually to the nonglycerol fraction at 5 C. A study of methods of glycerolization might lead to simplification of this procedure.

The objectives of the present study were to determine the effect of different glycerol levels and various methods of glycerol addition upon

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the fertility and livability of bovine spermatozoa in glycerol-containing skimmilk diluents stored at 5 C.

GENERAL PROCEDURE

Semen used in the livability studies was collected by artificial vagina from dairy bulls. Ejaculates used had at least an initial motility of 60% and a sperm concentration of 900 × 10^6/ml. Fresh, unfortified skimmilk was heated in a water bath and held between 92 and 95 C for 10 min. Diluents were prepared either as complete glycerol-containing diluents or as separate nonglycerol and glycerol fractions. In both laboratory and field experiments, 1,000 units of potassium penicillin G and 1,000 μg of dihydrostreptomycin sulfate were added per milliliter to the complete diluent or to both the nonglycerol and glycerol fractions. Semen was added to provide a final concentration of 15 × 10^6/ml motile sperm. The completely diluted semen or the partially diluted semen and glycerol fractions were cooled gradually to 5 C over a period of about 3 hr. The partially diluted semen was glycerolated at 5 C by different methods as described under the individual experiments.

Livability of coded semen samples during 5 C storage for 12 or 14 days was studied by estimating the percentages of progressively motile spermatozoa at 37 C. The data were analyzed by procedures outlined by Snedecor (13) and Steel and Torrie (14).

EXPERIMENTAL DESIGN AND RESULTS

Experiment 1. The effect of methods of glycerol addition on sperm livability was studied using a skimmilk–13% glycerol diluent. Methods of glycerolization tested were: (a) complete, semen added at room temperature to completely formulated skimmilk–13% glycerol diluent; (b) all at once, equal volume of skimmilk–26% glycerol added to partially diluted semen at 5 C in one step; and (c) stepwise, equal volume of skimmilk–26% glycerol added to partially diluted semen at 5 C, either in three portions (20, 30, and 50% by volume) at 10-min intervals or in six equal portions at 5-min intervals. A skimmilk control not containing glycerol also was tested.

Mean percentages of progressively motile sperm for 12 ejaculates from 11 different bulls are presented (Table 1). Superior livability ($P < 0.01$) was obtained when glycerol was added at 5 C, either all at once or by the stepwise procedures as compared to that in complete diluent or skimmilk alone. When the means within a particular day of storage were tested by Duncan's New Multiple Range Test (MRT), motility in skimmilk alone was higher than in the complete diluent during the first four days of storage ($P < 0.01$), but not different from any of the three treatments involving glycerolation at 5 C. However, between the fourth and sixth days of storage there was a severe drop in mean motility of sperm in skimmilk alone. Throughout the 12-day storage period, mean motilities in diluents glycerolated all at once or by the two stepwise methods were not statistically different.

Experiment 2. Relatively poor livability was obtained in Experiment 1 with a complete skimmilk diluent containing 13% glycerol. Thus, lower levels of glycerol were tested in complete skimmilk-glycerol (SG) diluents to determine whether a complete diluent could be formulated. Complete SG diluents containing 5, 7, 9, 11, and 13% glycerol by volume were tested. A stepwise skimmilk–13% glycerol diluent (three equal portions added at 5 C at 10-min intervals) and a nonglycerolated skimmilk diluent were included as controls.

TABLE 1

Effect of glycerolization method on the livability of spermatozoa in skimmilk–13% glycerol diluent

(Mean percentage of motile sperm for 12 ejaculates)

Glycerolization method	Days of storage at 5 C								Mean
	1	2	3	4	6	8	10	12	
Complete	49 [b]	46 [b]	39 [b]	35 [b]	21 [c]	15 [b]	5 [b]	2 [a, b]	26.7
All at once	61 [a]	59 [a]	58 [a]	55 [a]	51 [a]	40 [a]	19 [a]	8 [a, b]	43.8
Stepwise [d]	65 [a]	65 [a]	64 [a]	64 [a]	56 [a]	40 [a]	18 [a]	11 [a]	48.0
Stepwise [e]	66 [a]	66 [a]	62 [a]	60 [a]	54 [a]	43 [a]	22 [a]	12 [a]	48.2
Skimmilk alone	68 [a]	68 [a]	66 [a]	60 [a]	38 [b]	13 [b]	4 [b]	0 [b]	39.5

[a, b, c] Means within a particular day followed by different superscript letters are different at the 1% level of probability (Duncan's New Multiple Range Test).

[d] Three additions (20, 30, and 50%) at 10-min intervals.

[e] Six equal additions at 5-min intervals.

The mean percentages of motile sperm for ten ejaculates from eight bulls (Table 2) generally declined in complete SG diluents as the percentage of glycerol increased from 5 to 13%. Mean livability in diluents containing 5 and 7% glycerol did not differ significantly, but each was highly significantly superior ($P < 0.01$) to complete diluents containing 9, 11, or 13% glycerol. The latter three complete diluents also were inferior ($P < 0.01$) to the stepwise skimmilk–13% glycerol control. Based on the MRT, within any particular day the complete SG diluents containing 5 and 7% glycerol and skimmilk alone also maintained motility during the first eight days of storage not significantly different from that in the stepwise control. Beyond the eighth day, the 5 and 7% glycerol levels were superior to the skimmilk control ($P < 0.01$).

Approximately 10 to 20% of the sperm diluted in complete SG diluents containing higher glycerol levels exhibited abnormal motility. Affected sperm moved in circles or backwards as a result of bending of the flagellum at the junction of the tail and midpiece. Defective motility was most evident in complete 11 and 13% glycerol diluents. However, no abnormal motility was noted in the complete 5% glycerol, stepwise–13% glycerol, or skimmilk control diluents. Abnormal motility appeared in two and four of the ten ejaculates diluted in complete SG diluents containing 7 and 9% glycerol, respectively.

Experiment 3. Since sperm livability was significantly lower in complete SG diluents containing more than 7% glycerol, the effect of glycerol level on sperm livability was determined when partially diluted semen was glycerolated in a cold room rather than at room temperature. Glycerol levels of 5, 10, and 20% by volume were tested with the following methods of glycerolization: (a) stepwise, glycerol fraction added at 5 C in three equal portions at 10-min intervals; (b) dropwise, glycerol fraction added during the cooling process by allowing it to drip slowly from a separatory funnel suspended over the partially diluted semen; and (c) all at once, glycerol fraction added at 5 C in one step. Total volume of the glycerol fraction was 25 ml.

Analysis of variance for ten ejaculates from ten bulls showed highly significant differences among glycerolization methods and among glycerol levels (Table 3). The levels × methods, methods × days of storage, and levels × methods × days of storage interactions were not significant.

In view of the highly significant levels × days of storage interaction, MRT was used to compare motilities for the three glycerol levels at each day when averaged for the four methods of glycerolization (Table 3). The 20% glycerol level was consistently inferior ($P < 0.01$) to the 5 and 10% levels throughout the 14-day storage period. A concentration of 5% glycerol was superior to 10% glycerol at the 1 or 5% probability levels only during the middle of the storage period (third to the eighth days). None of the treatments showed abnormal motility, as was encountered in the previous experiment with complete SG diluents.

Comparison among the three levels of glycerol revealed a highly significant improvement in mean livability with each reduction in glycerol from 20 to 10 to 5%. At the 5% level, livability apparently was not affected materially by method of glycerolization. However, a comparison of mean livability among the three methods of glycerolization showed the stepwise method to be highly significantly superior to both the dropwise and all-at-once methods ($P < 0.01$).

Experiment 4. Because 5% glycerol maintained motility superior to the 10% glycerol level in routine field use at that time, a split ejaculate fertility trial was conducted from June 2 to July 18, 1958. After diluting to one-half the final volume in heated skimmilk and cooling to 5 C, each ejaculate was divided into two aliquots. Skimmilk–10% glycerol was added to one aliquot and skimmilk–20% glycerol to the other, using three equal additions at 10-min intervals. To insure greater use of semen on Days 3 and 4, part of the semen involving both treatments was shipped the day after collection and not used on Day 1.

The average 60- to 90-day nonreturn rates for the 112 ejaculates from the two Guernsey and five Holstein bulls are shown in Table 4. The mean fertility for skimmilk–5% glycerol was 1.3 percentage units lower than that for skimmilk–10% glycerol. Neither the mean difference nor any of the differences by day of use were significant.

DISCUSSION

These data show that the effectiveness of glycerol in the maintenance of sperm motility in skimmilk diluent is dependent on both the level of glycerol and the method of glycerol addition. In Experiment 1, livability was prolonged in skimmilk–13% glycerol diluent as compared to a nonglycerolated skimmilk diluent. However, it was necessary to glycerolate the partially diluted semen at 5 C to obtain comparable sperm motility during the first

TABLE 2

Effect of glycerol level on the livability of spermatozoa in complete skimmilk-glycerol diluents (Mean percentage of motile sperm in ten eqaculates)

Glycerol level	Days of storage at 5 C									Mean
	1	2	3	4	6	8	10	12	14	
(%)										
5	68 [a]	68 [a]	65 [a]	60 [a]	53 [a]	46 [a, b]	41 [b]	32 [a, b]	24 [a, b]	50.8
7	65 [a, b]	63 [a, b]	61 [a, b]	58 [a, b]	48 [a, b]	45 [a, b, c]	39 [b, c]	33 [a, b]	23 [b]	48.3
9	59 [b, c]	58 [b, c]	58 [b]	52 [b, c]	45 [b, c]	39 [b, c]	35 [b, c, d]	27 [b, c]	21 [b, c]	43.8
11	59 [b, c]	52 [c, d]	49 [c]	47 [c]	42 [b, c]	38 [c, d]	33 [c, d]	26 [b, c]	18 [b, c]	40.4
13	54 [c]	50 [d]	49 [c]	46 [c]	39 [c]	32 [d]	29 [d]	22 [c]	20 [b, c]	37.9
13-stepwise	69 [a]	66 [a]	65 [a]	61 [a]	53 [a]	51 [a]	49 [a]	37 [a]	30 [a]	52.9
Skimmilk alone	70 [a]	69 [a]	67 [a]	61 [a]	55 [a]	44 [a, b, c]	31 [d]	24 [c]	16 [c]	48.6

[a, b, c, d] Means within any particular day followed by different superscript letters are different at the 1% level of probability (Duncan's New Multiple Range Test).

TABLE 3

Effect of glycerol level and method of glycerolization on the livability of spermatozoa in skimmilk-glycerol diluents

(Mean percentage of motile sperm for ten bulls)

Glycerol-ization method	Glyc-erol level	Days of storage at 5 C										Mean
		1	2	3	4	5	6	8	10	12	14	
	(%)											
	5	71	67	64	61	59	55	48	39	29	18	51.1
Stepwise	10	68	68	65	57	55	50	42	37	28	16	48.6
	20	60	61	52	49	45	43	29	26	18	9	39.2
	Mean											46.3
	5	67	66	63	62	56	52	45	37	25	17	49.0
Dropwise	10	66	65	58	53	52	49	42	36	27	18	46.6
	20	53	49	43	42	35	31	24	19	13	7	31.6
	Mean											42.4
	5	70	67	64	62	57	53	48	39	29	18	50.7
All at once	10	65	61	58	54	51	48	41	34	23	15	45.0
	20	54	48	44	41	38	32	26	19	13	6	32.1
	Mean											42.6
	5	69 [a]	67 [a]	64 [a*]	62 [a]	57 [a]	53 [a*]	47 [a]	38 [a]	28 [a]	18 [a]	50.3
Mean	10	66 [a]	65 [a]	60 [a*]	55 [b]	53 [b]	49 [a*]	42 [b]	36 [a]	26 [a]	16 [a]	46.7
	20	56 [b]	53 [b]	46 [b]	44 [c]	39 [c]	35 [b]	26 [c]	21 [b]	15 [b]	7 [b]	34.3

[a, b, c] Means within a particular day followed by different superscript letters are different at the 1% level of probability; * indicates statistical difference within a group at the 5% level of probability (Duncan's New Multiple Range Test).

TABLE 4

Fertility of semen diluted with heated skimmilk containing 5 and 10% glycerol

Age of semen	No. first services		Per cent nonreturns		
	5% Glycerol	10% Glycerol	5% Glycerol	10% Glycerol	Diff.
Day 1 [a]	2,099	2,119	73.4	74.0	+0.6
Day 2	1,109	1,480	73.6	70.2	−3.4
Day 3	599	637	64.1	67.5	+3.4
Day 4	740	823	58.2	67.1	+8.9
Total	4,547	5,059			
Mean			69.7	71.0	+1.3

[a] Day after semen collection.

four days of storage in the two diluents. In Experiment 2, there was a general gradual improvement in sperm livability as the level of glycerol in complete SG diluents was reduced from 13 to 5%. In addition, sperm diluted in complete SG diluents containing more than 5% glycerol showed abnormal motility, due to a bending of the flagellum at the junction of the mid-piece and tail.

De Groot (5) suggested that the phenomenon of bent tails may be caused by the rapid plasmolysis of a protoplasmic remnant in a hypertonic glycerol diluent. The report by O'Dell et al. (11), that glycerol enters the sperm cell, offers a possible explanation for the absence of abnormally motile sperm in diluents glycerolated at 5 C. It is possible that when glycerol is added slowly to partially diluted semen at 5 C, or added in low concentrations, that sufficient time is allowed for establishment of osmotic equilibrium before sperm ultrastructure or motility is affected.

Comparison of published reports on inclusion of glycerol in liquid semen diluents is confounded by variations in glycerolization procedure. Albright et al. (1) reported that sperm in various milk and egg yolk diluents containing 7.5% glycerol generally showed lower motility immediately after dilution, but that motility declined at a slower rate during storage than in the same diluents without glycerol. They added semen directly to the completely formulated diluents at 32 C. A similar depression of initial motility in complete milk-glycerol diluents was obtained in Experiment 2. Later, Albright et al. (2) reported no significant difference in sperm motility at seven days of storage at 5 C when 7.5% glycerol was added stepwise at 5 C or present in the diluent prior to semen addition at 32 C. In Experiment 1, when 13% rather than 7.5% glycerol was studied, stepwise addition at 5 C maintained highly significantly superior motility during 14 days of storage than direct addition of semen to the completely formulated diluent at room temperature. A recent fertility study (8) provides indirect support for the finding in Experiment 1. A marked reduction in conception rate occurred for semen diluted in reconstituted skimmilk powder containing 10% glycerol at the time of semen addition, as compared with the powdered skimmilk without glycerol. This finding conflicts with other reports (4, 16) which show that inclusion of 10% glycerol in milk diluents improved fertility. In the latter research, however, the glycerol fraction was added stepwise to partially diluted semen at 5 C rather than adding the semen directly to the complete diluent.

In Experiment 3, livability following stepwise addition of glycerol at 5 C was highly significantly superior to that for either the dropwise or all-at-once methods. The relatively poor livability results obtained with the dropwise method may be accounted for partially by the difficulty encountered in regulating the rate of addition with the small volumes (25 ml) used under experimental conditions. Irrespective of method of glycerolization, livability improved with each reduction in the glycerol level from 20 to 10 to 5%. This finding is in contrast to work with glycerolated egg yolk–citrate diluents in which glycerol levels as high as 30% had little effect on sperm livability (9).

The first reports on improved fertility with milk diluents when glycerol was added involved only the 10% level of glycerol (3, 6, 16). In the present study, skimmilk diluent containing 5% glycerol maintained significantly better motility than higher concentrations of glycerol. However, in a fertility trial comparing 5 and 10% glycerol, the 10% level averaged 1.3 percentage units higher than the 5% level; a nonsignificant difference. This result is in direct contrast to laboratory findings that 5% glycerol maintained a higher level of motility than 10% glycerol on the third and fourth days of storage. In a recent report from this laboratory (4), there was no significant difference in fertility of semen in homogenized milk diluent containing 10 and 15% glycerol.

Although the livability data presented in this study substantiate other reports (1, 4, 6, 12, 15) that glycerol enhances sperm livability in liquid semen diluents, little information is available to adequately explain the specific role of glycerol in diluents stored at 5 C. Studies in this laboratory (11) and elsewhere (7) have shown that sperm can metabolize glycerol. O'Dell et al. (10) have shown that the addition of glycerol to commonly used semen diluents has a depressing effect on lactic acid production by sperm. However, whether the beneficial role of glycerol in liquid semen diluents is primarily metabolic or physical in nature remains to be demonstrated.

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SPERM OUTPUT OF DAIRY BULLS WITH VARYING DEGREES OF SEXUAL PREPARATION [1]

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SUMMARY

The sperm output value of false mounting relative to other sexual preparation stimuli was assessed by ejaculating 151 mature dairy bulls weekly for ten consecutive weeks after imposition of zero, one, two, or three false mounts during a planned duration of 0, 5, or 10 min of active sexual preparation.

The average values for motile sperm per ejaculate obtained from the bulls with a planned duration of 0 min were 4.95, 8.35, 10.12, and 9.84 billion for zero, one, two, and three false mounts, respectively. The comparable averages for the bulls with a planned duration of 5 min were 10.22, 10.14, 10.99, and 12.66, respectively; and for the bulls with a planned duration of 10 min were 11.61, 12.35, 10.94, and 14.31, respectively.

The averages of 8.32, 11.00, and 12.30 billion motile sperm for 0, 5, and 10 min of preparation, respectively, differed significantly ($P < .01$). Similarly, the averages of 8.93, 10.28, 10.68, and 12.27 billion motile sperm for zero, one, two, and three false mounts, respectively, differed significantly ($P < .01$). This result is primarily a factor of volume of semen per ejaculate, which was significantly ($P \cong .02$) affected by the number of false mounts; whereas, the concentration of sperm was not ($P \cong .29$).

The evidence supports the contention that the various sexual preparation stimuli are additive and that they conform to Fechner's Law.

Early in the history of practical artificial insemination of dairy cattle, Lagerlöf (9) observed that bulls frequently yielded first ejaculates with few or no sperm. An improvement on this situation was reported by Hellstrom (8), who found that restraining bulls for a few minutes before ejaculation resulted in larger seminal volumes.

Collins et al. (4) were the first to publish quantitative information concerning pre-ejaculation sexual preparation of bulls. They concluded that about 40% more motile sperm could be obtained by imposing one false mount before ejaculation. Branton et al. (2) obtained a similar increase as a result of imposing one false mount and found that one false mount was essentially as efficient as two in terms of sperm output. Subsequently, Almquist et al. (1) and Crombach (5) presented data to support the view that restraining bulls in the vicinity of a stimulus animal for several minutes may increase the sperm output by a quantity similar to that expected from one or more false mounts.

Although false mounting is apparently an efficient and effective method of sexual preparation in terms of sperm output, it increases the possibility of injury to bulls. Provided that one could obtain quantities of sperm comparable to those expected from false-mounted bulls, a restrained method of sexual preparation would seem preferable, since it reduces the risk of bull injuries.

The experiment reported herein was undertaken to more accurately evaluate the sperm output effect of false-mounting mature dairy bulls within various durations of sexual preparation.

EXPERIMENTAL PROCEDURES

A previous study (7) indicated that, in a two-treatment sperm output experiment, one should include at least ten bulls per treatment, with at least ten ejaculates per bull, in order to have a 90% chance of detecting a treatment difference of seven billion sperm per ejaculate at the 5% level of significance. Since only 20 to 30 mature bulls from the Michigan Artificial Breeders Cooperative were available for the

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experiment at any given time, the experiment was conducted two treatments at a time and the order was selected at random. As a consequence, most of the bulls were used on more than one treatment, although the analysis of the results ignored this fact. Age of bull was not considered in the design of the experiment.

Available bulls were randomly allotted to treatment ignoring breed, since previous work (6, 7) had shown negligible differences among breeds with regard to sperm output. Each bull was maintained on a particular treatment for ten consecutive weeks, during which first ejaculates were collected at weekly intervals. A second ejaculate was taken only when the first contained a quantity of sperm insufficient to meet the requirements of the Cooperative. After ten first ejaculates had been obtained from any bull on a particular treatment, at least one month was allowed to elapse before that bull was assigned to another treatment.

The design of the sexual preparation treatments involved comparison of zero, one, two, or three false mounts imposed during an intended 0, 5, or 10 min duration of time. False mounts were distributed throughout the period of preparation and the intervals between false mounting were occupied by active encouragement of the bulls in the vicinity of stimulus animals. This encouragement amounted to movement of the stimulus animal, exchanging stimulus animals, changing the location of preparation, or combinations of these. These three factors were varied among bulls according to recent experience with each bull and according to the planned duration of preparation. If, in the opinion of the men handling the bulls, a bull was not being adequately stimulated by the procedure then in use, one or more of the three factors was varied until adequate stimulation was achieved. The number of stimulus animals and the number of preparation locations were recorded during sexual preparation previous to each ejaculation.

As a measure of the libido of the bulls, the vigor of thrust was rated one when neither rear foot moved, two when one rear foot moved, or three when both rear feet moved at the moment of ejaculation. Ejaculation time, defined as beginning at the end of sexual preparation and ending at the moment of ejaculation, was recorded for a similar purpose.

Immediately after the semen samples were collected, the volume of semen, per cent of motile sperm, and sperm concentration were determined according to the procedures described by Willett and Salisbury (10) and by Bratton et al. (3). The analyses of variance of the results considered five sources of variation: the duration of preparation, the number of false mounts, bulls, ejaculates, and an interaction between the first two. The duration of preparation and the number of false mounts were considered to be fixed and factorial, whereas bulls were considered to be random and nested within treatment, and ejaculates were considered to be random and nested within bulls. Identical analyses were made for volume of semen, per cent of motile sperm, sperm per milliliter of semen, number of sperm per ejaculate, motile sperm per ejaculate, vigor of thrust, and ejaculation time.

RESULTS

The availability of bulls resulted in the distribution of bulls among treatments shown in Table 1. The total of 151 bulls was composed of 93 Holsteins, 20 Guernseys, 19 Jerseys, 12 Brown Swiss, and seven Ayrshires, ranging in age from 2 to 15 yr.

TABLE 1
Numbers of bulls per treatment

Planned *minutes* of preparation	Number of false mounts				
	0	1	2	3	Total
0	11	12	9	12	44
5	11	13	13	12	49
10	14	15	15	14	58
Total	36	40	37	38	151

The observed duration of preparation was computed by adding the time required to obtain the planned number of false mounts and the ejaculation time. The averages of these sums are presented in Table 2. The average numbers of stimulus animals and stimulus locations are also recorded in Table 2.

Averages for the sperm output criteria for each of the 12 treatments are presented in Table 3. A summary of the probabilities obtained in the analyses of variance for these five sperm output criteria is presented in Table 4. That the differences among the averages for the three durations of preparation were significant ($P < .02$) for each of the five sperm output criteria is illustrated by the fact that those bulls given a planned 5 min of preparation yielded 30% more sperm than those given a planned 0 min of preparation. Ten minutes of preparation resulted in a 12% average increase in sperm output as compared to 5 min.

Sperm output differences among the four numbers of false mounts were significant for

TABLE 2

Averages for the characteristics of sexual preparation

Planned minutes of preparation	No. of false mounts per ejaculate	Observed minutes of preparation	No. of stimulus animals per ejaculate	No. of stimulus locations per ejaculate
0	0	1.0	1.1	1.2
	1	2.9	1.1	1.4
	2	3.6	1.3	1.4
	3	3.7	1.2	1.3
	Avg	3.1	1.2	1.3
5	0	5.4	1.3	1.4
	1	6.0	1.4	1.6
	2	6.7	1.4	1.4
	3	6.4	1.3	1.5
	Avg	6.1	1.4	1.5
10	0	11.1	1.5	1.3
	1	10.4	1.6	1.7
	2	11.6	1.5	1.6
	3	10.3	1.3	1.2
	Avg	10.8	1.5	1.5
Avg	0	5.9	1.3	1.3
	1	6.4	1.4	1.6
	2	7.3	1.4	1.5
	3	6.8	1.3	1.4
	Avg	6.6	1.3	1.4

TABLE 3

Averages of the sperm output criteria

Planned minutes of preparation	No. of false mounts per ejaculate	Volume of semen	Motile sperm	Sperm per milliliter of semen	Sperm per ejaculate	Motile sperm per ejaculate
		(ml)	*(%)*	———	*(× 10^9)*	———
0	0	5.30	69	1.30	7.08	4.95
	1	7.00	71	1.74	11.69	8.35
	2	7.81	71	1.92	14.23	10.12
	3	7.98	72	1.79	13.50	9.84
	Avg	7.02	71	1.69	11.62	8.32
5	0	8.22	72	1.68	14.10	10.22
	1	7.16	73	1.97	13.83	10.14
	2	8.90	73	1.79	15.10	10.99
	3	9.72	73	1.79	17.42	12.66
	Avg	8.50	73	1.81	15.11	11.00
10	0	8.09	72	2.04	16.21	11.61
	1	8.95	73	1.93	16.85	12.35
	2	8.66	73	1.77	15.01	10.94
	3	9.43	73	2.07	19.61	14.31
	Avg	8.78	73	1.95	16.92	12.30
Avg	0	7.20	71	1.68	12.46	8.93
	1	7.70	72	1.88	14.12	10.28
	2	8.46	72	1.83	14.78	10.68
	3	9.04	73	1.88	16.84	12.27
	Avg	8.10	72	1.82	14.55	10.54

TABLE 4

Level of significance (P_1) for each measurable source of variation for each sperm output analysis

Source of variation	Degrees of freedom	Volume of semen	Motile sperm	Sperm per milliliter of semen	Total sperm per ejaculate	Motile sperm per ejaculate
Minutes of preparation	2	$<.01$	$<.01$	$\cong.02$	$<.01$	$<.01$
No. of false mounts	3	$\cong.02$	$\cong.02$	$\cong.29$	$<.01$	$<.01$
Treatment interaction	6	$\cong.44$	$\cong.52$	$\cong.04$	$\cong.09$	$\cong.14$
Bulls within treatment	139	$<.01$	$<.01$	$<.01$	$<.01$	$<.01$

volume of semen ($P \cong .02$), per cent of motile sperm ($P \cong .02$), total sperm per ejaculate ($P < .01$), and motile sperm per ejaculate ($P < .01$), but not for sperm per milliliter of semen ($P \cong .29$). The values in Table 3 indicate that, on the average, each additional false mount resulted in nearly 1.5 billion additional sperm per ejaculate. This result is primarily a factor of volume of semen per ejaculate, since the number of false mounts did not significantly affect the concentration of sperm in the semen ($P \cong .29$). However, this result may not be accepted at face value, because the interaction between number of false mounts and duration of preparation was significant ($P \cong .04$) in the analysis of the concentration of sperm in the semen. This reflects the fact that additional false mounts did increase the concentration of sperm in the semen from bulls given a planned 0 min of preparation, but not those given a planned 10 min of preparation.

In contrast, the treatment interaction did not approach significance for volume of semen ($P \cong .44$) or for per cent of motile sperm ($P \cong .52$). In view of these facts, the significance levels of the treatment interaction for total sperm per ejaculate ($P \cong .09$) and motile sperm per ejaculate ($P \cong .14$) largely reflect the significant treatment interaction in the analysis of the concentration of sperm. Bulls differed significantly ($P < .01$) in each of the five sperm output criteria analyses.

The average ejaculation times are shown in Table 5. The duration of preparation had no effect on this criterion ($P \cong .23$), but those bulls given no false mounts had significantly longer ejaculation times ($P < .01$). The analysis of the data for thrust potential revealed no significant treatment effects ($P > .50$), with the 12 treatment averages ranging only from 2.84 to 2.98.

DISCUSSION

Examination of the data for thrust potential revealed that, for each of the 12 treatments, more than 90% of the ejaculations were rated three. Apparently, either much more drastic preparation techniques are required to alter this measure of libido, or this response is a poor measure of libido.

The data showing a significantly longer ejaculation time for those bulls given no false mounts were examined to find whether this libido response changed from the first to the tenth ejaculate, and no apparent trend could be established. Although these and other libido criteria should be measured over longer periods of time, the data in the present experiment reveal no detrimental psychological effects resulting from any of the 12 methods of sexual preparation imposed.

The fact that the concentration of sperm in the semen was not significantly affected by the number of false mounts but was affected by

TABLE 5

Average ejaculation times (minutes)

Planned minutes of preparation	Number of false mounts				
	0	1	2	3	Avg
0	1.03	0.40	0.38	0.41	0.56
5	0.35	0.34	0.49	0.26	0.36
10	0.97	0.25	0.51	0.23	0.49
Avg	0.78	0.33	0.46	0.30	0.50

TABLE 6

Coded sexual preparation values and sperm output per ejaculate for each of the sexual preparation treatments

Planned minutes of preparation	Observed minutes of preparation	No. of false mounts per ejaculate	Coded preparation value	Logarithm of coded value	Billions of sperm per ejaculate
0	1.0	0	1.0	0.000	7.0
	2.9	1	3.9	0.591	11.7
	3.6	2	5.6	0.748	14.2
	3.7	3	6.7	0.826	13.5
5	5.4	0	5.4	0.732	14.1
	6.0	1	7.0	0.845	13.8
	6.7	2	8.7	0.940	15.1
	6.4	3	9.4	0.973	17.4
10	11.1	0	11.1	1.045	16.2
	10.4	1	11.4	1.056	16.8
	11.6	2	13.6	1.134	15.0
	10.3	3	13.3	1.124	19.6

the duration of preparation, leads one to suspect that there may be a qualitative difference between the preparation stimulus provided by the false mount and other preparation stimuli provided in the intervals between false mounts. On the other hand, one could interpret this result as meaning that the physiological mechanism involved requires the application of stimuli over several minutes before exhibiting appreciable response. In this case, the false mount stimulus would not be expected to have much effect, since it occupies so little time.

Inspection of the 12 treatment averages for total sperm per ejaculate (Table 3) reveals decreasing increments in sperm output with increasing intensity of sexual preparation. In an effort to quantitate this apparent trend in the data, a false mount was given an arbitrary value of one, and these were added to the observed minutes of preparation (Table 2) to obtain the coded values for methods of sexual preparation shown in Table 6. Plotting the coded preparation values against average sperm output per ejaculate resulted in the curve shown in Figure 1. Since the curve in Figure 1 appeared to be logarithmic, the logarithms of the coded values were taken as shown in Table 6. The correlation coefficient between the logarithms of the coded values and the averages of sperm per ejaculate was 0.92 ($P < .01$), indicating that the coded values for method of sexual preparation may be of value in predicting sperm output. The regression of sperm output (Y) on the logarithm of the coded method of preparation (X) is shown in Figure 2.

These considerations seem to support the contention that the various sexual preparation

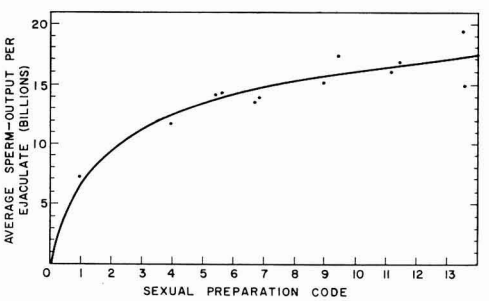

FIG. 1. Graph of average sperm output on coded sexual preparation values.

stimuli imposed upon the bulls in this experiment were additive. Furthermore, the logarithmic nature of the curve in Figure 1 leads one to suspect that these responses obey Fechner's Law: "The intensity of a sensation produced by a varying stimulus varies directly as the logarithm of that stimulus." However, proof of

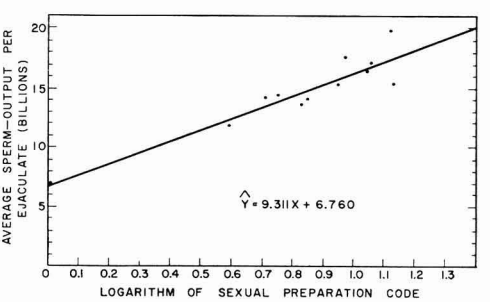

FIG. 2. Regression of average sperm output (Y) on the logarithm of the coded sexual preparation values (X).

these principles must come from another experiment, because the present considerations constitute reasoning after the fact.

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TECHNICAL NOTES

EFFECT OF NEURAMINIDASE ON κ-CASEIN

Interest in sialic acid (N-Acetyl Neuraminic Acid, NANA) has extended into the field of milk protein chemistry. This carbohydrate has been identified as a constituent of human milk oligosaccharides and appears in dialysates of bovine colostrum (4). NANA appears in the glyco-macropeptide (GMP) which is released from whole casein by the action of rennin (2, 7, 9), and exists in several minor protein fractions of bovine milk (11). Dumas and Alais (3) recently isolated, crystallized, and characterized NANA obtained from bovine casein.

The presence of strongly hydrophilic NANA in κ-casein (1) has prompted us to determine the possible role of this carbohydrate in the stabilization of α_s-casein in the presence of Ca^{++}. This paper reports the effects of neuraminidase (NANAse) on the release of NANA from κ-casein and its effect on the stabilizing power of κ-casein.

κ-Casein was prepared by the improved method of McKenzie and Wake (8). The κ-casein obtained by this method was essentially free of contamination in starch-gel-urea electrophoresis (8), possessed an S_{20} of 14 at pH 6.98, $\Gamma/2 = 0.20$ in the phosphate buffer of Waugh and von Hippel (15), and was an excellent stabilizer of α_s-casein in the presence of Ca^{++}. The κ-casein contained 0.22% phosphorus and 2.5% NANA. Three hundred milligrams of κ-casein was dissolved at pH 8.5 in a volume of 58 ml and adjusted to pH 7.0. One and two-tenths milliliters of V. cholerae NANAse, supplied by Behringwerke A. G.,[1] Marburg-Lahn, Germany, with an activity of 200 units per milliliter, was added to the protein solution at 37 C. The final pH of the solution was adjusted to 6.0 with 0.1 N HCl. Aliquots in which NANA was to be determined were withdrawn after the reaction had proceeded for 0, 15, 30, 60, and 180 min, heated to 80 C for 2 min, cooled, and dialyzed at 4 C against several changes of distilled water. NANA concentrations were determined using the resorcinol method of Svennerholm (10), with α_1 acid glycoprotein (orosomucoid) as a secondary standard for NANA. After the above reaction times, aliquots were also withdrawn for determination of stabilizing power by the method of Zittle (16), and the tests run immediately.

Measurements of the concentration of NANA remaining after dialysis revealed that V. cholerae NANAse liberated NANA from κ-casein at a rapid rate (Figure 1). Thompson et al. (12) observed electrophoretic alterations in

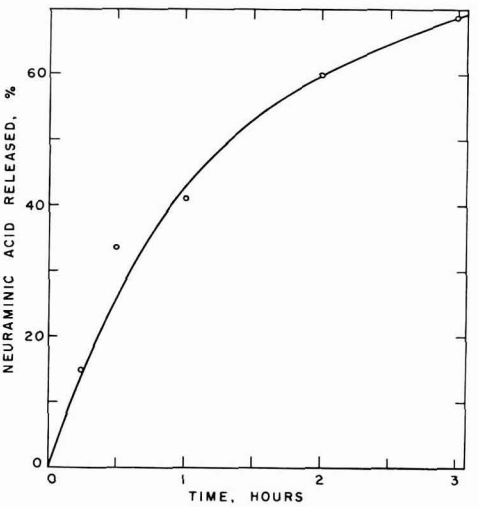

FIG. 1. The release of NANA from κ-casein by NANAse as a function of time.

influenza virus receptor destroying enzyme (RDE) -treated (4) sodium caseinate which they attributed to a release of NANA. Although Thompson et al. did not demonstrate NANA release from whole casein, Jollés and Alais (1, 7) showed that V. cholerae (RDE) attacked the GMP obtained from whole or κ-casein and liberated 80 and 66% NANA, respectively, from the two peptides. Jackson (6) observed that NANAse releases NANA from a glycopeptide obtained from the interfacial protein of bovine milk (5). Thompson and Brunner (11) had discovered earlier that a similar protein contained about 2% NANA, but did not ascertain the effect of NANAse on its release.

The action of NANAse on κ-casein NANA discloses the position of this carbohydrate in the κ-casein molecule. First, the release of NANA is evidence that it occupies a position terminal to an adjacent sugar residue in a disaccharide, trisaccharide, or polysaccharide because the action of NANAse is contingent upon this condition (4). The studies of Alais and Jollés (1) have shown that NANA is terminal in the GMP. Secondly, NANA is probably joined to either D-galactose or D-galactosamine through an α-glycoside (α-ketoside) linkage (4). Both of these carbohydrates are constituents of κ-casein (1). Studies currently in progress in this laboratory on the GMP are designed to determine the partner of NANA and the carbohydrate–amino acid linkage between the polysaccharide moiety and polypeptide portion of the molecule.

[1] It is not implied that the U. S. Department of Agriculture recommends the above company or its product to the possible exclusion of others in the same business.

Figure 1 shows that within 1 hr of reaction approximately 40% of the NANA had been released from κ-casein by NANAse. After 3 hr, about 69% of the total NANA had been released, whereas after 4 hr (not seen in Figure 1) 82.5% of the total NANA had been released. After longer reaction times, a 100% release of the carbohydrate could not be realized. Examination of the enzyme-reacted κ-casein by starch-gel-urea electrophoresis (13) revealed no change in the pattern as a result of the enzyme treatment. The protein continued to smear, as did the untreated control. At pH 6.98, $\Gamma/2 = 0.10$, phosphate buffer, and pH 8.6, $\Gamma/2 = 0.10$, veronal buffer, in free boundary electrophoresis, the enzyme-reacted κ-casein migrated as one symmetric component. The enzyme did not appear to alter the protein, except for a slight decrease in electrophoretic mobility which resulted from the loss of negatively charged NANA.

When NANAse treated κ-casein was examined for its ability to stabilize α_s-casein (Figure 2), it was observed that a loss of stabiliz-

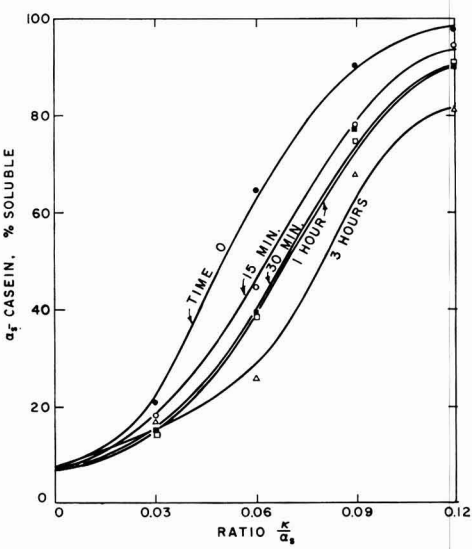

FIG. 2. Stabilization of α_s-casein by κ-caseins treated with NANAse for various periods of time.

ing power of κ-casein occurred within the first hour of enzyme reaction where the greatest amount (40%) of NANA was released. However, the protein still stabilized approximately 80% (κ/α_s ratio of 0.12) of the α_s-casein even after removal of 69% of the NANA.

The decrease in stabilizing power of κ-casein as a result of the release of NANA was not unexpected considering Waugh's (14) proposed structure of the casein micelle consisting, in part, of α_s-κ-casein. Waugh has depicted that a portion of the κ-casein monomer projects from the α_s-κ complex into the surrounding medium. Presumably, this portion of κ-casein is the hydrophilic or glyco-macropeptide portion of the molecule. The release of any hydrophilic groups from this portion of κ-casein, namely NANA, would result in a less soluble κ-casein which would lead to a decreased complex solubility.

In addition to NANA, we feel that other moieties of κ-casein, for example, the carbohydrate remaining after NANA removal, phosphate groups, and possibly –S–S groups contribute to the ability of κ-casein to form micelles with α_s-casein in the presence of Ca^{++}.

The observation that a loss of NANA alters a particular property of a protein is not peculiar to κ-casein. NANAse treatment of the follicle-stimulating hormone, for example, causes this mucoprotein to lose 97% of its biological activity (4).

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MEASUREMENT OF METAL LOSS FROM STAINLESS STEELS CAUSED BY ALKALINE DETERGENTS[1]

Davis (2) emphasized that the only completely satisfactory way of testing detergents and methods for cleaning was to try the method and the detergent under the existing conditions for the detergent operation concerned. The desirability of evaluating metal removal by detergents under similar conditions is evident.

A consideration of a combination of factors suggested the colorimetric diphenylcarbazide method for chromium VI to be a promising technique for measuring metal removal from stainless steels. The postulation was to measure removal by analyzing used detergent solutions for chromium, done after the detergent solutions had been used to clean stainless steel surfaces. Chromium was selected as the index of metal removal because of its presence in stainless steels. When found in the used detergent solutions, chromium would presumably have been removed from the stainless steel surface. Unlike iron, chromium is not commonly present in water supplies. Therefore, chromium would not be inherently present as a contaminant in testing procedures. Also, the diphenylcarbazide method has been reported to be quantitatively sensitive to small microgram quantities of chromium (5) and the composition of the surface oxide films of Types 302B, 304, 316, 347, and 446 stainless steels has been reported by Rhodin (4) to contain appreciable amounts of chromium.

EXPERIMENTAL PROCEDURE

Three Vollrath seven-liter Type 304 stainless steel beakers, bearing the factory identification no. 7878, were used as the test surfaces. A preliminary run of 24 hr with the following experimental conditions served to prepare the beaker surfaces for test: Four liters of 0.3% trisodium phosphate solution was prepared in each beaker, using deionized distilled water and reagent-grade trisodium phosphate. Sodium hypochlorite was added to the phosphate solutions in varying amounts, except for the preliminary trial, when the concentration was 100 ppm in all beakers. The solutions were exposed to the stainless steel surfaces at a temperature of 47 C for 48 hr. Temperature control was accomplished by placing the beakers simultaneously in a 24-liter water bath. Continuous and uniform agitation was furnished by glass agitators, which were of an electrically-driven propeller type. At the end of the 48-hr exposure period, the solution in each beaker was quantitatively transferred to a clean four-liter Pyrex beaker for evaporation. The four-liter volume was reduced, without boiling, to 100 to 150 ml over a low-temperature hot plate. These evaporated solutions were, in turn, washed quantitatively into 250-ml Pyrex beakers.

The diphenylcarbazide procedures for chromium analyses of The American Public Health Association, American Waterworks Association, and Federation of Sewage and Industrial Wastes Association (1), and Urone and Anders (6) were modified as follows:

(a) Clean all glassware by exposure to aqua regia for 15 min. Rinse the glassware seven times in tap water and three times in distilled water free from chromium contamination.
(b) Make chromium standards by placing 12 g $Na_3PO_4 \cdot 12H_2O$ and 25 ml NaOCl in a 250-ml Pyrex beaker containing approximately 150 ml deionized distilled water. Add varying amounts of chromium standard solution (5 γ Cr/ml, $K_2Cr_2O_7$, aqueous).
(c) Acidify the standard and steel samples to pH 2 with conc. H_2SO_4.
(d) Add 2 ml 5% hydrazinedihydrochloride (w/v, aqueous), made daily, to each sample. Bring just to a boil.
(e) Bubble air, filtered through water, into the sample until a negative qualitative

[1] Journal Article Number 2953, Michigan Agricultural Experiment Station.

test for available chlorine by starch iodine paper is obtained.

(f) Oxidize Cr to State VI adding enough K_2MnO_4 crystals to give a residual pink upon bringing to a brief boil.

(g) Cool the samples to room temperature in a water bath.

(h) Add a small amount of sodium azide (NaN_3) crystals to reduce the residual K_2MnO_4 color.

(i) If necessary, filter out any precipitates with asbestos supported by a coarse sintered glass filter.

(j) Adjust pH to exactly 1.9 with a Beckman (Model G) pH meter and 1:10 H_2SO_4 (v/v, aqueous).

(k) Add 1 ml of 0.25% diphenylcarbazide solution (1:1 v/v, acetone and water) to each colorless sample. Allow 30 min for color development before reading the optical density (O.D.).

(l) Quantitatively transfer samples to 200-ml volumetric flasks and make to volume with deionized distilled water. Five hundred-milliliter volumetric flasks may be substituted if color development is intense. Invert three times to mix.

(m) Check O.D. of all samples, using a Beckman (Model B) spectrophotometer employing a wavelength of 543 mμ and sensitivity of 3. The machine is adjusted to zero O.D., with the blank standard having no chromium.

Michigan State University Dairy Plant detergents were also analyzed for chromium after being used to clean the stainless steel plant equipment by recirculation cleaning. The procedures outlined above were employed, except that three permanganate oxidations were substituted in place of one. The precipitate was filtered off between each permanganate oxidation.

RESULTS AND DISCUSSION

Metal removal from stainless steel was indicated in this investigation to be in the order of small magnitude for any one alkaline detergent exposure period. The data in Table 1 show the results obtained from a laboratory study of Type 304 stainless steel. The data in Table 2 show the results when the method was employed to analyze used chlorinated alkaline proprietary detergents used in cleaning a commercial system of stainless steel piping automatically. Chromium loss in both experiments was small. However, in the laboratory study, the diphenylcarbazide test revealed marked differences in chromium loss proportional to the amount of hypochlorite present.

The test appears to have distinct advantages, namely, its sensitivity and practical use under field conditions of detergency. To eliminate the problems of interferences in analysis, future study should be made of a distillation procedure to effect a separation of the chromium from the detergent samples. Chromyl chloride has been reported by Hoffman and Lundell (3) to be volatilized at 116 C from mixtures of chromium, sulfuric acid, and hypochlorites. Alkaline detergent samples would appear to fit, or could be made to fit, the conditions for the formation of volatile chromyl chloride. If the chromyl chloride distillation was efficient in removing chromium, a diphenylcarbazide determination for chromium could be made on the distillate. Extreme sensitivity would then be attained without the interferences of detergent salts, heavy metals, organic matter, and hypochlorites. Success in developing the distillation procedure would make available a relatively

TABLE 1

Chromium losses from Type 304 stainless steel exposed to chlorinated 0.3% trisodium phosphate distilled water solutions measured by the diphenylcarbazide method

Trial	Chromium removed[a]		
	No. chlorine (μg Cr)	100 ppm chlorine (μg Cr)	500 ppm chlorine (μg Cr)
A	4.0	17.0	107.0
B	2.0	22.0	136.0
C	....	26.0	103.0
D	4.0	26.0	152.0
E	0.0	48.0	136.0
F	0.0	47.0	174.0
G	0.0	35.0	168.0
H	2.0	37.0	111.0
I	4.0	31.0	107.0
Totals	14.0	289.0	1,194.0
$\bar{x}$	1.5	32.1	133.0
σ	1.7	10.5	27.5

[a] Represents μg Cr removed from 1,120 sq cm steel by 48-hr exposure at 47 C.

TABLE 2

Chromium removed from 446 ft of 1.5-inch stainless steel pipeline by a chlorinated proprietary alkaline detergent as measured by diphenylcarbazide method

Trial	Available chlorine (ppm)	Chromium removed (μg)[a]
A	35	0
B	7	0
C	234	1–2
D	56	0
E	56	0
F	56	0
G	49	0
H	42	0
I	49	0

[a] Represents chromium in a four-liter sample, taken at an elapsed time of 30 min in the washing cycle, 135 ± 5 F.

simple, rapid method for evaluating corrosion of stainless steels.

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COMPARISON OF BITTER FLAVOR IN CHEESE WITH QUININE SULFATE SOLUTIONS

Differences in intensities of bitterness among cheeses have been determined by comparison with other cheeses which served as standard reference points on a numerical scale (2, 3). The interpretation of such results implies the following: (a) the reader must accept the accuracy of the descriptions of the intensities applied to the various points on the scale; (b) these descriptions are, in turn, a reflection of the acuities for bitterness of the tasters on the panel; acuities for bitterness in the population as a whole vary widely (1, 4). It would be desirable to be able to express the intensities of bitterness in terms of a chemical reference standard. This note will describe the comparison of aqueous solutions of quinine sulfate with the cheeses used as reference standards in a recent study (3).

Four tasters were presented with the following solutions in a random order: two samples of distilled water and solutions containing quinine sulfate at 20D, 19D, 18D, 17D, and 16D according to Dove's method (1) of expressing concentration of taste-stimulating substances; for example, 16D is $M/2^{16}$ g/liter, where M is the molecular weight of quinine sulfate. The tasters were asked to score the solutions in comparison with cheese Standards 3 and 5 on the whole-number scale of 1 to 6, the score increasing with bitterness (3). Each taster scored each sample of quinine sulfate once during each of four tasting sessions. Samples were presented at 7-min intervals rather than the 5-min intervals used for cheese, because the after-taste of the quinine sulfate was more persistent.

The results of the comparisons are presented in Figure 1. Transformation of the quinine sulfate concentrations to negative logarithms

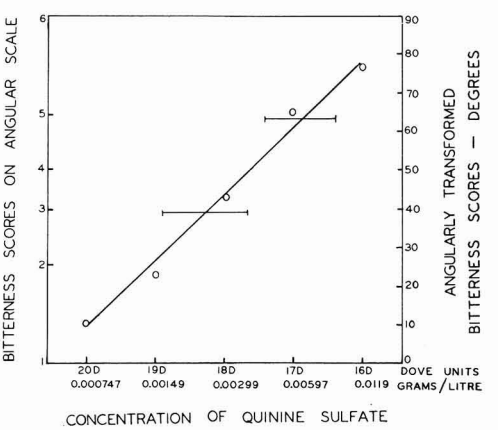

FIG. 1. Comparison of solutions containing various concentrations of quinine sulfate with the cheese standards, showing the 95% confidence limits at Scores 3 and 5.

(base of 2) and of the bitterness scale to angles via percentages (1 = 0%; 6 = 100%) yielded an approximately linear relationship between the bitterness scores and the concentrations of quinine sulfate examined. A regression analysis on the transformed data indicated significant differences ($P < 0.05$) among the tasters in their scoring of the different solutions. From the regression analysis, the concentrations of quinine sulfate corresponding to Scores 3 and 5 were estimated to be 18.3 and 16.9D, respectively. In spite of the variation among tasters, the 95% confidence limits were 17.7 to 18.9D

and 16.4 to 17.4D, respectively. There was, thus, a clear distinction, on the Dove scale, between the bitterness of the two standard cheeses.

According to Dove (1), tasters have high, medium, and low acuities for bitterness, if they can detect bitter flavor in solutions of quinine sulfate at 20, 17, and 14D, respectively. Some individuals, not on the taste panel, did not detect bitterness in any of the experimental cheeses, to some of which the panel gave scores as high as 5. Most people, however, found much of the bitter cheese to be objectionably so.

Although the taste of the quinine sulfate and some of the cheeses would both be described as bitter, the bitter flavor seemed to be different in character; one definable area of difference was that the bitterness of quinine sulfate lingered longer in the mouth after expectorating than did the bitterness of the cheese. Tasters on the panel commented that they had more difficulty comparing intensities of bitterness between cheese and a solution of quinine sulfate than between two cheeses. The addition of quinine sulfate to cheese did not help this tasting problem; furthermore, in order to taste as bitter as in distilled water, the quinine sulfate had to be added to the cheese at about 16 times the concentration.

Our results show that cheese can be compared with bitter-tasting substances such as quinine sulfate to obtain an absolute estimate of their intensities of bitterness. However, for estimates of relative intensities of bitterness in cheese, comparison with standard cheeses appears to be more satisfactory.

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OUR INDUSTRY TODAY

REMOVING RADIOSTRONTIUM FROM MILK—CURRENT STATUS OF A PILOT PLANT PROCESS

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Strontium^{90}—the long-lasting contaminant that results from fallout—can be removed from milk. A tentative process for removing it has been developed to the pilot-plant stage. The work is the result of a cooperative project by the Public Health Service of the Department of Health, Education, and Welfare, the Agricultural Research Service of the U. S. Department of Agriculture, and the U. S. Atomic Energy Commission. The pilot plant has been set up in Beltsville, Maryland, at the Dairy Products Laboratory of the ARS Eastern Utilization Research and Development Division. The present installation is the result of over 2 yr of research at this laboratory (2, 3) and at the Robert A. Taft Sanitary Engineering Center of the Public Health Service in Cincinnati, Ohio (5). The process is based on evidence presented earlier by the University of Tennessee (1) and the Canada Department of Agriculture (4) that strontium^{90} could be removed from milk by an ion-exchange process.

Research with this pilot plant indicates that the process could be adopted commercially in the event that radiostrontium in milk should reach dangerous levels. However, study of engineering problems and further research to establish the most economical procedure are needed before the fluid milk supply of the United States could be treated expeditiously, if such treatment should ever become necessary. This report is not intended to encourage premature entrance into this technical field by the dairy industry; it is presented only as an interim report to acquaint the dairy industry with the present status of the research.

PRINCIPLE OF THE PROCESS

The process is based on the ion-exchange principle. Milk contaminated with radiostrontium is passed through a bed of synthetic resin charged with a mixed solution of metallic ions—calcium, potassium, sodium, and magnesium—which are present in milk. As the milk passes through the column, the strontium ions in the milk change places with the metallic ions on the resin.

An important feature of the process is that the acidity of the milk is adjusted, before passing it through the resin column, from its normal pH of 6.6 down to 5.3 or 5.4. At the normal pH of milk, most of the strontium is bound by other milk constituents and is slow to be exchanged. Passing 25 resin bed volumes of milk at normal pH through the column removes an average of less than 50% of its strontium. At the low pH, strontium is largely converted to a soluble and more readily exchangeable form.

EFFECTIVENESS AND ESTIMATED COST OF PROCESS

With this pilot-plant process, about 90% of the radioactive strontium in milk can be removed. The process has been developed to secure maximum removal of strontium^{90}, inducing only a minimum of adverse changes in the milk's chemical composition, physical stability, or flavor. The appearance of the treated product is normal after homogenization and pasteurization. Flavor scores of the treated milks, as judged by an expert panel, compare favorably with the control samples. They usually average about 1.0 point below the controls on the A.D.S.A. score card. Procedures for minimizing changes in the composition of the major cations in milk have been worked out. Studies on nutritional changes as a result of the ion exchange treatment are in progress.

The cost of materials used for this process is primarily that of the salts used to regenerate the columns after they become exhausted (i.e., when the amount of strontium^{90} removed falls to some predetermined level). The cost of these salts, plus that of the citric acid and potassium hydroxide used to adjust and then restore the milk's normal pH, is estimated at about 2¢ per quart of milk processed. This estimate is based on a process which reuses the salt solutions so that maximum efficiency is obtained, and on the use of USP grades of salts. If the regenerating solution is not recovered, the cost may be as high as 5¢ per quart. Since the salts used for regeneration are not a direct addition, investigation is now under way to determine whether less expensive grades may be suitable.

The efficiency of this process is closely related to the amount of milk that can be passed through a column before the column becomes

exhausted. This depends on a predetermined level of strontium removal. It can be considered acceptable if 25 resin bed volumes of milk can be processed before a column must be regenerated.

PRESENT PILOT-PLANT EQUIPMENT

The accompanying figure shows schematically the operations involved in the process. There are two glass ion-exchange columns, 5 ft long and 6 inches in diameter. They are fitted with sufficient valves for directing the milk and the necessary solutions through the resin bed in controlled sequence for a continuous operation.

While milk is flowing through one column, the other is being cleaned and regenerated for reuse. The drawing depicts a batchwise method for adjusting the milk's acidity with citric acid. On a large scale this solution could be added with a metering pump to a continuous flow of milk, as is shown for neutralization with potassium hydroxide.

These pilot-plant facilities were designed for a capacity of about 100 gal per hour. The columns contain about two-thirds of a cubic foot of resin. This allows for sufficient head space for upflow washings.

STEPS IN THE PROCESS

The process for removal of radiostrontium from milk includes the following operations:

1. *Charging the resin bed.* The resin bed is charged with a mixed salt solution containing the chlorides of calcium, potassium, sodium, and magnesium. The quantities of each are selected so that the resin will be equilibrated with the proper ratio of cations. Thus, a minimum change in the composition of the milk is assured when it is passed through the resin. The composition of the salt solution found suitable for this operation is as follows (in grams per liter of solution):

Calcium chloride ($CaCl_2 \cdot 2\ H_2O$)	53.5
Potassium chloride	23.1
Sodium chloride	8.5
Magnesium chloride ($MgCl_2 \cdot 6\ H_2O$)	15.1

2. *Acidification of the milk.* Cold, raw whole milk is adjusted to a pH of 5.3-5.4 with 0.75 molar citric acid. The acid is added slowly and with thorough mixing. About 7% more strontium could be removed if the pH of the milk were lowered to 5.2 instead of 5.4. But at this acidity, laboratory experience has shown a greater likelihood of protein precipitation. This may require excessive pressure in the resin column to keep the milk flowing. At pH 5.4, milk temperatures from 40 to 80 F have been found to have no effect on the amount of strontium removed. Temperatures above 80 F cannot be used as the milk is sensitive to coagulation at low pH.

3. *Putting the milk through the column.* The acidified milk is filtered and pumped downflow through a column of cationic ion-exchange resin at a rate of about 2.5 gal per minute per cubic foot of resin. The resin should be of the nuclear sulfonic acid type, such as Amberlite IR-120 or Dowex 50W.[1] A small positive pressure of 2 psi or greater is usually required to maintain a constant flow rate through the column. Too fast a flow rate should be avoided, since the amount of strontium removed decreases with increasing flow rate. A drop of 8 to 10% can result from increasing the rate from 0.25 to 0.5 resin bed volume per minute.

4. *Neutralizing the milk.* The effluent milk is collected in a surge tank, where it is readjusted to normal pH by adding dilute potassium hydroxide or a mixed solution of sodium and potassium hydroxide. If the milk is neutralized entirely with sodium hydroxide, it acquires a salty flavor.

5. *Processing the treated milk.* After neutralization, the milk is pasteurized, homogenized, and deodorized to remove the water added in the acidification and neutralization steps.

6. *Cleaning and sanitizing the resin.* As the resin in one column becomes exhausted, milk flow is diverted to another column, while the first is being cleaned. This is done by rinsing with warm water, followed by an upflow wash with a nonionic cleaning solution at 140 to 160 F. The resin may then be sanitized with hypochlorites at a chlorine concentration of about 50 ppm. Sanitizing is not necessary with every cleaning, but it may be desirable at the end of each day's run or if the resin is idle for a considerable period. Another means of controlling bacterial growth is through the use of heat, since the resins will withstand temperatures up to the boiling point of water. Washing or sanitizing solutions are immediately followed by rinse water.

7. *Regeneration of the resin.* The clean resin is then regenerated with a mixed salt solution of the same composition as that used to charge it initially (Step 1). When applied to a resin column that has been in use for the processing of contaminated milk, the salt solution simultaneously strips the radiostrontium from the resin and regenerates the resin for the next cycle. Essentially 1.0 volume of salt solution is required per volume of milk processed. Most of this quantity may be recovered and used again. In this case from ¼ to ⅓ of the stripping solution would be discarded and replenished with fresh solution after each cycle. When excess salts have been removed with a water rinse, the column is ready for another milk cycle of 25 bed volumes.

RESEARCH STATUS OF THE PROCESS

A great deal of research has been required to bring this process to its present state of

[1] The use of trade names is for the purpose of identification only, and does not imply endorsement of the product or its manufacturer by the U. S. Department of Agriculture.

development. Much more is still to be done to improve the method, to assure a nutritionally satisfactory product, to evaluate possible sanitation problems, and to reduce its cost. The current research status of the process can be summarized briefly by mentioning the work done in establishing operating conditions for the equipment and then in evaluating the efficiency of the pilot-plant process by actual use with milk intentionally contaminated with known amounts of radiostrontium.

Establishment of operating conditions. The process described above was arrived at through a careful study of the ion-exchange principle of removing radiostrontium from milk. Several different types of resins have been evaluated for their effectiveness. Some basic research has been done in the area involving cationic equilibria of resin-milk systems and resin-salt solution systems. This work was necessary to develop optimum procedures for cleaning the resin, removing strontium[90] from the exhausted resin, and regenerating the resin for reuse. Work has also been in progress on the nature of the strontium complexes in the milk. Such basic information should aid in explaining the results obtained and may lead to further improvements in the process.

Work in progress designed to further reduce the cost of the process is primarily concerned with the salts used to regenerate the resin. Techniques for using reverse flow regeneration may reduce slightly the quantity of salts required. Studies on reclaiming a portion of the regenerating salts are being continued.

Production of contaminated milk for evaluation of the process. The pilot-plant has been used experimentally in the processing of milk obtained from a cow fed measured doses of radiostrontium. Because quick analysis of the strontium content of the milk samples before and after processing was essential to an accurate evaluation of the process, the cow is fed strontium[85] rather than strontium[90]. Unlike strontium[90], which requires a long and tedious procedure for analysis, strontium[85] is a gamma-emitting radionuclide that can be assayed with a minimum of sample preparation.

Strontium[85] is fed to a Holstein cow either orally or intravenously to obtain levels in the milk of about 1 μc per quart. This is a much heavier concentration of radiostrontium than has been found to occur in fallout, where levels of the order of 10 $\mu\mu$c of strontium[90] per quart have been detected. The higher levels for this research have been necessary for rapid assay of a large number of samples. Since both levels represent much smaller weights of radiostrontium than the stable strontium in milk, the amount removed by an exchange process should be the same on a percentage basis. This has been confirmed by preliminary work with levels of about 200 $\mu\mu$c per quart.

Work is now in progress at this laboratory to apply the techniques to the removal of environmental levels of strontium[90].

ACKNOWLEDGMENT

The authors acknowledge assistance from their colleagues: E. Dodson, F. W. Douglas, Jr., W. A. Mattingly, and C. D. Mitchell.

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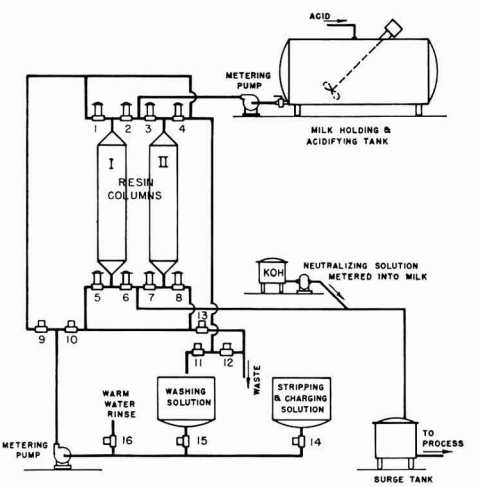

FIG. 1. Flow diagram showing strontium[90] removal by ion exchange resin.

FIGURE KEY

Flow Operation of Equipment with Milk Flowing Down Column I and Regeneration Process for Column II.

A. Valves No. 2 and No. 6 are opened for 60 min, allowing raw milk to flow down Column I. At the same time that the milk is flowing through Column I, Column II is being regenerated in sequence as follows:

B. Valves No. 16, 9, 4, 8, and 13 are opened from 2-4 min, allowing warm rinse water to flow down Column II.
C. Valves No. 15, 10, 8, 4, and 11 are opened for 8 min, allowing washing solution to flow up Column II.
D. Valves No. 16, 9, 4, 8, and 13 are opened from 2-4 min, allowing rinse water to flow down Column II.
E. Valves No. 14, 9, 4, 8, and 13 are opened for 25 min, allowing stripping and charging solution to flow down Column II.
F. Valves No. 16, 9, 1, 5, and 13 are opened from 2-4 min, allowing rinse water to flow down Column II until chloride-free.

Flow Operation of Equipment with Milk Flowing Down Column II and Regeneration Process for Column I.

A. Valves No. 3 and 7 are opened for 60 min, allowing raw milk to flow down Column II. At the same time that the milk is flowing down Column II, Column I is being regenerated in sequence as follows:
B. Valves No. 16, 9, 1, 5, and 13 are opened from 2-4 min, allowing warm rinse water to flow down Column I.
C. Valves No. 15, 10, 5, 1, and 11 are opened for 8 min, allowing washing solution to flow up Column I.
D. Valves No. 16, 9, 1, 5, and 13 are opened from 2-4 min, allowing rinse water to flow down Column I.
E. Valves No. 14, 9, 1, 5, and 13 are opened for 25 min, allowing stripping and charging solution to flow down Column I.
F. Valves No. 16, 9, 1, 5, and 13 are opened from 2-4 min, allowing rinse water to flow down Column I until chloride-free.

Notes:
All valves are normally closed and only opened as indicated.
The flow rate of the milk pump is 96 gal per hour.
The flow rate of the regeneration pump is 6 gal per min.

ASSOCIATION AFFAIRS

MEETING OF THE EASTERN DIVISION OF THE AMERICAN DAIRY SCIENCE ASSOCIATION

R. E. MATHER, Secretary-Treasurer, Eastern Division A.D.S.A.
Department of Dairy Science, Dairy Research Farm, Sussex, New Jersey

The Eastern Division of the American Dairy Science Association will meet on August 14 and 15, 1962, at the University of Maine, Orono. This is the third annual meeting to be held at a land-grant institution following meetings at the Universities of New Hampshire and Massachusetts. All members and friends of the American Dairy Science Association in the Eastern United States and Canada are invited to attend.

Abstracts for this meeting should be prepared according to the Call for Papers for the 1962 Annual Meeting of the American Dairy Science Association by M. E. Senger, Chairman, Program Committee, A.D.S.A. Instructions for preparing abstracts were printed in issues of the JOURNAL from September, 1961, through January, 1962.

PRICE SCHEDULE FOR REPRINTS OF PAPERS THAT APPEAR IN THE JOURNAL OF DAIRY SCIENCE

H. F. JUDKINS, Secretary-Treasurer
32 Ridgeway Circle, White Plains, New York

The Executive Board, at the time of the Annual Meeting of the American Dairy Science Association at the University of Wisconsin, increased the price of reprints 25%, effective July 1, 1961. The new reprint schedule follows:

No. of reprints	Number of pages								
	2	4	8	12	16	20	24	28	32
	(Cost in dollars)								
50	17.50	20.00	36.25	51.25	67.50	78.75	97.50	115.00	125.00
100	20.00	22.50	41.25	61.25	77.50	92.50	112.50	132.50	145.00
200	22.50	28.75	51.25	76.25	97.50	117.50	143.75	162.75	185.00
300	28.00	33.75	62.50	91.25	117.50	143.75	173.75	205.00	226.25
400	30.00	40.00	72.50	107.50	137.50	170.00	205.00	241.25	266.25
500	33.75	45.00	83.75	122.50	157.50	195.00	236.25	277.25	306.25
600	37.50	51.25	93.75	137.50	177.50	221.25	266.25	313.75	346.25
700	41.25	56.25	105.00	153.75	197.50	246.25	297.50	350.00	387.50
800	45.00	62.50	115.00	168.75	218.75	272.50	328.75	386.25	427.50
900	48.75	67.50	126.25	185.00	238.75	298.75	358.75	422.50	467.50
1,000	57.25	73.75	136.25	200.00	258.75	323.75	390.00	458.75	507.50

If covers for reprints are desired, the cost of 50 covers will be $21.18, and for each additional 100 covers, the cost will be $8.75. Back copies of the JOURNAL will cost $2 each.

The reprints are made from standing type within 30 days after the papers appear in the JOURNAL. Requests for a few reprints of a paper should be sent to the authors, whose names and addresses appear with the title. The Secretary and the Editor's office do not keep supplies of the various reprints. Orders for large numbers of reprints should be sent to The Garrard Press, 510 North Hickory Street, Champaign, Illinois. These orders must be received within 30 days after the papers are published in the JOURNAL; otherwise, the type will have been destroyed.

In case the original type has been destroyed, it is possible to supply reprints by a special photographic process, and their cost will be 50% more than the regular ones. For example, 100 reprints of 32 pages will cost $217.50.

It is hoped that the publication of this reprint schedule will make it easier for interested people to obtain reprints in any number desired and, at the same time, aid in disseminating useful information to the dairy and related industries.

BOOK REVIEWS

FOOD PLANT SANITATION. M. E. Parker and J. H. Litchfield. Reinhold Publishing Corp., New York. 401 pp. 1962.

Those familiar with a prior book of the same title by the senior author will find this publication to be a substantial improvement. This is a good book on the title subject, covering the central subject and touching on most of the related disciplines. In dealing with such broad material, this book must be considered to be written in a survey style, although the basic principles of some topics are well developed. Of particular merit are the cited references, which are extensive, contemporary, carefully chosen, and supplemented by an author index. These references allow the reader to effectively pursue any phase of the subject in greater detail.

The first three chapters on quality control, buildings and equipment, and pest control are written within the framework of the various federal acts and laws. Pertinent excerpts from the Food, Drug and Cosmetic Act are found in the appendix. The next three chapters are on effective control of insects, pests, and microorganisms. Further chapter topics included in this publication are: water supply, waste disposal, cleaning technology, establishing and maintaining sanitary practices, and packaging materials and methods. The volume is thoughtfully indexed.

While the subject of food plant sanitation is well handled in this book, the already sanitation-conscious dairy industry reader will probably not find it very provocative. However, this book would serve as a fine text for an introductory course, be of value to a food technologist, and be an excellent source of reference material.

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ATTACK ON STARVATION. Norman W. Desrosier. Avi Publishing Co., Inc., Westport, Conn. 1961. 312 pp.

This book contains four parts, with 16 chapters. The first part covers man's requirements for food and the adequacy of his diet. The next part concerns the over-all world potential for food production. Part Three covers the problem of food storage and distribution from harvest to harvest. The last part is concerned with the general subject of world starvation and its control. No specific food commodity or product is emphasized.

There is a great need for a regular review and discussion of this subject, to consider the problems in light of current developments and future predictions. This book adequately reviews the subject and delineates the current and future problems. The author presents a number of plans for producing more food, especially protein, for the hungry people of the world.

The subject matter in this text should be of interest and concern to all food scientists. However, it is specifically recommended to all concerned with the adequacy of the diet of the people of the world today and in the future.

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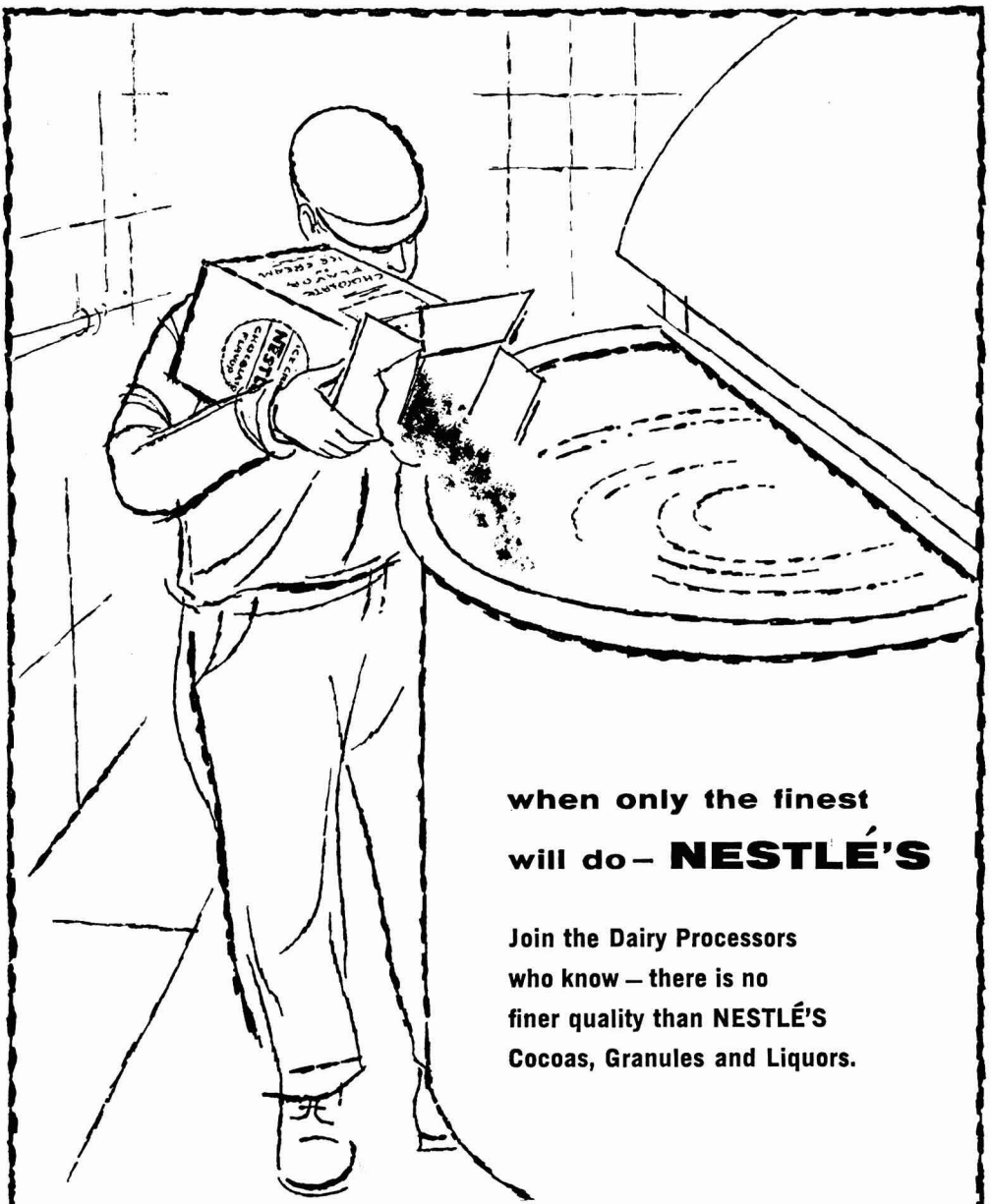

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