Journal of

DAIRY SCIENCE

Vol. 45 July, 1962 Number 7

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Acknowledgment

The special cover for the June JOURNAL, commemorating the establishment of the Land-Grant Colleges and Universities, and the U. S. Department of Agriculture was originally proposed by DR. R. E. HODGSON when he was president of the American Dairy Science Association during 1960-61, His suggestion was forgotten until DR. H. F. JUDKINS revived it in May, 1962. The Journal Management Committee of which J. F. CAVANAUGH was Chairman, approved this special recognition in the JOURNAL.

Dr. Hodgson contributed a suggested format, and with help from Cavanaugh's committee, and with the competence of Claude Cruse of the Garrard Press, it was possible within a short time to formulate a cover which, it was hoped, would symbolize the CENTENNIAL. Dr. R. F. Davis supplied material for the inside front cover.

This statement was intended for the June JOURNAL, but the Editor-in-Chief forgot to submit it.

JOURNAL OF DAIRY SCIENCE

OFFICIAL PUBLICATION OF THE
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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.
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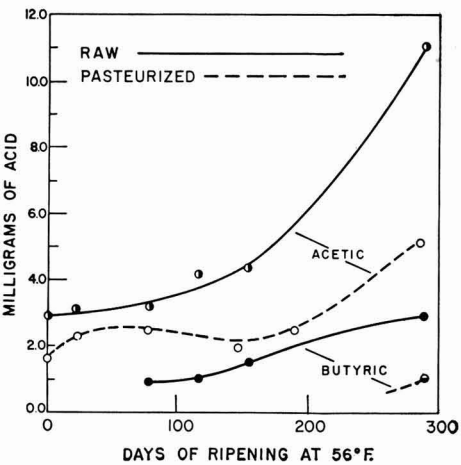

FIG. 1. Acetic and butyric acids in raw and pasteurized milk Cheddar cheese during ripening (milligrams in distillate obtained from 150 g of cheese oil).

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Tables are costly. Use graphs whenever possible.

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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

Memorial services were held April 23 for W. M. REGAN, 78, emeritus professor of animal husbandry at the University of California, Davis. He died April 14 after an illness of several weeks. Services were held also at the Davis Community Church. K. A. RYERSON, dean of the College of Agriculture, emeritus, and long-time colleague and friend of Professor Regan, was the speaker. The family requested that any remembrances be sent to the Cancer Research Fund, UC Medical Center in San Francisco, care of The Regents of the University of California.

W. M. Regan

A resident of Davis for 40 yr, where he was on the staff of the University, Professor Regan was a pioneer in experimentation with dairy cattle, the initiator of the nation's first large-scale breeding experiments.

Equally important, according to his colleagues in a eulogy delivered at Dairy Cattle Day, held earlier this year, "Professor Regan endeared himself to hundreds of students, who found in him a sympathetic friend as well as a competent instructor and who were influenced by his character and ideals while they were taught by his knowledge and experience . . . A man whose scholarly interests were balanced by his deep feeling for people and their problems. . . ."

Born at Joplin, Missouri, February 18, 1884, Regan was granted his bachelor's and master's degrees at the University of Missouri and did his first teaching at that institution. After a brief period as assistant professor at Nevada and as professor and head of the Dairy Department at Rutgers University, he came to the College of Agriculture at Davis. He retired in 1951.

Professor Regan was known throughout California for his devoted promotion of the work of the Agricultural Extension Service. He was a member of the scholastic fraternity Phi Beta Kappa, the scientific fraternities Sigma Xi and Gamma Alpha, the agricultural scholastic fraternity Gamma Sigma Delta, the social fraternity Phi Delta Theta, the American Dairy Science Association, the American Society of Animal Production, the American Genetic Association, and the New York Academy of Sciences, as well as the standing committee for Animal Development in the Pacific Area, Ninth Pacific Science Congress. He was a classification judge for the American Jersey Cattle Club.

He is survived by his widow, Susan, who is Dean of Women at Davis, and three children, William, Jr., of San Jose, Susan (Mrs. Allan McKillop) of Davis, and Mary, a graduate student at the University of Wisconsin, Madison.

Harry Alexis Harding

When DR. H. A. HARDING, 580 Haverhill, Bloomfield Hills, Michigan, passed away at the Stevens Nursing Home, Royal Oak, November 16, 1961, after a long illness, the American Dairy Science Association lost one of its long-time illustrious members. He lacked 12 days of being 90 yr of age.

Perhaps through fate Dr. Harding was not one of the founders of the American Dairy Science Association, in Urbana, July 17, 1906. Nevertheless, at the time he had already distinguished himself as a dairy bacteriologist at the New York Agricultural Experiment Station, Geneva.

H. A. Harding

Yet, as chairman of the committee including M. MORTENSEN, secretary, and W. A. STOCKING, president, to study the future name of the Official Dairy Instructors' Association, he announced, 1918, "the 'Official Dairy Instructors' Association' has become the 'American Dairy Science Association.'" To his leadership, the society is indebted for the name held in high esteem throughout the scientific world.

Many of the fundamentals of dairy bacteriology taken for granted today were established by Dr. Harding, his associates, and professors such as H. L. RUSSELL, E. G. HASTINGS, L. A. ROGERS, W. A. STOCKING, R. S. BREED, and M. J. PRUCHA, to name a few. His cooperative report, What is Meant by Quality in Milk, 1918, challenging then, is vital today. Bacteria were a near obsession to him; his bounteous energy was spent in knowing and controlling them in the milk supplies. Few scientists at annual meetings defended their fundamental knowledge of bacteria as did Dr. Harding. His presence and discussions at scientific meetings was a challenge and inspiration to young scientists.

Dr. Harry Alexis Harding, born in Oconomowoc, Wisconsin, November 28, 1871, died after a year's illness, on November 16, 1961, ending a long career in bacteriology and public health.

He took his bachelor's and master's degrees from the University of Wisconsin, and then spent 2 yr in research in phytopathological studies and as laboratory assistant. He also for 2 yr was assistant in charge of meteorological service of the Washburn Observatory.

He studied at Massachusetts Institute of Technology and took his doctorate at Cornell. He went abroad for a year's study in Western Europe and returned to become Commissioner of Health, and Bacteriologist for New York Experimental Station at Geneva. He took up the problem of an epidemic in Geneva, traced it to water pollution north of the city, and after several years of political opposition brought it to a close, with well planned editorial assistance from the leading newspaper.

In 1913 the University of Illinois called him to take charge of dairy bacteriology and become head of the department of dairy husbandry. This was a new field, made attractive with new problems. With characteristic vigor and thoroughness he correlated his experience with more research and launched a large activity into public health and sanitary problems that brought him pre-eminence as an authority and took him into every state in the Union except Alabama. He was called to make a survey of an epidemic in Portland, Oregon, and from there proceeded to give the same service in other states and in Canada.

In 1926 he became vice-president and chief of dairy research for the Mathews Company in Detroit, until his retirement in 1940 to his estate at North Bay. He was the author of numerous books and articles on bacteriological subjects and at the time of his death was planning a book on city milk supplies. A genial personality and caustic wit, combined with a thoroughness and tenacity to get the job done, won him popularity as a lecturer, and unbudging faithfulness to principle the respect and confidence of all who knew him.

He was an authority on bacteriology, bacterial plant diseases, causes of cheese ripening, soft rot in plants, fixation of nitrogen, and city milk supplies. He was a serious student of religions and education. His private funds were always available to any needy and seri-

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ous student. Fly fishing, reading, and bear hunting engaged his free moments.

Dr. Harding was one of the founders of the American Society of Public Health and the American Society for the Advancement of Science.

Surviving are four children, Gordon, Glenview, Illinois, Esther Patrick, Elletsville, Ind., Helen Holscher, Toledo, Ohio, and Ruth Kasdorf, Florida; his widow, Jessica, Bailey Harbor; two stepchildren, Muriel Lockwood, Bloomfield Hills, Michigan, and Trevor Hay, Birmingham, Michigan.

G. M. TROUT, Historian
American Dairy Science Association

SPECIAL RECOGNITIONS

Dr. J. L. Strominger Receives Paul-Lewis Award

DR. J. L. STROMINGER of Washington University, St. Louis, was the 1962 recipient of the Paul-Lewis Award in Enzyme Chemistry. The award, consisting of $1,000 and a gold medallion, was presented to Dr. Strominger by DR. VINCENT BAVISOTTO of Paul-Lewis Laboratories, Inc., at the general meeting of the American Chemical Society in Washington, D. C.

Dr. Strominger was recognized for his outstanding work on uridine nucleotides and for elucidating the mechanism of cell wall synthesis. This work demonstrated that penicillin acts by blocking cell wall syntheses. Dr. Strominger has also studied mucopolysaccharide formation in the hen's oviduct and has isolated several previously unknown nucleotides from this source. He also discovered how the liver enzymatically forms glucuronides from various drugs, metabolites, and similar materials.

Dr. Strominger joins the select circle of 16 enzyme chemists previously honored with this award. Established in 1946 to recognize and stimulate research, the Paul-Lewis award is offered annually under the administration of the American Chemical Society.

Dr. Vincent S. Bavisotto (left) presenting the award, consisting of $1,000 and a gold medallion, to Dr. Jack L. Strominger.

Labor Secretary Goldberg Presents Milk Industry Foundation's Louis Pasteur Gold Medal to Ohio Milkman

D. C. RIGGS, 33, an Ohio milkman, was presented the Milk Industry Foundation's 1962 Gold Medal Pasteur Award for heroism by Secretary of Labor Arthur J. Goldberg on June 14. In an impressive ceremony held at the Secretary's Washington office, Riggs was honored for the quick thinking and personal courage that saved a mother and her two children from an attack by her mentally deranged husband.

Riggs, a mild-mannered man of five feet eight inches and a hundred and forty-six pounds, disregarded his personal safety on December 18 when he grappled with a mentally unbalanced man who was armed with a knife. Riggs disarmed the man and held him until help arrived.

Secretary Goldberg paid tribute to Riggs's heroism in his citation that accompanied the presentation of the medal. "Your determined action in the face of physical danger, your willing acceptance of great personal risk in order to avert a potential tragedy, is truly service above and beyond the call of duty. I am proud to present to you the Louis Pasteur Gold Medal of the Milk Industry Foundation in gratitude and in recognition for outstanding heroism."

Arthur J. Goldberg, Secretary of Labor, presents the Milk Industry Foundation's Pasteur Gold Medal Award to Donald C. Riggs, driver-salesman for Harmony Farms Dairy of Columbus, Ohio, while Mrs. Riggs looks on.

STUDENT CHAPTER NEWS

W. W. Snyder, Editor
Section Devoted to News of Student Members

Utah State University Dairy Club Sells 168 Gallons of Ice Cream

During Agathon, an annual event for high school seniors, at Utah State University, Dairy Club members dipped and sold 168 gal of ice cream. After subtracting the cost of cones and the ice cream, the club earned about $100. The money from this project and previous projects will be used to finance the Steak Fry, for which plans are nearly complete. Dairy Club members and partners, and staff members and partners, all go to one of the choice Rocky Mountain picnic spots for an evening of recreation and feast. Dairy Club members will cook the steaks this year.

For the second consecutive year, the returning Dairy Club president has been named valedictorian of the College of Agriculture. Congratulations are in order to Clayton Huber this year.

Ferron Perkes is chairman of this year's Dairy Cattle Judging Contest, sponsored annually. The breed organizations are making an award to the top judge of each breed, and the Dairy Club will make an award to the top judge of the contest.

The election of officers for 1962-63 was held at the last regular meeting, with the following being elected: president, Harry G. Markos; vice-president, Leo Israelesen; secretary, Daryush Valanejad; treasurer, Frank Markos; advisor, Professor Robert C. Lamb. Retiring officers are: president, Clayton S. Huber, vice-president, Ronald Boman; secretary, Harry G. Markos, treasurer, Lewis Wiser.

Los Lecheros Dairy Club Holds Fitting and Showing Contest

Each year the Los Lecheros Dairy Club conducts a Fitting and Showing Contest during the Poly Royal on the San Luis Obispo campus of California State Polytechnic College. This year the event was judged by Warren VanderHule of California Dairy Breeders, Inc., Davis, California. The Mistress of Ceremonies was Annabelle Alberti, a dairy student from Los Banos.

The California Dairy Princess, Marilyn Jacobs, was in attendance to present the awards. Competition was keen for the top awards in this year's contest held April 28. The four winners were: first, John Winters, Wapato, Washington; second, Fred Silva, Los Banos; third, George Nunes, Point Reyes; fourth, Richard Cotta, Salinas, California.

Ohio State University Holds Honors Banquet

The Ohio State University Student Branch of the American Dairy Science Association held its annual Honors Banquet April 27, 1962. Robert D. Stewart, Secretary of the American Guernsey Cattle Club, was present to honor Howard J. Ziegler, Clyde, Ohio, an outstanding Guernsey breeder and director of the American Guernsey Cattle Club. Ziegler's portrait is now in the Dairy Science Hall of Fame.

Judging team keys were presented to the judging team members, consisting of Dick Soldner, Virginia Garrett, Bob Fleming, Myron Wehr, Bill Young, Warren Briggs, and Jim Patterson.

The winners of the Student Judging Contest were also recognized. The high individual was Art Smallseed, and Joe Miller received the high score for oral reasons. The high individuals in each of the breeds were as follows: Jersey, Joe Miller; Ayrshire, Joe Miller; Brown Swiss, Joe Miller; Holstein, Bob Bauman; Guernsey, Don Isler.

Dr. William Brakel presented the Salsbury Scholarship to Dick Soldner, Virginia Garrett, and Bob Bauman. Dr. Fordyce Ely presented the Borden Award to Frank Bonie. Joe Miller presented a trophy to Jackie Miller, the 1962 Dairy Queen.

Buckeye Dairy Show

The Student Branch members sponsored a very successful Dairy Show on April 28, 1962, in Plumb Hall Arena. Dick Soldner chaired the program during the judging and Jackie Miller, the queen, presented the trophies. The official judges were Purebred Dairy Cattle breeders who were on the campus for their annual Purebred Dairy Cattle Association Judging Conference. The breed winners in the showmanship classes were as follows: Ayrshire, Kent Bone; Brown Swiss, Charles Wallace; Guernsey, Ron Renner; Holstein, Bob Haskins; Jersey, Roger Smith. At the conclusion of the individual breed championships, Bob Haskins was declared the winner over all breeds.

As an added feature to the show, the best cow of each breed in the university dairy herd was paraded before the spectators, to show how these cows had contributed to their respective breeds.

1962-63 Officers

At a recent Dairy Science Club meeting, the following officers were elected for 1962-63: president, Bill Crist; vice-president, Jerry Smollen; secretary, Don McAllister; treasurer, Jim Patterson; Directors, Dick Soldner and Joe Miller.

Kentucky Dairy Science Club Reports Activities

The University of Kentucky Dairy Science Club assisted with a Career Day program sponsored by the staff of the Department of Dairy Science and the Kentucky Dairy Industry held April 26. As a climax to the day's activities, the Dairy Science Club sponsored their annual Honors Banquet in the evening.

Mr. C. O. Ewing II was honored by the Dairy Science Club as the outstanding dairyman of the year in Kentucky. His portrait will be hung in the Dairyman's Hall of Fame in the Dairy Products Building. Awards were also made to students who participated in the Dairy Cattle and the Dairy Products Judging Contests. The winner of the Senior Division in the Dairy Cattle Judging Contest was Mendel Howard, Elkton; top award for the Junior Division was claimed by Neal Owen, Butler; and winner of the Dairy Products Judging Contest was Carl Johnston, Custer, Kentucky.

Dairy Club Active in Tennessee

The second Tennessee Dairy Service Award was made at the Annual Dairy Club Banquet, April 25, to L. O. Colebank, official classifier for the American Guernsey Cattle Club. Colebank has been very active in the promotion of dairy programs for young people in dairy cattle judging and in dairy extension work.

The Purina Bowl for outstanding scholar-

ship and leadership during the past year was awarded to Steve Cates, a junior majoring in dairy production.

The University of Tennessee Dairy Club initiated a new activity this year. A judging contest was held for all university agricultural students. Four classes of animals and two classes of dairy products made up the contest. Awards were presented to the winners at the banquet. The all-class winner was John Housley; Steve Cates was the products winner and Herbert Lackey was high in cattle.

This is to be an annual event sponsored by the University of Tennessee Dairy Club.

Washington State Dairy Club Conducts Open House

Nearly all of the Dairy Club members participated in a very successful open house conducted by the Washington State University Student Chapter on April 28. The open house consisted of a tour of the Washington State University creamery and a tour of the J. C. Knott Dairy Center about six miles out of Pullman. The tour started at the creamery where the Dairy Manufacturing students explained the holding tank, pasteurizers, homogenizer, plate cooler, separator, bottler, ice cream mix tank, fruit feeder, freezer, cheese vat, cheese knives, cheese press, can scrubber, can washer, bottle washer, and several pieces of laboratory equipment, including the cryoscope, Babcock test bottles, pH meter, titratable acidity apparatus, and a laboratory manual.

At the J. C. Knott Dairy Center, the Dairy Production students led visitors through the milking parlor, milk room, bull barn, silage bunker, yearling shed, hay barn, calf barn, and freshening stalls. The visitors were also shown the bulk tanker. Since the tour was in the afternoon, they also saw the cows being milked.

Refreshments, including milk, were served at the creamery and transportation was provided to the dairy farm.

The tour was successful and the Dairy Club will probably continue to make it an annual event.

Babcock Club at Wisconsin Holds Annual Banquet and Makes Awards

The Marschall Dairy Laboratory Award was presented to Thomas C. Everson, a student in the Department of Dairy and Food Industries, University of Wisconsin, by Bruno Stein, Chairman of the Board, Marschall Dairy Laboratory, Madison, Wisconsin, on May 12, at the Annual Spring Banquet of the Babcock Club, the student organization of the Department of Dairy and Food Industries, University of Wisconsin, Madison.

The Virginia Dare Award was presented to Everson by Professor H. E. Calbert, Chairman of the Department of Dairy and Food Industries. He was chosen by the staff of the Department on the basis of his scholastic achievement, interest in the dairy industry, and his activities in college, such as participation in the Babcock Club and other student organizations.

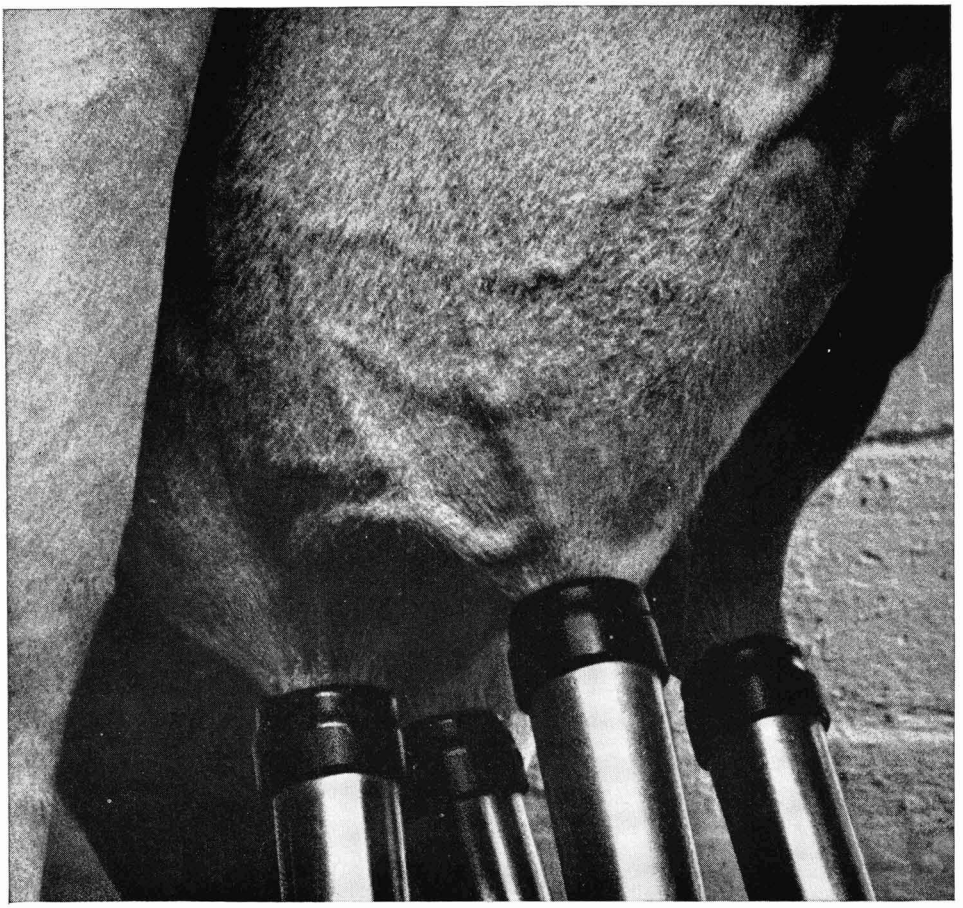
Louisiana State University Awards Fellowship

Ronnie Blanchard, a junior majoring in Dairy Production Science, at Louisiana State University, Baton Rouge, has been named as the recipient of the Danforth Summer Fellowship award for 1962. Blanchard has also been elected to serve as president of the Louisiana State University Dairy Science Club for the 1962-63 period.

NEWS FROM THE UNIVERSITIES

Cornell Dairy Industry Conference to be Held at Niagara Falls

The dates of the 1962 Joint Conference of the Cornell Dairy Industry Conference and the New York State Association of Milk Sanitarians are September 24-26, 1962. The meetings will be held in Niagara Falls, New York, at the Hotel Niagara.



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The following Cornell University staff members will present papers: R. D. APLIN, Department of Agricultural Economics; B. L. HERRINGTON, Department of Dairy and Food Science; J. K. LOOSLI, Department of Animal Husbandry; J. C. WHITE, Department of Dairy and Food Science; and P. J. VANDEMARK, Department of Dairy and Food Science. Also on the program will be: ALEC BRADFIELD, Department of Animal and Dairy Husbandry, University of Vermont; L. C. THOMSEN, Department of Dairy and Food Industries, University of Wisconsin; F. W. LENGEMANN, Atomic Energy Commission, Washington, D.C.; H. J. BARNUM, Dairy Products Improvement Institute; and DAROLD TAYLOR, U. S. Public Health Service, Washington, D.C.

NORTH CAROLINA

June Dairy Month Activities in North Carolina

Staff members in the Department of Food Science at North Carolina State College assisted and participated in the June Dairy Month activities throughout the state. A June Dairy Month Kick-off Breakfast was held on June 1 in Raleigh to start activities. L. L. RAY, Director of Foundations at North Carolina State College, and State Chairman of June Dairy Month, presided at the breakfast. State Dairy Princess BARBARA MORGAN was on hand to add her charm to the event.

STATE SENATOR RALPH SCOTT, who is also president of Melville Dairy Company at Burlington, North Carolina, introduced GOVERNOR TERRY SANFORD in his usual incomparable fashion.

Governor Sanford made several significant remarks about the new Department of Food Science at North Carolina State College. He told the group that the 1963 Legislature will be urged to provide a $2.3 million building for the new Food Science Department. The Food Science Department of the College deserves recognition, Sanford asserted. "It will work more effectively in a building of its own . . . I pledge this administration will give this building top priority."

Sanford said the new building when completed, "will symbolize the single greatest effort being made to improve the farm economy and the total economy of North Carolina."

The dairy industry provides an excellent example of what can be done in food processing in North Carolina, the Governor said. "Not only has the dairy industry contributed to the health and economy of the State; of special significance this year, it has shown the way for the larger industry of food processing and marketing."

A Frozen Food Handlers Conference, sponsored by the Food Science Department, was held June 14 at the Sir Walter Hotel in Raleigh. A North Carolina–produced food luncheon highlighted the meeting.

Ice cream cake was presented to Governor Terry Sanford by the North Carolina Dairy Industry at the June Dairy Month Kick-Off Breakfast. The presentation was made by Miss York Kiker, Dairy Marketing Specialist, North Carolina Department of Agriculture.

OHIO

Adult Education Program Discussed

A general review of the Adult Education Programs in the Department of Dairy Technology, Ohio State University, was conducted during June through a series of meetings with industry representatives. The Education Committee of the Ohio Dairy Products Association, composed of ANTON KELLER, Columbus, Chairman; L. R. STAUFFER, Warren; CARL BROUGHTON, Marietta; O. A. DEAN, Cleveland; J. I. KLINGENBERG, Athens; RALPH PALMER, Columbus; JOHN SPAHN, Steubenville, met with staff members to explore the needs of industry. A luncheon meeting of presidents and vice-presidents of the four Ohio Dairy Technology Societies with staff members provided an opportunity for planning the 1962-63

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programs of the Societies. Representatives of the State Departments of Health and Agriculture met with members of the Department to plan for the expansion of joint programs in public health and sanitation during the coming year. Early in the month the governing committee of the Ohio Dairy Products Fund, composed of FRED MEYER, Cleveland, ARTHUR MUSSETT, New Bremen, and WILLIAM BICHSEL, New Philadelphia, met at the Department to review research and consider suggested expenditures from the fund.

Nikolai King Lectures

NIKOLAI KING, on leave from his duties as Principal Dairy Research Officer, CSIRO, Melbourne, Australia, and a member of the Dairy Technology Staff at the University of Illinois, discussed the physical chemistry of milk system at the Research Seminar sponsored recently by Ohio State University's Institute of Nutrition and Food Technology.

At a dinner given in his honor at the University Faculty Club following the Seminar, King discussed scientific matters of mutual interest with staff members and graduate students of the Department of Dairy Technology and interested industry personnel in the Columbus area.

Ice Cream Sanitation Standards Reviewed

As an outgrowth of a recent conference between Ohio State Department of Health officials, municipal health authorities, dairy industry personnel, and representatives of Ohio State University's Department of Dairy Technology, a committee has been appointed to study the matter of ice cream sanitation standards.

The findings of the committee will be utilized by the parent group in developing a State-recommended frozen desserts standard ordinance and code. A Dairy Technology Staff member will assist the committee in an advisory capacity.

Recommendations Made for Manufactured Milk

The Manufacturing Milk Division of the Ohio Dairy Products Association went on record at a recent meeting as favoring those standards for manufacturing milk regulations recommended by the Evaporated Milk Association and The American Dry Milk Institute. Their action has been submitted to the Ohio Department of Agriculture for its consideration, and indications are that the standards in question will be implemented.

Future Farmers of America Hold Contest

Fifty-eight three-man teams competed in the Annual FFA Milk Judging Event, held at Ohio State's Department of Dairy Technology in May. The contest, coordinated by DR. T. KRISTOFFERSEN, was won by the team from Prospect, coached by J. W. WATKINS. Runners-up in the contest were teams from Pleasant View and Canal Winchester.

Frank Wish Passes

FRANK WISH, former owner, president, and general manager of the Hopewell Dairy of Bellefontaine, Ohio, died on May 8, 1962. After entering the dairy business in 1927 with his father, Wish developed the Hopewell Dairy into a sizable operation embodying many pioneer developments in automation, prior to being sold in 1961 to the Hawthorn-Mellody Company. At the time of his death Wish was serving as the president of the Ohio Milk Distributors Division of the Ohio Dairy Products Association. A prominent and progressive dairyman, who was also active in civic affairs, his passing constitutes a serious loss to the dairy industry.

Dairy Technology Societies

Central Ohio—The May meeting was highlighted by a talk by R. P. Zelm, in charge of quality development for the American Dry Milk Institute, on Skim Milk Powders and Their Application in the Manufacture of Fluid Milk Products. New officers elected for the 1962-63 year were: president, F. Bright, Waterford; vice-president, R. Flowers, Lancaster; secretary, J. Peters, Marysville; treasurer, F. Marsh, Columbus; and sergeant-at-arms, J. Salabino, Dayton.

Cincinnati—Dr. G. P. Gundlach spoke at the May meeting on Rebuilding the Image of the Dairy Industry. The following new officers were elected: president, H. Carl, Jr., Erlanger, Ky.; vice-president, D. Appel, Cincinnati; secretary, C. Hannekan, Covington, Ky.; treasurer, D. Meyer, Lockland.

Maumee Valley—After an afternoon of golfing, the Society met at Orchard Hills Country Club for its May meeting, which featured an election of officers and a discussion on dairy economics by C. A. Hilt, Pet Milk Co. New officers include: president, P. Lind, Toledo; vice-president, R. Heindricks, Wauseon; secretary, N. George, Toledo; treasurer, N. Stahl, Toledo; sergeant-at-arms, M. Goldsmith, Wauseon.

Northeastern Ohio—The program for the May meeting was designed to acquaint management personnel of dairy plants with the Society and consisted of a review of the year's

activities and accomplishments by President Sibert. Dr. I. A. Gould, Chairman, Department of Dairy Technology, The Ohio State University, introduced the winners of the Freshman Dairy Technology Scholarships made available by the Society, and B. F. Forbes was presented with a briefcase in recognition of his outstanding service as chairman of the Society's Education Committee. Newly elected officers include: president, M. Swinehart, Akron; vice-president, G. Johnson, Cleveland; secretary, Norman Berg, Orrville; treasurer, Robert Black, Mogadore; sergeant-at-arms, Abe Sandler, Youngstown.

WISCONSIN

Joachim von Elbe Joins Staff at University

JOACHIM VON ELBE, a native of Rostock, Germany, currently a graduate student at the University of Wisconsin, joined the staff of the Department of Dairy and Food Industries as an instructor on July 1.

He will work in extension and research in food science. At the same time he will complete his thesis for the Ph.D. His current research deals with insecticide residues and color stability in canned fruits. He will work with commercial food processors in Wisconsin on processing and quality problems.

INDUSTRY NEWS

Far Eastern Fair Visitors to See American Style Dairying

Modern milk production, as achieved in the United States in 1962, will be viewed by Far Eastern visitors at the International Trade Fall opening this fall at Izmir, Turkey. A milking panorama, using Turkish cows, will be presented several times a day.

G. D. Regan of the Babson Bros. Co. demonstrating a milking machine to an interested child.

Four cows will be milked at a time in individual, elevated stalls by a white-clad attendant. The milk will be visible as it is taken by transparent breaker cup machines and flows through a low milk line, mounted at the operator's knee-level. It will then pass into a glass receiver in an ultra-modern milkroom.

There the milk will be cooled in a 250-gal Minnesota-made bulk tank, where it will be stored until it is processed. An automatic washing device will show how the modern American dairy farmer's equipment is cleaned at the push of a button.

In charge of installation and operation of the milking panorama will be G. D. REGAN of East Point, Georgia, Divisional Sales Manager of Babson Bros. Co. Unusual assignments are nothing new to Regan, who has had extensive experience in the dairy production, artificial insemination, and dairy farm equipment fields.

Regan has helped promote America's dairy industry by milking cows before school children in such unusual places as department stores and has trained men working with the dairy farm equipment industry.

New Additions to Carnation Research Staff

Recent additions to the Carnation Company Research Staff are DR. P. MELNYCHYN, University of Minnesota, formerly employer as a collaborator with USDA Fruit and Vegetable Chemistry Laboratory in Pasadena, Calif., and M. M. MAXWELL, B.S., College of Idaho and who has done graduate work at Washington State University.

William F. May to Fill Key Post with American Can

American Can Company's new key corporate post of vice-president, planning and development, will be filled by a 46-yr-old executive, W. F. MAY, who began his career with the company as a research chemist and subsequently served in nearly every other function of the business, including sales, manufacturing, industrial relations, and general management.

May, a native of Chicago, graduated from the University of Rochester in 1937 with a degree in chemical engineering. He began his career with American's research center in 1938.

J. M. Jackson Assumes Two Presidential Offices

DR. J. M. JACKSON of American Can Company's Research Center in Barrington, Illinois, has been elected president of the Research and

Development Associates, Food and Container Institute. The Associates is an industry-supported organization which serves as a link between the research and development activities of the food and container industries and those of the Armed Forces.

He is also president of the Institute of Food Technologists, thus becoming the first person to hold these two presidential offices simultaneously. He assumed his duties as IFT president when the group met for its 22nd annual meeting in Miami Beach, June 10-14.

A graduate of the University of Chicago with a bachelor of science degree, Jackson received his doctorate in chemistry from that institution in 1932. He joined American Can the same year.

Degrees Awarded

M.S. Degree:

MOHAMMED ABUL HASNATH—Relative merit of duck egg-yolk compared to hen egg-yolk as a bovine semen extender. A & M College of Texas.

BENEDICTO A. PARKER—Some factors affecting milk fat and protein levels in milk from dairy herds in central Texas. A & M College of Texas.

KENNETH O. WILSON—De-fatted fish meal as a source of protein in a milk replacer for dairy calves. A & M College of Texas.

Ph.D. Degree:

C. N. GRAVES—Studies on the metabolism of bovine spermatozoa. University of Illinois.

ADVERTISERS' INDEX

RESEARCH PAPERS

κ-CASEIN—β-LACTOGLOBULIN INTERACTION IN SOLUTION WHEN HEATED

C. A. ZITTLE, M. P. THOMPSON, J. H. CUSTER, AND J. CERBULIS
Eastern Regional Research Laboratory,[1] Philadelphia, Pennsylvania

SUMMARY

Heating a mixture (0.5% of each) of β-lactoglobulin and κ-casein at pH 7 at 90 C 15 min led to interaction between the two proteins. Electrophoresis at pH 2.1 showed that, before heating, the components moved independently; whereas, after heating, a single component of intermediate mobility was observed. Ultracentrifuge examination of the heated mixture showed a new component with an S_{20} value of 45, three times greater than the S_{20} value of κ-casein. The ability of the κ-casein to stabilize calcium-sensitive casein was considerably reduced in the heated mixture. The clotting time of κ-casein by rennin was increased by the addition of β-lactoglobulin and was increased still further when the mixture had been heated. The clot formed by the action of rennin on the heated mixture contained the β-lactoglobulin as well as the κ-casein.

The effect of heat on solutions of mixtures of β-lactoglobulin and whole casein has been described in an earlier publication (2). In systems containing calcium chloride in which the β-lactoglobulin precipitated, there was no evidence for complex formation. In systems in which precipitation did not occur, the use of electrophoresis at pH 6.5 suggested that complex formation had occurred. The results of electrophoresis are equivocal, however, for demonstrating complex formation since, under certain conditions of heating, the electrophoretic mobility of β-lactoglobulin increases to about the same value as for α-casein. Thus, it could not be decided whether the single peak obtained after heating represented a complex of α-casein—β-lactoglobulin or a mixture of the two proteins, each with about the same mobility. Electrophoresis in acid solution, which has been useful in resolving the components of the α-casein complex (7, 12), has been used in the present experiments in the hope that an unambiguous answer to the question of casein–β-lactoglobulin interaction might be obtained. κ-Casein was used in the present experiments. The electrophoresis experiments were supplemented with ultracentrifuge studies. In addition, the effect of heating the κ-casein—β-lactoglobulin mixture on clotting time and precipitation by rennin, and the ability of the κ-casein in the mixture to stabilize the calcium-sensitive (α_s) casein, were explored.

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

MATERIALS AND METHODS

Where not described, the materials and methods are the same as in the previous paper (2). The protein solutions were heated at 90 C 15 min. The concentrations (0.25 to 1.0% total) are indicated for each experiment.

Several κ-casein preparations were used. All gave a symmetrical peak in free-flow electrophoresis (see later), and all were good stabilizers (11) (i.e., 50% stabilization of α_s-casein at κ/α_s ratio of 0.04 to 0.05). Some were prepared by fractionation in urea solution with trichloroacetic acid (8). Others were prepared by fractionation in ethanol (6). This procedure was simplified by precipitating the calcium-κ-casein by saturating with sodium chloride instead of using the potassium oxalate–sodium sulfate steps. Although seemingly homogeneous in free-flow electrophoresis, the κ-casein preparations contained a small amount of material that sedimented more rapidly than did the κ-casein. This heavy fraction did not stabilize α_s-casein. The preparation used in the ultracentrifuge and some of the electrophoretic experiments had had this heavy fraction removed. The very sensitive starch gel electrophoresis in 7 M urea (9) showed that the κ-casein preparations were contaminated with small amounts of the lambda-casein components.

The β-lactoglobulin (Species A) was crystallized from the albumin fraction of Species A pooled milk (3) and recrystallized twice. A suspension of the crystals was freeze-dried.

Solutions of casein and β-lactoglobulin were prepared at pH 7.0 by the addition of NaOH.

The rennin was a commercial preparation rated 1:30,000.

1. *Electrophoresis.* The free-flow Tiselius electrophoretic technique was used with Perkin-Elmer [2] equipment, Model 38. All experiments were performed in a mixture of 1% formic acid and 6% acetic acid at pH 2.1 (ionic strength 0.01). The protein solutions (0.6% of each) at pH 7.0 were acidified with HCl to about pH 2 and were then dialyzed for 24 to 48 hr against the acid mixture.

2. *Ultracentrifugation.* The protein solutions were centrifuged in a Spinco Model E analytical ultracentrifuge.[2] The centrifuge was operated at 25 C, with a 4-degree sector cell. The sedimentation values obtained at 25 C were recalculated to 20 C (S_{20}). The protein solutions were at a total concentration of 1% and adjusted to pH 7.0 with 0.1 N NaOH. These solutions, heated or not heated, were dialyzed against phosphate buffer, pH 7.0 (two one-liter portions in a period of 24 hr) until equilibrium was attained. The phosphate buffer was that described by Waugh and von Hippel (10), but the concentrations were one-half as great, i.e., the ionic strength was 0.1.

3. *Stabilization test.* This test has been described (11). It measures the amount of κ-casein required to maintain α_s-casein in solution with 0.020 M $CaCl_2$ at pH 6.8. The test was applied to heated and unheated mixtures of κ-casein and β-lactoglobulin.

4. *Clotting time with rennin.* The rennin was permitted to act on the κ-casein, or the mixture with β-lactoglobulin, at pH 6.4 and 30 C in the presence of 0.020 M $CaCl_2$. The rennin was added to the protein solution in a ratio of 1:2,000 of casein; 0.1 M $CaCl_2$ was added to give the designated concentration. The clotting time was determined by a visual method (1). Maintenance of a constant pH is very important. The solutions were heated at pH 7.0; after the $CaCl_2$ was added, the pH was adjusted to 6.4 with 0.01 N HCl or NaOH.

5. *Estimation of amount of protein clotted by rennin.* In these experiments the concentration of κ-casein was 1.0%, of β-lactoglobulin, 0.8%. These solutions, heated and unheated, were mixed with water and 0.1 M $CaCl_2$; 5 ml protein solution, 3 ml water, and 2 ml 0.1 M $CaCl_2$. The pH was 6.6. Two milligrams of rennin in 0.1 ml of water were added at 30 C. The solutions were stirred occasionally to observe clot formation. After clotting was complete (5 to 10 min), the solutions were centrifuged in 15-ml graduated centrifuge tubes 5 min. The volume of the sediment was recorded and the protein remaining in the supernatant solution was determined from the ultraviolet absorption at a wave length of 280 mμ. For this purpose, the solutions were clarified by the addition of a drop of 0.5 N NaOH.

[2] It is not implied the USDA recommends the above company or its product to the possible exclusion of others in the same business.

RESULTS

1. The electrophoretic behavior at pH 2.1 of κ-casein and of β-lactoglobulin, and of their mixtures, both unheated and heated, is shown in Figure 1. The tracings shown were obtained after about 35 min electrophoresis at 130 v and 4.5 ma (field strength 10.0 v cm^{-1}). The descending boundaries in the cell are illustrated. The κ-casein gives a sharp peak, with a slight suggestion of inhomogeneity on the advancing limb of the peak. The mobility is 7.3 units (7.3×10^{-5} cm^2 v^{-1} sec^{-1}). The mobility is positive, i.e., all these proteins are positively charged at pH 2.1. The average mobility of the heated κ-casein is 8.3. For β-lactoglobulin the mobility is 15.5, and when heated 13.5. In the unheated mixture the mobilities are 7.3 and 15.4. When the mixture is heated, the major component has a mobility of 10.1. When κ-casein alone is heated and then mixed with β-lactoglobulin, electrophoresis gives mobilities of 8.4 (average) and 15.5, values expected for an

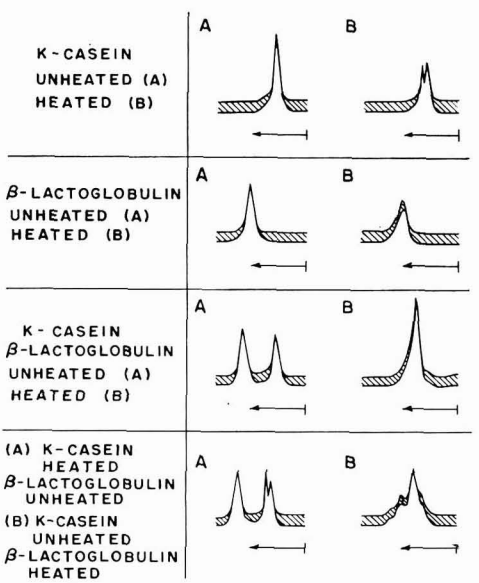

FIG. 1. Electrophoresis at pH 2.1 of κ-casein and of β-lactoglobulin, and of mixtures, unheated and heated.

unreacted mixture from the results given above. When β-lactoglobulin alone is heated and then mixed with κ-casein, the major component has a mobility of 9.8, i.e., similar to that in the heated mixture above. Minor components may be unreacted κ-casein (8.8) and heated (12.8) β-lactoglobulin.

2. Sedimentation values (S_{20}) of 2.7 and 15.1 were obtained for β-lactoglobulin and κ-casein, respectively. When these proteins were heated individually the κ-casein contained a fraction with an S_{20} of 32, but the bulk of the κ-casein sedimented with the characteristic value of 15; β-lactoglobulin A sedimented with an S_{20} of 5.3. In the unheated mixture of these two proteins, the S_{20} values were 2.7 and 17.4, respectively. When the mixtures were heated, however, and this was done for κ-casein:β-lactoglobulin ratios of 1:2 and 1:1, ultracentrifugal examination showed a rapidly sedimenting component with an S_{20} value of 45. Components with sedimentation values corresponding to uncomplexed β-lactoglobulin and κ-casein were still present in both instances.

3. A test of the ability of κ-casein to stabilize α_s-casein was made of an unheated and heated mixture of κ-casein and β-lactoglobulin. The results are shown in Figure 2.

4. The influence of β-lactoglobulin on the clotting time of κ-casein, in unheated and heated mixtures, is shown in Table 1. A marked influence on the clotting time is apparent.

5. The precipitation by rennin of various combinations of κ-casein and β-lactoglobulin, unheated and heated, is given in Table 2. The light absorbance of κ-casein is 1.0 for a concentration of 1 mg per milliliter; for β-lactoglobulin, the value is 0.93. The soluble products resulting from rennin acting on κ-casein, however, have a negligible ultraviolet absorption. Thus, the ultraviolet readings do not assess the total soluble proteinaceous material, but they do show that the β-lactoglobulin in the heated mixture was precipitated by rennin action, together with the κ-casein. Some β-lactoglobulin apparently has been coprecipitated in the clot from the unheated mixture. The nature of this association is not apparent from the present experiments. The volumes of the clots were markedly larger for the heated solutions than for the unheated. This was true even for κ-casein alone. Thus, heat had altered the κ-casein, even though with this preparation the ability to stabilize α_s-casein had remained the same.

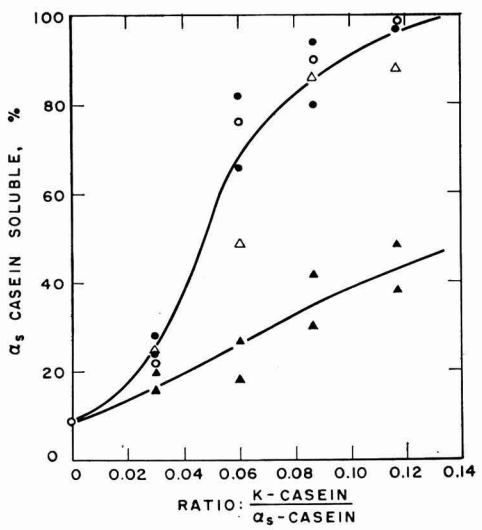

FIG. 2. The stabilization of α_s-casein in the presence of 0.020 M $CaCl_2$ by κ-casein, and κ-casein mixed with β-lactoglobulin, both unheated and heated (90 C for 15 min). κ-Casein (○), κ-casein, heated (●), κ-casein plus β-lactoglobulin (△). These three are represented by a single curve. κ-Casein plus β-lactoglobulin, heated (▲).

TABLE 1

Rennin clotting time of κ-casein (κ), of κ-casein with β-lactoglobulin (β), unheated and heated, pH 6.4, 30 C, with 0.020 M $CaCl_2$

Sample	Treatment	Concentration	Clotting time
		(%)	*(sec)*
κ	Unheated	0.25	645
κ	Heated	0.25	250
κ + β	Unheated	0.25 each	1,040
κ + β	Heated	0.25 each	1,240

TABLE 2

Precipitation of κ-casein (κ), of κ-casein with β-lactoglobulin (β), unheated and heated, by the action of rennin, pH 6.6, 30 C, measured by UV_{280} absorption

Sample	Vol of sediment	UV_{280} absorption per milliliter	
		Original	Supernatant
	(ml)		
κ, unheated	0.1	5.0	0.25
β, heated	0	4.35	4.35
κ + β, unheated	0.1	9.35	3.75
κ + β, heated	0.6	9.35	0.35
κ, heated	0.4	5.0	0.35

DISCUSSION

Electrophoresis at pH 2.1 provides unequivocal evidence that β-lactoglobulin and κ-casein interact when heated together at pH 7 at 90 C for 15 min. Electrophoresis shows also that heating β-lactoglobulin before mixing with κ-casein brings about interaction when subsequently mixed with κ-casein at 20 to 25 C. Long (5) also, in his extensive studies of β-lactoglobulin—κ-casein interaction found that heating β-lactoglobulin alone was sufficient for interaction. Presumably, the β-lactoglobulin is activated by heating, perhaps by the exposure of SH groups, so that interaction occurs at the lower temperature. Ultracentrifuge examination of the heated mixtures shows that a new component has been formed which sediments rapidly with an S_{20} of 45, three times larger than the S_{20} value of κ-casein, the largest component of the mixture. Long (5) observed a complex with an S_{20} value of the same magnitude. Heating κ-casein alone does not lead to interaction when subsequently mixed with β-lactoglobulin. κ-Casein is altered by heating, as shown by the split in the electrophoretic pattern, by decrease in the clotting time (Table 1), and increase in the volume of the clot (Table 2). Heated κ-casein solutions, however, frequently retain their ability to stabilize calcium-sensitive casein (11).

It is evident from the results in Figure 2 that the ability of κ-casein to stabilize the calcium-sensitive casein has been considerably reduced by its interaction with β-lactoglobulin. This does not necessarily mean that heating milk would lead to a destabilization by such a mechanism, for there the κ-casein is already associated with the calcium-sensitive casein before heat is applied. The work of others (4) indicates that β-lactoglobulin does complex with the caseins when milk is heated.

The mere presence of β-lactoglobulin lengthens the clotting time of κ-casein by rennin (Table 1). This may be a competitive effect, since rennin is a protease and presumably will have a tendency to associate with all proteins concomitant to peptide bond splitting. Heating the β-lactoglobulin—κ-casein mixture brings about a still further increase in the clotting time, presumably because of the combination of the β-lactoglobulin with the κ-casein, with perhaps spatial interference. Kannan and Jenness (4), in their studies on the clotting time of the calcium caseinate–calcium phosphate complex heated with β-lactoglobulin, observed an increase in the clotting time and considered this evidence for interaction between casein and β-lactoglobulin. The clot obtained by the action of rennin on the heated mixture of κ-casein and β-lactoglobulin contains both proteins (Table 2), as would be expected if heat had caused κ-casein and β-lactoglobulin to combine. Presumably, if conditions are found for satisfactory clotting of heated milks, the β-lactoglobulin, and perhaps other whey proteins also, would be found with the casein clot.

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APPLICATION OF POLARIZATION OF FLUORESCENCE TECHNIQUE TO PROTEIN STUDIES. I. THE ROTATORY PROPERTIES OF β-LACTOGLOBULIN [1]

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SUMMARY

A study was conducted using the polarization of fluorescence method to characterize β-lactoglobulin. The method involved forming a fluorescent dye conjugate of the protein molecule with a dye molecule (1-dimethylaminonaphthalene-5-sulfonyl chloride) which imparted fluorescent properties to the conjugate. Only minor alteration in the physical properties of the protein was produced by the conjugation process.

Curves of reciprocal polarization vs. T/η for β-lactoglobulin yielded an average slope of 1.02×10^{-4} at 0.02 ionic strength and 0.72×10^{-4} at 0.10 ionic strength. The apparent molecular volume of a spherical protein molecule, having the same relaxation time as the slightly elongated β-lactoglobulin molecule, was 98,310 cm^3 at 0.10 ionic strength and 64,580 cm^3 at ionic strength 0.02. The mean harmonic relaxation time for β-lactoglobulin was 6.85×10^{-8} sec at 0.02 ionic strength and 10.91×10^{-8} sec at 0.10 ionic strength. Addition of sucrose to protein solutions to increase the viscosity and, thereby, reduce the rate of rotatory diffusion of the fluorescent β-lactoglobulin molecule, resulted in substantial reduction in the apparent molecular volume.

A relatively new technique known as polarization of fluorescence has been employed by several workers to study the molecular dimensions of various proteins from widely different sources. Proteins included in these studies have been bovine serum albumin (4, 10), actin (8), lysozyme and egg albumin (10), horse serum albumin (1), and soybean trypsin inhibitor (7).

Weber (9) has pioneered the development of the theory for this technique. In practice, a fluorescent dye such as 1-dimethylaminonaphthalene-5-sulfonyl chloride is linked to the protein molecule by covalent bonding. The partial polarization of the fluorescent light emitted by such protein-dye conjugates in solution is a function of the relative size and rotatory diffusion characteristics of the protein molecule.

This paper deals with the application of the polarization of fluorescence method to study the effect of altering the ionic strength, protein concentration, and sugar concentration upon the relaxation time and the apparent molecular volume of β-lactoglobulin.

Received for publication April 5, 1962.

[1] Article 262, Department of Dairy Technology. Supported in part by University grants-in-aid and by a grant from the National Institutes of Health (U. S. Public Health Service).

EXPERIMENTAL PROCEDURE

The β-lactoglobulin used (Nutritional Biochemical Corporation) was found to possess typical electrophoretic and ultracentrifugal properties.

To prepare the β-lactoglobulin-dye conjugate, a quantity of the fluorescent dye, 1-dimethylaminonaphthalene-5-sulfonyl chloride, equivalent to 1-2% of the β-lactoglobulin, was dissolved in acetone, added slowly with stirring to the protein solution (0.5 g per 100 ml), and the mixture stored at 0-5 C for 5-12 hr (10). At this point, the unreacted dye, present in the free sulfonyl form, was removed from the solution by repeated passing of the solution through a Dowex 2 ion-exchange column as measured by paper chromatography (11). The β-lactoglobulin-dye conjugate solution was stored in polyethylene bottles at about −10 F.

Reagent-grade chemicals were used. Buffers utilized were sodium phosphate (pH 6.5, ionic strength 0.02 and 0.10) and potassium phosphate (pH 6.5, ionic strength 0.02). Final pH adjustments for these buffers were made at 23-25 C.

Electrophoresis experiments were conducted at 2 C in a Tiselius-Klett instrument equipped with a Longsworth schlieren scanning device.

Sedimentation determinations were made by utilizing a Spinco Model E Analytical Ultracentrifuge, equipped with a rotor designed to accommodate a standard and a wedged cell simultaneously. All determinations were conducted at rotor temperatures regulated between 20 and 26 C, with a centrifugal force of about 259,600 × G. Sedimentation constants were calculated without correcting for the small changes in density of the protein at these temperatures. The protein concentration was maintained at about 0.5 g per 100 ml, the pH was 6.5, and the ionic strength at either, 0.02 or 0.10.

A schematic diagram of the fluorescence microphotometer, used to measure the intensity of fluorescent radiation emitted by the protein-dye conjugate, is presented in Figure 1. The

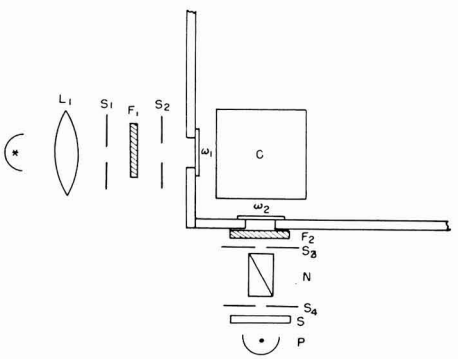

FIG. 1. Diagram of the fluorescent microphotometer.

light source was a G.E. H-100 A4 mercury vapor lamp. The quartz lens (L_1) produced parallel light which passed through two eliminating slits (S_1 and S_2). The incident filter (F_1) had a maximum transmittance at 365 mμ wave length, used for the excitation radiation. The unpolarized radiation entered the cell compartment (C), which contained the fluorescent protein sample supported in a temperature-regulated water bath, through a quartz window (W_1). The fluorescent radiation emitted from the excited fluorescent molecules passed through the thin glass window (W_2) at an angle of 90 degrees from the incident radiation. The intensity of the fluorescent radiation was measured with a no. 931-A photomultiplier tube (P). The vertical and horizontal components of the fluorescent radiation were measured separately by rotation of the nicol prism (N) through an angle of 90 degrees. Also, an optical shutter (S) was mounted in front of the phototube so that the cell compartment could be opened without turning off the voltage to the phototube. The current output from the phototube was measured on a Rubicon galvanometer whose sensitivity was 6×10^{-4} amp/mm deflection. Voltage regulators were used to maintain a constant voltage in the light and phototube circuits.

It was found necessary to correct for the generally small amount of scattered, nonfluorescent radiation entering the phototube. Blank values were determined for nonconjugated β-lactoglobulin solutions at each of the temperatures and protein concentrations that were to be used with the conjugated protein solutions. These blank values for horizontal and vertical intensities were subtracted from the observed experimental values before calculation of partial polarization values.

RESULTS AND DISCUSSION

Effect of fluorescent conjugation reaction upon the electrophoretic and sedimentation properties of β-lactoglobulin. The conjugation process whereby one or more fluorescent dye molecules were linked with a molecule of β-lactoglobulin by means of a covalent sulfonamide linkage might be expected to alter slightly the properties of the protein. Therefore, a comparison of electrophoretic and sedimentation data was made for nonconjugated and conjugated β-lactoglobulin at pH 6.5 and ionic strength 0.10 (see Table 1). These results indicate that relatively minor changes occurred in these quantities as a result of conjugation. However, examination of the electrophoretic and sedimentation patterns showed that slight polydispersity was produced in the protein by the conjugation process, as evidenced by some

TABLE 1

Comparison of electrophoretic mobilities and sedimentation constants for nonconjugated and conjugated β-lactoglobulin

Sample	Descending mobility [a]	Ascending mobility [a]	Sedimentation constant [b]
Nonconjugated β-lactoglobulin	−4.94	−4.94	3.00
Conjugated β-lactoglobulin	−4.83	−4.96	3.26

[a] Electrophoretic examination at 2 C mobility × 10^{-5} cm^2 v^{-1} sec^{-1}.
[b] $S_{20} \times 10^{13}$.

flattening and broadening of the respective peaks.

Previous workers have reported that other proteins such as ovalbumin, bovine serum albumin, lysozyme, and others were not altered to an appreciable amount, based upon electrophoretic, sedimentation, enzymatic, and other fundamental properties (1, 4, 8, 10).

Polarization of fluorescence—theory and calculations. Partial polarization (P) is defined by the equation

$$P = \frac{I_h - I_v}{I_h + I_v} \tag{1}$$

where (I_h) is the component of fluorescent radiation emitted with its direction of vibration in a horizontal plane, and (I_v) is the component of radiation with its vibration in a vertical plane. Arbitrary galvanometer units were used for the radiation intensities applied to Equation (1).

It has been shown (9) that the partial polarization (P) for a spherical molecule in solution is related to its molecular volume (V) by the expression

$$\left[\frac{1}{P} + \frac{1}{3}\right] = \left[\frac{1}{P_o} + \frac{1}{3}\right]\left[1 + \frac{RT}{\eta V}\tau\right] \tag{2}$$

where P_o is the value for (P) when $T/\eta = 0$, at which time no depolarization due to molecular rotation takes place, (R) is the gas constant $(8.314 \times 10^7$ erg mole^{-1} deg$^{-1})$, (T) is absolute temperature of the protein solution during the polarization of fluorescence measurements. These temperatures ranged from 5 to 35 C for all trials, (η) the viscosity of the solvent in poises, (τ) the lifetime of the excited state of the fluorescent molecule, and (V) the molecular volume of the molecule in cm^3. According to this equation, the intercept of the graph of $\left[\frac{1}{P} + \frac{1}{3}\right]$ vs. T/η is equal to $\left[\frac{1}{P_o} + \frac{1}{3}\right]$ and the slope is equal to $\left[\frac{1}{P_o} + \frac{1}{3}\right]\frac{R\tau}{V}$. From this it follows that the volume of the macromolecule, assuming spherical shape, is obtained from the simplified expression

$$V = R\tau \times \frac{\text{Intercept}}{\text{Slope}} \tag{3}$$

The value for the relaxation time of the macromolecule (ρ) can be calculated from the following relationship (9)

$$\rho = \frac{3\eta V}{RT} \tag{4}$$

To determine the relaxation time for ellipsoidal molecules, whose axial ratio is sufficiently large, requires the use of a more refined equation that takes into consideration the relaxation time about each major axis of revolution. In the present study, a value for the harmonic mean of the principal relaxation times (ρ_h) was obtained from the equation

$$\left[\frac{1}{P} + \frac{1}{3}\right] = \left[\frac{1}{P_o} + \frac{1}{3}\right]\left[1 + \frac{3\tau}{\rho_h}\right] \tag{5}$$

According to Weber (9), if a straight line is obtained in plotting $\left[\frac{1}{P} + \frac{1}{3}\right]$ vs. T/η, the relaxation time of the rotation is (ρ_h). In the case where curvature of the graph is experienced, the value of the initial slope where $T/\eta \rightarrow 0$ is used to calculate (V) and (ρ_h) for the ellipsoid.

The curve for an elongated ellipsoid will exhibit curvature of the graph only if the axial ratio of the protein is six or greater (9). Since the reported value for the axial ratio of β-lactoglobulin is less than six (2, 6), curvature of the graph would not be expected. On this basis, the value of the mean harmonic relaxation time for β-lactoglobulin was calculated from the simplified equation

$$\rho_h = \frac{3\eta V}{RT} \tag{6}$$

Polarization of fluorescence—β-lactoglobulin. (a) *Effect of ionic strength.* The partial polarization of fluorescence was determined for fluorescent, conjugated β-lactoglobulin in phosphate buffer, pH 6.5 and at ionic strength 0.02 (Figure 2) and 0.10 (Figure 3). Although some variation was noted in the intercept values among trials at each ionic strength, this variation is considered to be relatively small and probably due to uncertainties in measurement of the fluorescent intensities. Average values for intercepts for trials at 0.02 ionic strength were 5.66 ± 0.77 and the value for 0.10 ionic strength was 6.08 ± 0.61. The slopes of the graphs were significantly greater on the average for ionic strength 0.02 (1.02×10^{-4}) than at ionic strength 0.10 (0.72×10^{-4}).

(b) *Effect of protein concentration.* To determine the effect of protein concentration on the results obtained by the polarization of fluorescence method, determinations were con-

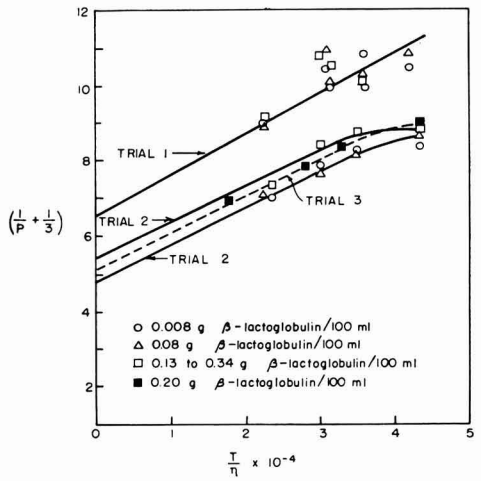

FIG. 2. The partial polarization of fluorescence of conjugated β-lactoglobulin in phosphate buffer as affected by protein concentration (pH 6.5, ionic strength 0.02).

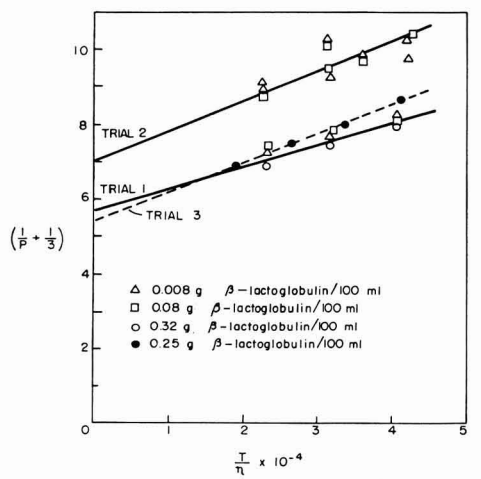

FIG. 3. The partial polarization of fluorescence of conjugated β-lactoglobulin in phosphate buffer as affected by protein concentration (pH 6.5, ionic strength 0.10).

ducted at ionic strengths of 0.02 and 0.10 and protein concentrations (β-lactoglobulin) ranging from 0.006 to 0.34 g/100 ml. The results are presented in Figure 2 (ionic strength 0.02) and Figure 3 (ionic strength 0.10).

In general, the shape and position of the curve were not greatly affected by the concentration of the protein, within each particular trial. One exception was noted; two curves were necessitated to represent the data for Trial 2 at ionic strength 0.02 (Figure 2). No particular significance is attached to this observation.

(c) *Molecular volume.* The average values for slope and intercept for curves of trials at ionic strength 0.02 and 0.10 were employed for estimation of the molecular volume (V) of a sphere whose relaxation time is that of the slightly elongated β-lactoglobulin molecule. τ was assumed to be 1.4×10^{-8} sec (10). The values obtained for (V) were 64,580 cm^3 $mole^{-1}$ at 0.02 ionic strength and 98,310 cm^3 $mole^{-1}$ at 0.10 ionic strength.

To use these data to obtain information concerning the nature of the rotational behavior of β-lactoglobulin molecules, the following assumptions and considerations were made with a hypothetical molecular model possessing the known characteristics of β-lactoglobulin.

The molecular weight of β-lactoglobulin has been shown to be about 36,000 (3), its apparent specific volume 0.746 (5), and its axial ratio about 4 (2). Using this information, the true molecular volume for β-lactoglobulin was estimated to be in the order of 26,860 cm^3 $mole^{-1}$.

The radius of a model sphere of equal volume would be about 18.6 cm; and the major and minor axes of an oblate spheroid fitting these conditions ($a/b = 4$) would be about 29.5 and 7.4 cm, respectively.

The larger experimental values for (V) in the present study than is predicted from these calculations is probably due to the random rotation of the ellipsoidal β-lactoglobulin molecule about its two axes. Due to the uncertainty of the configuration of β-lactoglobulin, no attempt was made to relate the polarization of fluorescence data to the true molecular weight of the β-lactoglobulin molecule.

(d) *Mean harmonic relaxation times.* The mean harmonic relaxation time at 25 C was calculated from Equation (4). The value for $\left[\frac{1}{P}+\frac{1}{3}\right]$ 25° was interpolated from the curves in Figures 2 and 3 at T/η corresponding to 25 C. The average intercept value for $\left[\frac{1}{P_o}+\frac{1}{3}\right]$ was already available from these figures and the value for (τ) was again assumed to be 1.4×10^{-8} sec. The average for the mean harmonic relaxation time was found to be 6.85×10^{-8} sec at 0.02 ionic strength and 10.91×10^{-8} sec at 0.10 ionic strength. These values compare rather closely with those of

Oncley (6), who listed values which averaged to about 15.5×10^{-8} sec and 5.4×10^{-8} sec for the two principal relaxation times of lactoglobulin as calculated for each axis of rotation. Furthermore, the present findings reveal a significant difference in the mean harmonic relaxation time obtained in the two different ionic strength buffers, indicating a possible change in shape or size of the β-lactoglobulin molecule.

(e) *Effect of addition of sucrose.* Sucrose was added to β-lactoglobulin solutions, to increase the viscosity and thereby furnish points on the graph of 1/P vs. T/η at lower values of T/η. The concentration of β-lactoglobulin was maintained at 0.25 g/100 ml in sodium phosphate buffer at pH 6.5 and ionic strength 0.10. All solutions were adjusted to volume at 20 C, and the sucrose concentrations varied up to 40% by weight. Typical results reveal (Figure 4) that increasing sucrose concentrations resulted in a definite tendency to steeper slopes and lower intercept values.

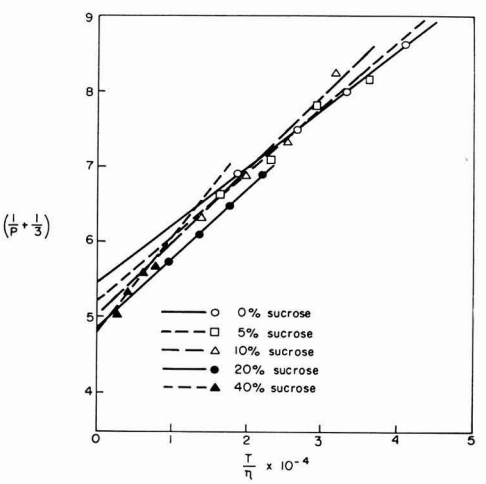

FIG. 4. The partial polarization of fluorescence of conjugated β-lactoglobulin in phosphate buffer as affected by the addition of sucrose (pH 6.5, ionic strength 0.10, protein concentration 0.25 g/100 ml).

The values for the intercepts and slopes of the curves and the calculated molecular volume (V) and mean harmonic relaxation time (ρ_h) for β-lactoglobulin, in each sucrose solution, were determined (Table 2).

The molecular volumes of β-lactoglobulin in sucrose solution show a pronounced and steady reduction as the sucrose concentration was increased. This was likely due to a reduction in the hydration of the protein molecule, caused by the affinity of sucrose for the free water of solution. Additional calculations revealed that nearly all of the water of hydration was removed from β-lactoglobulin in 40% sucrose solutions.

TABLE 2

Intercepts and slopes of the curves of $\left[\frac{1}{P}+\frac{1}{3}\right]$ vs. T/η, molecular volume and relaxation time for β-lactoglobulin in sucrose solutions [a]

Sucrose concentration	Intercept	Slope $\times 10^4$	V $\times 10^{-3}$	(ρ_h, 25°) $\times 10^8$
(%)				
0	5.38	0.798	78.49	8.56
5	5.20	0.871	69.47	8.24
10	5.02	0.940	62.14	8.97
20	4.92	0.902	63.47	13.07
40	4.80	1.152	48.58	27.24

[a] pH 6.5, sodium phosphate buffer, ionic strength 0.10.

Due to the large relative increase in viscosity from 0.9 to 5.2 cp, upon raising the sucrose concentration to 40% in the above solutions, the mean harmonic relaxation times increased by about 300%. This increase in relaxation time would no doubt have been larger except for the previously noted reduction in molecular volume at the high sucrose concentrations.

ACKNOWLEDGMENT

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APPLICATION OF POLARIZATION OF FLUORESCENCE TECHNIQUE TO PROTEIN STUDIES. II. THE ROTATORY PROPERTIES OF κ-CASEIN [1]

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SUMMARY

The polarization of fluorescence technique was used to study certain properties of κ-casein prepared either by the urea-fractionation method or a constant-pH method. Dye-conjugated preparations of each of these caseins responded similarly to the application of the polarization of fluorescence method, in that both yielded curves with negative slopes, but with the urea-fractionated κ-casein giving slightly steeper slopes. These κ-caseins were found to be generally similar, with only minor differences being observed in their electrophoretic and ultracentrifuge patterns.

The properties of constant-pH κ-casein as determined by the polarization of fluorescence method were altered slightly by addition of sucrose, disodium EDTA, calcium, and phosphate, but treatment with a cation exchange resin at pH 6.5 and 11.0 was essentially without effect. The polarization of fluorescence properties of this protein were shown to be dependent upon temperature and pH, yielding positive slopes for reciprocal polarization vs. temperature/viscosity curves at pH 10.9 and above.

The monomer-polymer interaction for κ-casein was investigated and values for the mole fraction of each, as a function of pH and temperature, were approximated.

The polarization of fluorescence method for studying the rotatory properties of fluoreseent β-lactoglobulin was described in the previous paper (4). The present study deals with the application of this procedure to κ-casein prepared by urea fractionation (1) or by a constant-pH method (3) for the purpose of investigating the characteristics of this protein in respect to molecular size and micelle formation, sensitivity to selected additives, pH, and response to cation exchange resin treatment.

EXPERIMENTAL PROCEDURE

Protein preparations were obtained from a commercial source of fresh, unheated skimmilk.

Constant-pH κ-casein (pH 6.5-7.0) was prepared essentially as described by Long et al. (3), with only one modification. The finely suspended αs-casein was removed by warming the solution to 35 C and centrifuging for 60 min at 10,000 × G in a Lourdes Model LR Centrifuge (VRA rotor).

Urea κ-casein was prepared by the method of Fox (1), with several modifications. αs-Casein and β-casein were removed from the 0.2 M calcium chloride solution by centrifuging at about 2,500 rpm in an International Centrifuge for 10-15 min. The supernatant was then adjusted to pH 4.7 at 0 C and dialyzed for 24 hr against a large excess of distilled water to precipitate the crude κ-casein. Further purification steps were conducted exactly as outlined (1).

The protein solutions were stored in polyethylene bottles at −20 C at pH 6.5-7.0.

Fluorescent-dye conjugates were prepared using the dye 1-diamino-naphthalene-5-sulfonyl chloride. The preparation and handling of the conjugates was identical to that for β-lactoglobulin (4).

In addition to the buffers previously described (4), a sodium phosphate buffer (pH 6.98, ionic strength 0.10) was prepared according to the directions of Waugh and von Hippel (5).

The methods for electrophoretic examination and ultracentrifugal analysis and polarization

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TABLE 1

Electrophoretic mobilities and sedimentation constants for κ-casein [a]

κ-Casein preparation	No. detns.	Mobility [b] Desc.	Asc.	No. detns.	$S_{20} \times 10^{13}$
Urea fractionated	6	−7.48	−8.07	7	13.69
Urea fractionated-dye conjugate	4	−7.90	−8.39	1	13.46
Constant pH	3	−7.45	−7.87	4	13.10
Constant pH-dye conjugate	2	−7.32	−8.06	1	15.36

[a] pH 6.98, ionic strength 0.10 sodium phosphate buffer.
[b] Mobility × 10^{-5} cm^2 v^{-1} sec^{-1} at 2 C.

of fluorescence have been previously described (4).

RESULTS AND DISCUSSION

Effect of fluorescent dye conjugation upon κ-casein. The electrophoretic mobilities of urea-fractionated and constant-pH κ-casein were obtained for the original and dye-conjugated preparations (Table 1). Relatively minor changes in the ascending and descending electrophoretic mobilities were produced by the conjugation process; however, it may be seen that the mobilities for the conjugated preparations were slightly larger than for the nonconjugated preparations.

Comparison of the values for sedimentation constants of the nonconjugated solutions reveals little difference between urea and constant pH κ-casein (Table 1). Furthermore, conjugation of the urea κ-casein caused no appreciable alteration in its sedimentation constant; however, on the basis of one trial, the conjugation reaction caused a significant increase in sedimentation constant with constant-pH κ-casein.

The sedimentation patterns revealed that urea κ-casein was a much more homogeneous preparation than constant-pH κ-casein. The conjugation process caused a slight broadening of the sedimenting peaks, which indicates that this treatment introduced some additional polydispersity to both urea and constant-pH κ-casein.

Polarization studies with urea-fractionated κ-casein. Urea-fractionated κ-casein solutions were examined by the polarization of fluorescence method at four different temperatures, in a manner identical to that described for β-lactoglobulin (4). Three trials were conducted; each trial included four different protein concentrations ranging from 0.005 to 0.50 g per 100 ml in phosphate buffer at 0.02 and 0.10 ionic strength at temperatures ranging from 5 to 35 C. Curves representing the averages for two trials at 0.10 ionic strength are presented in Figure 1, where reciprocal polarization is plotted against the ratio of temperature to viscosity. Considerable variation was exhibited in the values for the intercept (±1.37) and slope (±0.172) for these two trials.

Concentration effect. Intercept values for the curves for different κ-casein concentrations varied appreciably. Theoretically, the intercept value should be dependent only upon the wave length of the exciting radiation and independent of the protein concentration (6). It was noted that the more concentrated solutions of urea κ-casein possessed considerable turbidity, which may have altered the wave length of exciting and/or fluorescent radiation by differential scattering of the various wave lengths. Also, the effect of dilution may have caused some dissociation of the κ-casein polymers into smaller protein particle sizes, thereby altering the amount of such turbidity effects.

Temperature effect. Negative slopes were obtained for urea κ-casein for the curves obtained by plotting reciprocal polarization vs. T/η. Theoretical considerations indicate that the mac-

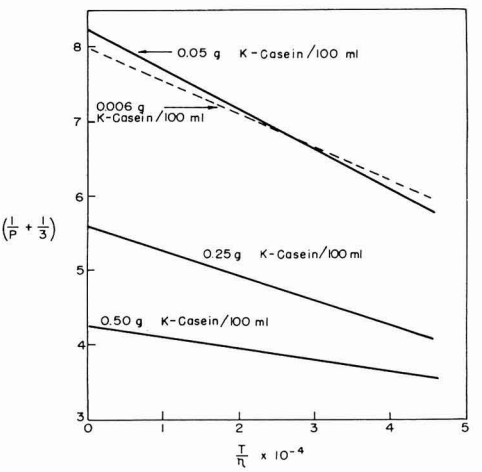

FIG. 1. The partial polarization of fluorescence of conjugated urea-fractionated κ-casein in phosphate buffer (pH 6.5, ionic strength 0.10).

romolecule would be expected to rotate at a faster rate at larger T/η values and produce larger values for the reciprocal of the partial polarization. Since the reverse behavior was observed, it may be concluded that the average molecular dimensions of the κ-casein aggregate were not constant but were increasing in size upon warming from 5 to 35 C. It was further determined that the slope and intercept of each of these curves was reproducible by making the four different polarization measurements, for any one sample, in the order of either increasing or decreasing temperature. Therefore, the change in size of the κ-casein micelle, which was observed, appears to be reversible. Since the sedimentation constant for κ-casein reportedly increases from 13.2 to 14.8 S upon raising the temperature from 1 to 32 C (5), it was concluded that the apparent increase in rotational size during such temperature changes was due to an association-dissociation reaction between κ-casein monomer and polymer, and not due simply to an unfolding of existing casein micellar components.

Ionic strength effect. Ionic strength, within the limits used, appeared to have virtually no effect upon the slope or intercept of the graph and, consequently, this variable may be considered ineffective in altering the previously described monomer-polymer association reaction.

Polarization studies with constant-pH κ-casein. Seven polarization of fluorescence trials were conducted for constant pH κ-casein, each with at least four different protein concentrations in 0.02 and 0.10 ionic strength phosphate buffer. Since no significant differences were observed for the results with the two ionic strengths, the results were averaged for each protein concentration and plotted in Figure 2. For all trials rather close agreement for intercept and slope values, within each protein concentration, was noted. For example, the seven samples containing 0.05 g κ-casein per 100 ml solution exhibited an average of 6.33 ± 0.11 for the intercept and -0.133 ± 0.0267 for the slope. This variation was considerably less than was obtained for urea-fractionated κ-casein trials.

Concentration effect. The figure shows that the concentration of constant pH κ-casein had a definite effect upon the intercept of the respective curves, although this effect is less pronounced than was observed with urea-fractionated κ-casein. Increasing the concentration of κ-casein from 0.005 to 0.25 g per 100 ml caused a progressive increase in the average intercept value; however, upon further increasing the protein concentration (to 0.50 g per 100 ml), a reduction was effected in the intercept value to a point below all the others. This latter alteration, i.e., reducing the intercept value by raising the protein concentration, is consistent with the concentration effects as previously noted at all concentrations of urea-fractionated κ-casein.

Temperature effect. The temperature effect upon the slopes in Figure 2 for constant-pH κ-casein were similar to those for urea-fractionated κ-casein but the magnitude was smaller (Figure 1). This indicates that the relative amount of monomer-polymer interaction is probably greater in urea-fractionated κ-casein than in constant-pH κ-casein.

Added sucrose. Since negative slopes were obtained in all preceding trials with κ-casein in which T/η was altered by temperature manipulation, attempts were made to alter T/η by adjusting the viscosity of the respective solutions by addition of sucrose while maintaining a constant temperature. It was hoped that this would provide a graph of reciprocal polarization vs. $1/\eta$ with a positive slope and permit estimation of the relaxation time and molecular volume for the κ-casein molecule.

Sucrose was added to constant-pH κ-casein in pH 6.5 sodium phosphate buffer at 0.10 ionic strength to a final sucrose concentration up to 60%. The partial polarization was measured at three or four different temperatures for each sucrose concentration. Solutions with higher viscosities (lower $1/\eta$) should cause the κ-casein

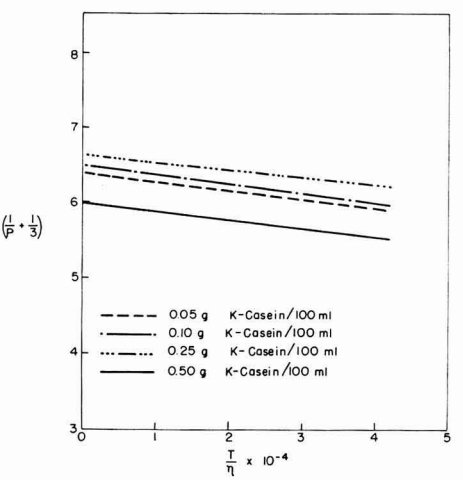

FIG. 2. The partial polarization of fluorescence of conjugated constant-pH κ-casein in phosphate buffer (pH 6.5, ionic strength 0.10).

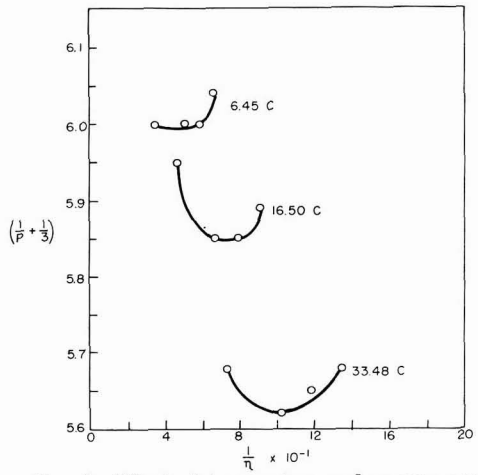

FIG. 3. Effect of temperature and sucrose concentration upon the partial polarization of fluorescence of constant-pH κ-casein in phosphate buffer (pH 6.5, ionic strength 0.10, casein concentration 0.05 g/100 ml).

molecule to rotate at a slower rate and thereby produce a larger value for partial polarization (P). The resulting curve for reciprocal polarization vs. $1/\eta$ would, therefore, be expected to yield a straight line with a positive slope. The results for determinations at three temperatures for κ-casein solutions containing 0, 20, 40, and 60% sucrose concentrations are shown in Figure 3. The three curves in Figure 3 are all convex toward the $1/\eta$ axis, reflecting a nonlinear relationship between viscosity and reciprocal polarization in the presence of such high sucrose concentrations. These curves tend to substantiate the previous findings that the κ-casein micelle is larger at higher temperatures and reveal an inverse relationship between temperature and reciprocal polarization values.

When the conventional plotting of reciprocal polarization against T/η is made (Figure 4), the results indicate that sucrose alters the properties of the protein in the same manner as that reported for β-lactoglobulin (4). In the present study, the addition of sucrose accentuates the temperature-induced casein monomer-polymer interaction, causing steeper curves of reciprocal polarization vs. T/η at higher sucrose concentrations.

The above results seem to justify the conclusion that sucrose addition does not permit satisfactory determination of the κ-casein micelle size by the polarization of fluorescence method.

Calcium effects. Since calcium had been used in the preparation of κ-casein, it was considered possible that incomplete removal of trace amounts of calcium might be responsible for the unusual temperature alteration of κ-casein, i.e., the increase in micelle size upon warming to 35 C.

To investigate this possibility, κ-casein solutions (0.50 g per 100 ml) were subjected to the following treatments prior to polarization of fluorescence determinations: (a) suspended in sodium phosphate buffer, pH 6.5, ionic strength 0.10 (ionic strength adjusted from 0.02 to 0.10 with and without NaCl); (b) suspended in sodium cacodylate buffer, pH 6.5, ionic strength 0.10 (ionic strength adjusted from 0.02 to 0.10 with NaCl); (c) suspended in each of the above buffers containing 0.05 or 0.50% disodium ethylenediaminetetraacetate (EDTA); (d) treatment of the κ-casein-water solution with Dowex-50 cation exchange column (sodium cycle at pH 6.5 and 11.0) prior to suspending in sodium phosphate buffer; and (e) addition of $CaCl_2$ (up to 0.1 M) to κ-casein solutions in cacodylate buffer, pH 6.5, and 0.10 ionic strength.

Only minor differences were produced by these treatments; no significant difference was obtained between the results with sodium phosphate and sodium cacodylate buffers. However, the addition of 0.50% disodium EDTA to this protein caused a slight reduction from the normal curves in slope and intercept of the curve of reciprocal polarization vs. T/η. The phosphate buffer (0.10 ionic strength–no NaCl) resulted in a slight reduction from the normal

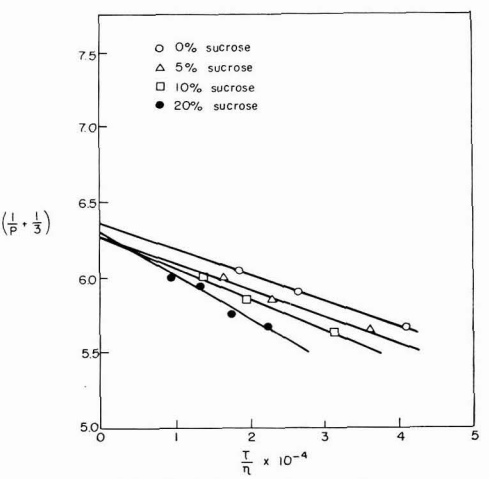

FIG. 4. Effect of temperature and sucrose concentration upon the partial polarization of fluorescence of constant-pH κ-casein in phosphate buffer (pH 6.5, ionic strength 0.10, casein concentration 0.05 g/100 ml).

curves in both the slope and intercept of the curve and the addition of disodium EDTA to this buffer solution of κ-casein caused an greater reduction of the slope of the curve. The ion-exchange treatment had no significant effect upon the properties of the solution as measured by polarization of fluorescence. Addition of calcium (0.001 to 0.05 M) to κ-casein solutions in sodium phosphate and sodium cacodylate buffers resulted in steeper curves than at 0.1 M calcium, indicating the possibility that a certain range of calcium concentrations may cause maximum aggregation of κ-casein monomers upon warming the protein system to 35 C (see Table 2).

TABLE 2

Effect of added calcium chloride upon the polarization of fluorescence properties of κ-casein solutions [a]

Calcium chloride added (Molar)	Intercept value	− Slope × 10^4
0	6.42	0.190
0.001	6.68	0.340
0.01	6.59	0.340
0.05	6.63	0.308
0.10	6.49	0.280

[a] Concentration of constant-pH κ-casein 0.05 g/100 ml in sodium cacodylate buffer, pH 6.5, ionic strength 0.10 (ionic strength 0.02 in respect to sodium cacodylate and 0.08 in respect to sodium chloride).

pH effects. κ-Casein solutions (0.05 g per 100 ml) were made up in sodium phosphate buffer, ionic strength 0.10 (0.02 ionic strength contributed by phosphate and 0.08 by NaCl), at various pH values between pH 3.2 and 11.3 by titration of their pH 6.5 solutions with either 0.10 N HCl or NaOH. The straight line curves for trials at pH 3.2, 5.2, 6.5, and 8.4 (Figure 5) are all similar to those previously obtained at pH 6.5. This figure reveals that each of these curves exhibits progressively larger values for its intercepts with increasing pH from 3.2 to 8.4. However, the results for pH 10.9 and 11.3 differ from other pH results in two major aspects: their intercepts values are larger but, more significantly, both high pH trials exhibit curves with positive slopes.

The observation that the high pH (pH 11.3) results in a steeper positive slope than when the pH is 10.9 and lower is explained by the increased resistance against the temperature-induced aggregation reaction of the κ-casein monomers produced by the greater dissociation force at the higher pH.

The apparent molecular volume of the κ-casein micelle was calculated for the pH solutions exhibiting positive slopes (pH 10.9 and 11.3), using the method previously described (4). The molecular volumes were estimated at 611,900 cm^3 and 104,400 cm^3 $mole^{-1}$ at pH 10.9 and pH 11.3, respectively.

Mole fraction of κ-casein monomer. pH effect. Based upon equations by Weber (6) and calculations of Westerberg (7), an expression was derived to estimate the mole fraction of fluorescent κ-casein in the monomer (free) and micelle (complex) species. The expression was:

$$\frac{1}{Y} = \frac{X_f}{Y_f} + \frac{X_c}{Y_c} \qquad (1)$$

where (Y) is the reciprocal of partial polarization for the solution of κ-casein containing both monomer and complex species, (X_f) the mole fraction of κ-casein monomer, (Y_f) the value for reciprocal partial polarization for the fluorescent monomer species, (X_c) the mole fraction of κ-casein complex species, and (Y_c) the value for reciprocal partial polarization for the fluorescent complex κ-casein species.

The above expression was used to calculate the mole fraction of conjugated κ-casein in the monomer form for solutions at various pH levels from about 4.5 to 11.3, assuming the total intensity of fluorescence was contributed by only two molecular species: monomer and complex (micellar). For these calculations, the values for (Y), for each pH employed, were obtained by interpolation from each re-

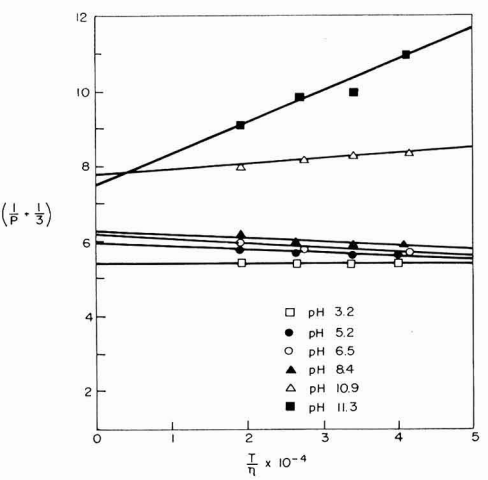

FIG. 5. Reciprocal polarization of constant-pH κ-casein in sodium phosphate buffers at several pH levels (ionic strength 0.10, κ-casein concentration 0.05 g/100 ml).

spective curve in Figure 5 at the value of T/η of 4.00×10^4 (33 C). A value of 10.85 was used for (Y_m), the experimentally obtained value for (Y) in the pH 11.3 determination, where it was assumed that all κ-casein was in the monomer species $(X_m = 1.0)$. (Y_c) was taken to be 5.00, since in all preceding experiments this appeared to be the limiting minimum value for the reciprocal of partial polarization for constant-pH κ-casein; the value that would be expected when all of the κ-casein is in the complex species form.

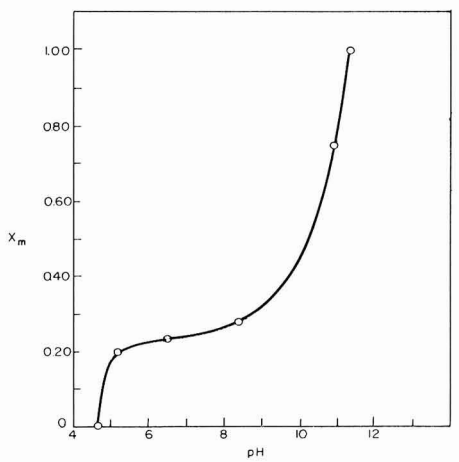

FIG. 6. Relationship between the mole fraction of κ-casein monomers and pH of the sodium phosphate buffer (ionic strength 0.10, κ-casein concentration 0.05 g/100 ml).

The values calculated for (X_m) were graphed vs. pH (Figure 6), from which it is seen that κ-casein monomer-polymer interactions are most sensitive to pH changes at each end of the curve, i.e., between pH 4.4 and 5.0 and again between pH 8.5 and 11.0. Thus, based upon these assumptions and calculations, the range of pH from about 5.0 to 8.5 is the region of the pH scale at which κ-casein monomer-polymer interactions are relatively insensitive to pH changes.

Temperature effect. Since it was shown that warming κ-casein solutions from 5 to 35 C caused a gradual increase in molecular weight for the polymer form, it was desired to calculate the mole fraction of κ-casein monomer (X_m) as a function of temperature. The values used for (Y) in these calculations were interpolated from the pH 6.5 curve (Figure 5) at values of T/η equivalent to each respective temperature in the range of 5 to 35 C. (Y_m)

TABLE 3

Calculated mole fractions of monomers for κ-casein solutions at several selected temperatures [a]

Temperature (C)	Y $\left(\frac{1}{P}+\frac{1}{3}\right)$	Y_m $\left(\frac{1}{P}+\frac{1}{3}\right)$ pH 11.3	X_m Mole fraction monomer
0	6.20	7.50	0.58
5	5.98	9.10	0.36
15	5.88	9.75	0.31
25	5.78	10.35	0.26
35	5.70	10.95	0.23

[a] Concentration of κ-casein (constant pH) 0.05 g per 100 ml, ionic strength 0.10.

values were obtained in the same manner from the pH 11.3 curve in Figure 5 at each of the above points. The values for (X_m) were calculated and are listed in Table 3. The values shown for (X_m) reveal a rapid decrease from 0 to 5 C and, thereafter, a steady but slow decrease up to temperatures of 35 C. This reduction in (X_m) further substantiates the observations made previously, that warming causes a gradual increase in molecular weight of κ-casein and that it does so by association of monomer forms to polymers with corresponding slower relaxation times.

ACKNOWLEDGMENTS

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APPLICATION OF POLARIZATION OF FLUORESCENCE TECHNIQUE TO PROTEIN STUDIES. III. THE INTERACTION OF κ-CASEIN AND β-LACTOGLOBULIN [1]

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SUMMARY

The interaction between κ-casein and β-lactoglobulin in phosphate buffer when held at 25 or 65 C for 1 hr was demonstrated by the polarization of fluorescence technique. The average apparent molecular volume of fluorescent β-lactoglobulin in a mixture with nonfluorescent κ-casein underwent approximately a 42.5% and a 96% increase as a result of holding them in phosphate buffer for 1 hr at 25 and 65 C, respectively.

The mole fraction of fluorescent β-lactoglobulin in the complex form (X_c) was most highly dependent upon the ratio (r) of the weight of β-lactoglobulin to κ-casein (β/κ) in the region where (r) is 1.0 or less.

Evidence was presented indicating that the interaction of β-lactoglobulin and κ-casein probably occurs in a manner that allows the β-lactoglobulin molecules to attach themselves at the κ-casein micelle–water interface to produce a complex whose properties are less temperature-dependent than those of pure κ-casein.

The preceding papers have dealt with the application of the polarization of fluorescence technique to fluorescent-dye conjugated κ-casein and β-lactoglobulin (3, 4). This paper will deal with the use of this procedure in determining the responses of fluorescent and nonfluorescent κ-casein and β-lactoglobulin when they are both present in a system subjected to different temperature treatments.

Considerable evidence has been presented dealing with the heat-induced interaction of κ-casein with β-lactoglobulin in milk (1, 5-7) as well as in purified, synthetic protein systems (2). The polarization of fluorescence method appears to offer another useful tool for studying this interaction, since it enables the determination, independently, of the rotatory properties of each protein in the mixture.

EXPERIMENTAL PROCEDURE

The preparation and treatment of β-lactoglobulin and constant-pH κ-casein and their fluorescent dye conjugates (the fluorescent 1-diamino-naphthalene-5-sulfonyl chloride has been chemically bonded to the protein molecule through a covalent sulfonamide linkage), as well as the preparation of the pH 6.5, 0.02 ionic strength sodium phosphate buffer have been described previously (3, 4).

For the protein interaction mixtures, pure solutions of conjugated β-lactoglobulin and nonconjugated κ-casein were mixed in varying weight ratios ($r = \beta/\kappa$) from zero to infinity in duplicate 25-ml volumetric flasks. All mixtures were prepared to contain 0.50 g total protein at a final volume of 10.0 ml in sodium phosphate buffer (pH 6.5, ionic strength 0.02). For each mixture, one duplicate was held in a water bath at 25 C for 1 hr and another was maintained at 65 C for 1 hr. Each mixture was agitated gently at regular intervals throughout the heating period. After heating, each sample was cooled rapidly, adjusted to 20 C, and made to volume with the sodium phosphate buffer. The final protein concentration was 0.20 g per 100 ml (in all samples). These samples were then subjected to the polarization of fluorescence measurements at several temperatures between 0 and 35 C as described earlier (3).

Three mixing ratios ($r = \beta/\kappa$) of pure solutions of conjugated κ-casein and nonconjugated β-lactoglobulin were also prepared; $r = 1.0$, 2.0, and 4.0. These were treated exactly as the mixtures of conjugated β-lactoglobulin and nonconjugated κ-casein, except that only a 65 C trial was conducted.

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RESULTS AND DISCUSSION

Conjugated β-lactoglobulin and nonconjugated κ-casein. The partial polarization of fluorescence was determined for reaction mixtures containing conjugated β-lactoglobulin and nonconjugated κ-casein in which the ratio (r = weight ratio of β-lactoglobulin/κ-casein) ranged from 0.111 to infinity. Results for reciprocal polarization ($1/P$) vs. the temperature/viscosity ratio (T/η) are presented (Figures 1 and 2).

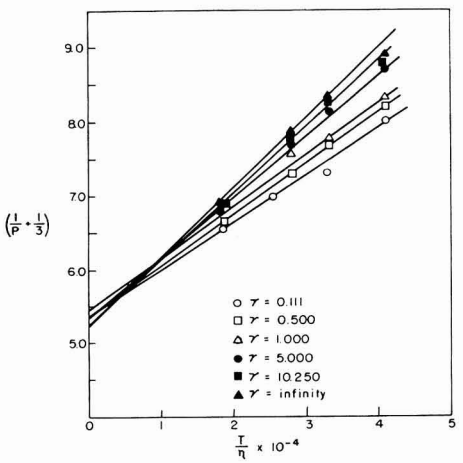

FIG. 1. Polarization of fluorescence of mixtures of fluorescent-dye conjugated β-lactoglobulin and nonconjugated κ-casein held at 25 C 1 hr in sodium phosphate buffer (pH 6.5, ionic strength 0.02, total protein concentration 0.20 g/100 ml).

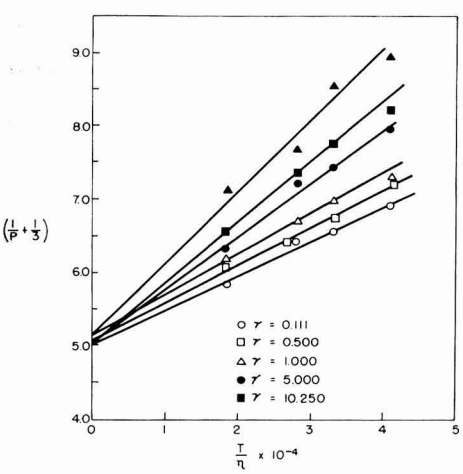

FIG. 2. Polarization of fluorescence of mixtures of fluorescent-dye conjugated β-lactoglobulin and nonconjugated κ-casein held at 65 C 1 hr in sodium phosphate buffer (pH 6.5, ionic strength 0.02, total protein concentration 0.20 g/100 ml).

In Figure 1 (25 C trial) all six curves exhibit a similar intercept value of 5.2 to 5.5; however, a direct relationship between (r) and the magnitude of the slope for all curves was observed. The slope increased progressively in magnitude from 0.638×10^{-4} ($r = 0.111$) to 0.900×10^{-4} (r = infinity).

Figure 2 (65 C trial) shows similar results; the degree of dependence of the slope of the curves to (r) was more pronounced. The slope increased progressively from 0.465×10^{-4} ($r = 0.111$) to 0.957×10^{-4} (r = infinity).

Apparent molecular volume. The increase in slope of the curves for the samples containing progressively larger proportions of conjugated β-lactoglobulin ($r = 0.111$ to infinity) were interpreted in terms of changes in molecular volumes and relaxation times for the heat-induced complex species (Table 1). The average apparent molecular volume for the fluorescent β-lactoglobulin dye conjugate decreased from 98.03×10^3 to 68.79×10^3 cm^3 mole^{-1} as the mixing ratio (r) was increased from 0.111 to infinity in the trial at 25 C. For 65 C, the corresponding decrease in apparent molecular volume was from 125.6×10^3 to 63.48×10^3 cm^3 mole^{-1}. The reduction of the average apparent molecular volume for both free and complexed conjugated β-lactoglobulin in those mixtures containing progressively larger mixing ratios (r) was interpreted as evidence of an interaction between the two proteins present.

TABLE 1

Values for the slope, molecular volume, and relaxation time of fluorescent-dye conjugated β-lactoglobulin and nonconjugated κ-casein mixtures [a]

r (β/κ)	Slope ($S \times 10^4$)	Apparent molecular volume ($V \times 10^{-3}$)	Mean relaxation time ($\rho_h \times 10^8$)
	25 C		
0.111	0.638	97.03	10.55
0.500	0.668	94.99	10.27
1.000	0.695	92.55	9.88
5.000	0.838	78.89	7.95
10.250	0.865	71.61	7.76
Infinity	0.900	68.79	7.45
	65 C		
0.111	0.465	125.60	13.60
0.500	0.508	116.60	12.65
1.000	0.540	112.10	12.14
5.000	0.688	86.97	9.47
10.250	0.740	82.74	8.98
Infinity	0.957	63.48	6.89

[a] pH 6.5 sodium phosphate buffer, 0.02 ionic strength.

This effect could be caused by two possible mechanisms: (a) a higher ratio of (β/κ) may cause formation of smaller average complex sizes between β-lactoglobulin and κ-casein by a stoichiometric relationship, or (b) higher ratios of (β/κ) may result in proportionately more free β-lactoglobulin (not complexed with κ-casein).

The more pronounced effect of mixing ratio (r) upon the apparent molecular volume in the 65 C trial indicates that more interaction was caused at this higher temperature than at 25 C.

Mean harmonic relaxation times. Trends in the relaxation times (Table 1) correspond to the changes in average apparent molecular volumes of the fluorescent β-lactoglobulin molecules, since larger molecular volumes produce correspondingly larger relaxation times. For example, the mean relaxation time of the fluorescent protein species (β-lactoglobulin in the free and complexed form) increased from 7.45 $\times$ 10^{-8} to 10.55 $\times$ 10^{-8} sec for the 25 C trial and from 6.89 $\times$ 10^{-8} to 13.60 $\times$ 10^{-8} sec for the 65 C trial, with the reduction of the mixing ratio from infinity to 0.111.

Mole fraction of free and complexed β-lactoglobulin. A relationship suitable for estimating the approximate proportion of dye-conjugated β-lactoglobulin in the free (X_f) and complexed form (X_c) has been described earlier (4).

$$\frac{1}{Y} = \frac{X_f}{Y_f} + \frac{X_c}{Y_c} \qquad (1)$$

For the present application of this expression, the value of reciprocal polarization (Y) was determined for each mixture by interpolation of the respective curves in Figures 1 and 2 from the value of $T/\eta = 4.0 \times 10^4$, which corresponds roughly to 30-32 C. A value of 6.88 was obtained for (Y_c) by interpolation from the curve for ($r = 0.111$) in the 65 C trial, as these experimental conditions resulted in the largest average molecular volume of the fluorescent species (Table 1). The value of (Y_f) was taken to be 9.04, since it corresponds to the value read from the curve for the mixture containing the smallest average molecular species for all of these mixtures (r = infinity for the 65 C trial). Results for these calculations are listed in Table 2.

When (X_c) is plotted against (r), the difference between the two temperature trials becomes more definite (see Figure 3). Although the shape of both curves is quite similar, the 65 C curve is positioned above the 25 C curve at all values of (r). These curves indicate that

TABLE 2

Mole fraction of free (X_f) and complexed (X_c) β-lactoglobulin in heated mixtures of fluorescent-dye conjugated β-lactoglobulin and nonconjugated κ-casein [a]

Mixing ratio [b] ($r = \beta/\kappa$)	Y at $T/\eta = 4 \times 10^4$	Mole fraction free (X_f)	Mole fraction complex (X_c)
	25 C		
0.111	7.86	0.522	0.478
0.500	8.12	0.639	0.361
1.000	8.24	0.690	0.310
5.000	8.67	0.866	0.134
10.250	8.78	0.908	0.092
Infinity	8.92	0.960	0.040
	65 C		
0.111	6.88	0.000	1.000
0.500	7.12	0.144	0.856
1.000	7.36	0.273	0.727
5.000	7.80	0.495	0.505
10.250	8.12	0.676	0.324
Infinity	9.04	1.000	0.000

[a] pH 6.5, ionic strength 0.02, total protein concentration 0.20 g per 100 ml.
[b] Weight ratio, g β-lactoglobulin/g κ-casein.

the mole fraction of conjugated β-lactoglobulin in the complexed form (X_c) increases at the greatest rate when (r) is in the order of 1.0 or less. Increasing the amount of nonconjugated κ-casein in the solution to furnish lower values of (r) causes the greatest increases in (X_c) values.

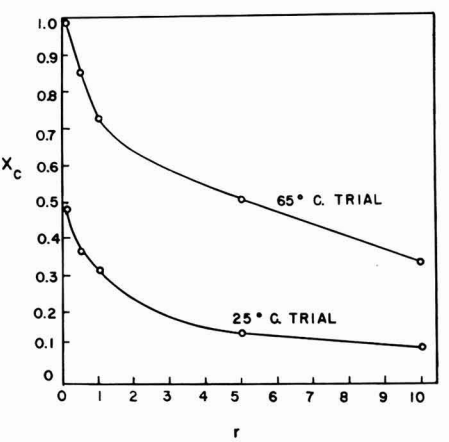

FIG. 3. Effect of mixing ratio upon the mole fraction of fluorescent-dye conjugated β-lactoglobulin in the complexed form (pH 6.5, ionic strength 0.02, total protein concentration 0.20 g/100 ml).

Conjugated κ-casein and nonconjugated β-lactoglobulin. Studies were also conducted using fluorescent, dye-conjugated κ-casein and nonconjugated β-lactoglobulin and three mixing ratios ($r = 1.0$, 2.0, and 4.0) of β-lactoglobulin/κ-casein were employed. The solutions of the two proteins were held at 65 C for 1 hr, to induce interaction.

The values obtained for the slope and intercept of each of these mixtures are listed in Table 3. Lower intercept values resulted from increases in the ratios of β-lactoglobulin/κ-casein. Straight-line curves with negative slopes were obtained which exhibited a trend toward smaller values for their slopes by increasing (r) from 1.0 to 4.0. This is interpreted as indicating a greater amount of interaction of the two proteins at the higher values of (r); the resulting protein complex possesses less ability to associate into larger micelle sizes upon warming to 35 C (during measurement of polarization of fluorescene values at these temperatures) than does conjugated κ-casein. In this respect, the protein complex appears to possess more of the properties of β-lactoglobulin and less of the properties of κ-casein.

It is assumed that the interaction of κ-casein monomers and polymers is responsible for causing negative slopes for the curves of reciprocal polarization vs. T/η in these interaction mixtures, as was the case for pure κ-casein solutions (4). Since higher values for (r) in the present study resulted in curves whose slopes exhibited smaller negative values, the role of nonconjugated β-lactoglobulin in these mixtures is thought to be one of competing with the fluorescent κ-casein monomers for interaction sites on the κ-casein polymer. The larger relative number of nonconjugated β-lactoglobulin molecules reacting with the κ-casein polymers, in the case of the higher values of (r), appears to cause a reduction in the number of available sites for interaction between polymer and monomer forms of κ-casein. The reduction in the number of these interaction sites was reflected by the smaller values for the negative slopes of the graphs of reciprical polarization vs. T/η in those samples with higher values of (r).

TABLE 3

Intercept and slope of reciprocal polarization vs. T/η for interaction mixtures of fluorescent-dye conjugated κ-casein and nonconjugated β-lactoglobulin [a]

Mixing ratio (β/κ)	Intercept	Slope ($-S \times 10^4$)
1.0	6.36	0.096
2.0	6.26	0.065
4.0	6.16	0.015

[a] Concentration of total protein 0.20 g per 100 ml, sodium phosphate buffer pH 6.5, ionic strength 0.02.

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INHIBITORY EFFECT OF NISIN UPON VARIOUS ORGANISMS [1, 2]

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SUMMARY

Two nisin-producing strains of *Streptococcus lactis* were found to vary in their ability to produce the antibiotic. In the milk medium containing 5% of nisin-broth, cell multiplication and acid production of two strains of *Streptococcus thermophilus* were delayed, but upon further incubation their total cell count and acid production were not affected appreciably. Nisin-broth did not inhibit three strains of *S. lactis*, two strains of *Lactobacillus bulgaricus*, two strains of *S. thermophilus*, a mixed lactic culture, and one strain each of *Bacillus subtilis* and *Bacillus cereus*. Nisin was not quite as inhibitory as several other antibiotics against the dairy cultures tested. Of 11 strains of *Staphylococcus aureus* used, the antibiotic seemed to be active only against the coagulase-negative and weak coagulase-producing organisms.

The data presented in this paper indicate that the antibiotic may be considered for use to inhibit certain staphylococci or lactic organisms. Additional work should be conducted to determine the applications of nisin and nisin-producing organisms in the dairy industry.

Nisin, an antibiotic, is a normal metabolite of certain strains of a commonly used cheese starter, *Streptococcus lactis*. The antibiotic is a large polypeptide with the molecular weight of about 10,000, and it consists of five polypeptide components which vary slightly in their chemical, physical, and biological properties (3, 4). Hawley (9) has reported that nisin is inhibitory against several streptococci, lactobacilli, clostridia, staphylococci, and bacilli; and Mattick and Hirsch (11) observed that the antibiotic has no activity against gram-negative organisms. Several toxicity studies (1, 6, 8) have shown that when the crystalline nisin or milk soured with nisin-producing *S. lactis* was fed to animals, it produced no toxic effects. O'Brien et al. (13) have reported that nisin lowers the thermal resistance of food spoilage organisms and suggested that consideration could be given to using nisin for food preservation.

In the dairy industry, considerable quantities of processed cheese and several other sweet or low-acid cheeses, for example, Swiss, Gouda, Edam, and Parmesan, are spoiled during storage due to gas-holes or inflation caused by growth of Clostridia or butyric acid fermentation. McClintock et al. (12) observed that the incorporation of small quantities of nisin in processed Gruyère cheese was found to retard or inhibit such undesirable fermentation. Hawley (9), in his review article, reports that the cheeses in which the antibiotic is produced naturally have been found to be quite resistant to certain bacterial spoilage defects. Also, some work has been done relative to the use of nisin-producing streptococci, singly or in combination with other cheese cultures, to prevent the spoilage of cheese (14). As far as is known, comparatively little thought has been given to the determination of the effects of nisin upon the commonly used dairy cultures. This work was initiated, therefore, to investigate the effects of nisin and nisin-producing streptococci upon the organisms generally found or used in dairy products.

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[2] Published with the approval of the Director as paper no. 1004, Journal Series, Nebraska Agricultural Experiment Station, Lincoln.

EXPERIMENTAL PROCEDURE

Several nisin-producing strains of *S. lactis*, obtained from France, Austria, and England, where much of the previous work on nisin has been done, were used in this work. Preliminary trials revealed that two strains multiplied at a faster rate than the others, and were chosen for further studies. The initial phase of this study was concerned with the comparison of nisin production of the two cultures. The method of Chevalier, Fournaud, and Mocquot (5), with minor alterations, was used to determine the

antibiotic production of the cultures. The method involves plating suitable dilutions of a nisin-producing culture and of a nisin-sensitive culture in the same plate, which is then incubated. It is imperative that the optimum temperature for growth of the sensitive organisms be considerably different from that of the nisin-producing organisms. In this case, the plate thus prepared is incubated at 22 C, or at the optimum temperature of the nisin-producing *S. lactis*, in order for the streptococcus to grow and produce nisin. At this temperature, *L. lactis* with an optimum temperature of 32-35 C cannot multiply. After the plate is incubated at 22 C for 24 hr, it is transferred to an incubator at 32 C for 48 hr to permit *L. lactis* to grow. During the second incubation, the test organism grows normally, except around the colonies of the nisin-producing organisms, and clear zones of inhibition are obtained, as is shown in Figure 1.

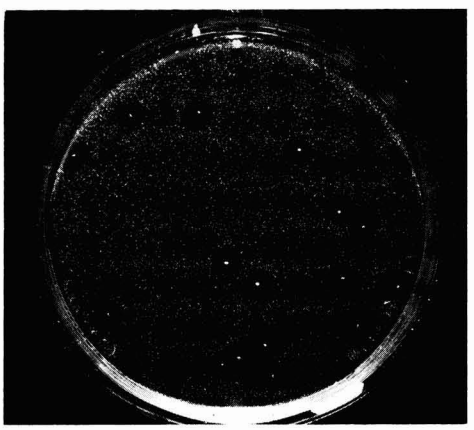

FIG. 1. A plate showing the inhibition of *Lactobacillus lactis* by nisin produced by *Streptococcus lactis*.

Using this technique, it was observed that when similar dilutions were used for the two nisin-producing organisms and for the test organisms, the two nisin-producing cultures produced nearly the same number of zones or colonies. However, one culture (*S. lactis* F) produced bigger zones of inhibition around the colonies than did the other culture (*S. lactis* T). This was felt to be due to the larger amount of nisin produced by F than by T. To investigate this further, nisin-broths were prepared from 14- to 16-hr old tomato juice broth suspensions of cultures F and T. Cells were removed by centrifugation, and the clear broth was heated at 80-90 C for 2 to 3 min to kill the remaining live organisms. This heat treatment was considered to have had no effect on the nisin content of the broth, since it has been shown that nisin is highly heat-resistant (7). Varying concentrations of F and T nisin-broths were introduced in separate broth tubes and were inoculated with 1% broth suspension of *L. lactis*. Turbidity determinations were made after 14 to 16 hr of incubation at 32 C. It was observed that broth of both the nisin cultures inhibited the growth of *L. lactis*, but nearly twice as much broth of T culture was required as F for the same amount of inhibition.

Effect of nisin-broth upon various lactic cultures. Studies were conducted to determine the effect of nisin-broth upon the acid production of three strains of *S. lactis*, two strains of *L. bulgaricus*, four strains of *S. thermophilus*, and a mixed lactic culture. Using the same technique as above, nisin-broth of the nisin-producing culture F was prepared and added at the rate of 1- to 18-ml aliquots of sterile milk in test tubes. The tubes were then inoculated with 1 ml of a 12- to 14-hr-old lactic culture prepared in milk. Thus, the tubes contained 5% by volume of the nisin-broth and 5% by volume of inoculum from a culture of the test-organism. The contents of the tubes were mixed thoroughly and incubated at the optimum temperatures of the test organisms. At periodic intervals of 6, 16, and 24 hr of incubation, tubes were taken out and titratable acidity was determined. Four to six trials were conducted with each culture, and average results are presented in Table 1. It was observed that except for two strains of *S. thermophilus*, Cultures 8 and 9, the amount of nisin in 1 ml of nisin-broth had no appreciable inhibitory effect upon the three strains of *S. lactis*, the two strains of *L. bulgaricus*, the two strains of *S. thermophilus*, and the mixed culture. The antibiotic inhibited slightly the acid production of two strains of *S. thermophilus* (no. 8 and 9) during the first 6 hr of incubation. However, upon longer incubation, the amounts of acid produced by the cultures in the presence and in the absence of the antibiotic were almost the same. The fact that nisin-broth showed some inhibitory effect upon the acid production of *S. thermophilus* Y and Z during the initial stages of incubation and showed no adverse effect upon extended incubation (24 hr) may be explained by a prolongation of the lag phase of the *S. thermophilus* cultures. This prolongation in the lag phase may be due either to the inhibitory effect of the antibiotic upon the young and/or sensitive bacteria or to the time

TABLE 1

Effect of nisin upon the rate of acid production by vvarious lactic cultures

Culture	0 hr	6 hr	16 hr	24 hr
		(% *Titratable acidity*)		
1. *Str. lactis* UN	0.22	0.50	0.70	0.81
Str. lactis UN + nisin	0.25	0.48	0.66	0.80
2. *Str. lactis* C	0.23	0.48	0.73	0.84
Str. lactis C + nisin	0.25	0.49	0.71	0.85
3. *Str. lactis* F_1	0.25	0.45	0.67	0.79
Str. lactis F_1 + nisin	0.28	0.45	0.68	0.77
4. *L. bulgaricus* C_1	0.28	0.79	1.42	1.88
L. bulgaricus C_1 + nisin	0.30	0.80	1.45	1.95
5. *L. bulgaricus b*	0.26	0.91	1.68	1.91
L. bulgaricus b + nisin	0.30	0.94	1.60	1.89
6. *Str. thermophilus U*	0.21	0.51	0.79	0.92
Str. thermophilus U + nisin	0.23	0.52	0.78	0.94
7. *Str. thermophilus X*	0.24	0.46	0.69	0.87
Str. thermophilus X + nisin	0.25	0.49	0.70	0.86
8. *Str. thermophilus Y*	0.23	0.51	0.72	0.91
Str. thermophilus Y + nisin	0.25	0.44	0.69	0.90
9. *Str. thermophilus Z*	0.23	0.49	0.73	0.89
Str. thermophilus Z + nisin	0.25	0.40	0.70	0.91
10. Mixed culture *F*	0.21	0.46	0.71	0.90
Mixed culture *F* + nisin	0.23	0.46	0.72	0.86

required for the culture to get accustomed to the new medium. Also, there exists a possibility that the antibiotic may have lost its activity upon prolonged incubation in the presence of milk solids and of the lactic culture, as found true in terramycin (15).

Effect of nisin upon cell-multiplication. Hirsch (10) has indicated that nisin is bactericidal rather than bacteriostatic and that it achieves a rapid killing of sensitive bacteria. Also, he suggests that nisin often forms an irreversible complex with the protein moieties of the organisms. Trials were made to determine the effect of nisin upon cell multiplication of the nisin-sensitive *L. lactis* and *S. thermophilus* when grown in sterile milk. In this work, the technique used for determining the effect of nisin upon cell multiplication was essentially the same as used in the preceding phase, except that pure nisin[3] was used instead of nisin-broth. Appropriate quantities of aqueous solutions of nisin were added to yield concentrations of 0.5, 1.0, 5.0, and 10.0 units per milliliter. Pure nisin is believed to contain 40 to 50 million Reading units per gram (2, 9). A Reading unit has been described as the amount that will normally just inhibit the growth of the test organism, *Streptococcus agalactiae*, in 1 ml of broth. Using the standard plate count method involving duplicate or triplicate plates for each dilution, viable cell counts were determined immediately after adding the antibiotic and then at the end of 6, 12, 18, 24, and 36 hr of incubation. In Figure 2 are presented data representing the average results of five to eight trials conducted with each organism and each concentration level of the antibiotic. Curves I are the control curves, or represent the cell numbers produced by the organisms in the absence of the antibiotic, and Curves II, III, IV, and V represent the cell numbers produced by the organisms in the presence of 0.5, 1.0, 5.0, and 10.0 units of nisin per milliliter, respectively. In the absence of the antibiotic, the organisms grew normally, reaching the maximum counts in 18 to 24 hr and declining thereafter. In the presence of the antibiotic, the viable counts of both the organisms decreased rapidly for a while. There was a direct but improportional relationship between the antibiotic concentration and the reduction in the counts, following which the cultures multiplied normally and paralleled the growth curves of the controls. As little as 0.5 or 1.0 unit of nisin per milliliter effected an immediate slight decrease in the counts, after which the cultures seemed to grow normally. The higher concentrations of nisin (5.0 and 10.0 units/ml) decreased rapidly the cell counts of the organisms during the first few minutes and continued to do so for the first 6 hr of incubation, but

[3] Crystalline nisin was supplied by Alpin and Barret Ltd. Co., England.

thereafter the surviving organism multiplied normally and proximated the counts of the control samples at the end of 24 to 36 hr. At the end of 36 hr, the total counts of the cultures in the presence of the antibiotic were either just about the same or slightly higher than the control counts. This could be expected, since the cultures in the absence of nisin reached the stationary or declining phase earlier than the cultures in the presence of the antibiotic.

Comparison of the inhibitory effect of nisin with other antibiotics. Trials were made to compare nisin with other antibiotics with respect to their inhibitory effect upon various lactic cultures. Individual test tubes containing 19 ml of sterile milk were inoculated with 1 ml of freshly prepared 12- to 16-hr-old lactic cultures. Appropriate amounts of penicillin, streptomycin, aureomycin, or erythromycin were added to obtain concentrations varying between 0.05 and 50.0 ppm of the antibiotics. In nisin, the concentrations varied between 0.5 to 150.0 units per milliliter. The tubes were then incubated for 14 to 16 hr at the respective optimum temperatures of the cultures under study. At the end of the incubation period the coagulation of the milk was taken as the criterion of the growth of the cultures. The samples coagulated were assumed to be uninhibited and the samples which showed only partial or no coagulation were considered to be inhibited. Average results of four to six trials with each antibiotic and each culture are presented in Table 2. The table shows the minimum levels of various antibiotics required for inhibiting the organisms. It may be observed that in general the organisms were more sensitive to penicillin, aureomycin, streptomycin, or erythromycin than to nisin. Also, there were observed wide variations in the sensitivities of these organisms to penicillin, aureomycin, streptomycin, erythromycin, or nisin. The concentrations of the antibiotics required for the inhibition of the cultures were 0.05 to 0.3 unit of penicillin per milliliter, 0.05 to 0.75 μg of aureomycin per milliliter, 0.2 to 1.5 μg of streptomycin per milliliter, or 0.2 to 5.0 μg of erythromycin per milliliter. In the case of nisin much higher concentrations of the antibiotic (2.0 to 150

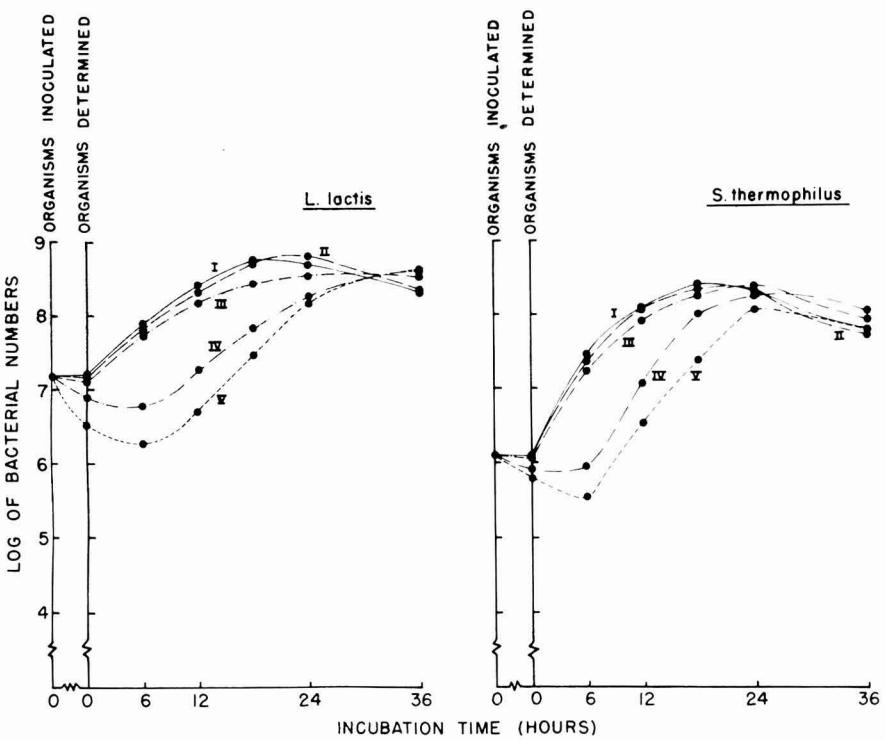

FIG. 2. Effect of nisin upon the cell-multiplication of *Lactobacillus lactis* and *Streptococcus lactis*.

TABLE 2

Minimum levels of penicillin, aureomycin, streptomycin, erythromycin, and nisin for inhibiting milk coagulation by various lactic cultures [a]

Culture	Penicillin	Aureomycin	Streptomycin	Erythromycin	Nisin
	(μ/ml)	*(μg/ml)*	*(μg/ml)*	*(μg/ml)*	*(μ/ml)*
1. *S. lactis UN*	0.10	0.50	0.3	0.6	24
2. *S. lactis C*	0.10	0.50	1.5	0.2	72
3. *S. lactis* F_1	0.05	0.75	0.6	0.5	24
4. *S. thermophilus U*	0.05	0.10	0.75	2.0	48
5. *S. thermophilus X*	0.10	0.05	0.15	5.0	100
6. *S. thermophilus Y*	0.30	0.05	0.3	0.8	4
7. *S. thermophilus Z*	0.10	0.25	0.5	0.5	4
8. *S. cremoris*	0.15	0.50	0.75	0.75	2
9. *L. bulgaricus* C_1	0.25	0.25	0.2	1.0	8
10. *L. bulgaricus b*	0.20	0.10	0.75	1.0	50
11. *L. lactis*	0.10	0.25	0.75	1.0	2
12. Mixed culture	0.15	0.30	1.5	1.25	150

[a] Noncoagulation of the milk at end of 16-hr incubation period was taken as the criterion of the inhibition of the cultures by the antibiotics.

TABLE 3

Effect of nisin-broth upon the growth of *Bacillus subtilis* and *Bacillus cereus*

Culture	0 hr	6 hr	16 hr	24 hr
	(Log of bacterial count)			
1. *Bacillus subtilis* ATCC 6633	5.954	7.708	8.827	8.638
Bacillus subtilis + nisin	5.929	7.740	8.786	8.601
2. *Bacillus cereus*	6.121	8.179	8.997	8.838
Bacillus cereus + nisin	6.127	8.121	9.041	8.860

TABLE 4

Effect of nisin upon the growth of different strains of *Staphylococcus aureus*

No.	Culture	Coagulase test		Transmission %	
		Nature	Time	Control	With nisin
			(hr)		
1. *S. aureus*	196	Positive	0.4– 1.4	22.5	25.0
2. *S. aureus*	UN	Positive	0.5– 2.1	30.0	29.5
3. *S. aureus*	209	Positive	0.5– 1.8	28.0	26.0
4. *S. aureus*	Roth	Positive	0.8– 2.6	28.5	24.0
5. *S. aureus*	Red	Positive	0.9–10.8	22.0	20.5
6. *S. aureus*	139	Positive	0.7–12.2	18.0	25.0
7. *S. aureus*	174	Positive	1.1–13.2	31.0	35.0
8. *S. aureus*	125	Weak	0.7–20.3	32.0	27.5
9. *S. aureus*	26	Weak	14.4–24.1	29.5	52.0
10. *S. aureus*	3	Weak	1.8–19.4	26.0	28.5
11. *S. aureus*	21	Negative or very weak	26.0	24.5	86.0

units/ml) had to be present in the medium to inhibit the organisms.

Effect of nisin upon B. subtilis and B. cereus. Another phase of the study was to determine the effect of nisin upon *B. subtilis* ATCC 6633 and *B. cereus*. Varying quantities of nisin-broth, ranging between 1 and 5% v/v, were added to whey broth tubes inoculated with *B. subtilis* or *B. cereus*. The tubes were incubated, and at periodic intervals the total counts of bacilli were determined using whey agar as the plating medium. The average results of four to five trials with each organism are presented in Table 3. It was observed that both the organisms had little or no sensitivity towards the antibiotic.

Effect of nisin upon Staph. aureus. Gowans et al. (8) have reported that some strains of *Staph. aureus* were very sensitive to nisin, whereas some other strains were relatively insensitive. The virulence of food-poisoning staphylococci has been reported to be directly related to their coagulase activity. A study was conducted, therefore, to investigate the effect of nisin upon several strains of *Staph. aureus* varying in their coagulase production. The antibiotic sensitivity of the cultures was determined by growing the cultures in whey broth containing 5 units of nisin per milliliter, and the turbidity development was measured and taken as an index of their growth. Their coagulase activity was determined by the standard plasma coagulase test. Eleven strains of *Staph. aureus* were graded according to the rapidity with which they produced the enzyme coagulase, and are listed in Table 4. Culture No. 1 was the most active coagulase producer and coagulated plasma in 25 to 80 min, and the last culture was almost negative, in that it took more than 24 hr, or in some trials it did not coagulate the plasma. When tested for their sensitivity to nisin, it was observed that out of the 11 cultures only two cultures, no. 9 and 11, were sensitive to the antibiotic. Both of these sensitive cultures were found to be either weak or negative coagulase producers. The results indicate that nisin might be active against only the coagulase-negative or weak coagulase-producing strains of *Staph. aureus*. However, further study is required to arrive at any definite conclusions.

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B-COMPLEX VITAMIN CONTENT OF CHEESE [1, 2]
II. NIACIN, PANTOTHENIC ACID, PYRIDOXINE, BIOTIN, AND FOLIC ACID

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SUMMARY

A large number of samples of 23 varieties of cheese and eight varieties of processed cheese or cheese foods and spreads were assayed for several B vitamins. The cheeses contained on an average 277 μg of niacin, 94 μg of vitamin B_6, 691 μg of pantothenic acid, 1.79 μg of biotin, and 22.3 μg of folic acid per 100 g. There were wide variations in vitamin content among individual samples of the same variety, as well as among the different varieties. The proteolytic type cheeses, Blue, Camembert, etc., had the most niacin, vitamin B_6, pantothenic acid, biotin, and folic acid. The hard varieties, except for biotin and folic acid, were next, followed by semihard or soft-unripened types. The proteolytic varieties were high in vitamin content, possibly because microorganism varieties synthesize vitamins during curing. In general, any cheese variety high in one B-vitamin was high in most of the other B-vitamins studied. The cheese contained 9 to 55% of the vitamin content of milk and, nutritionally, cheese may be considered a fairly good source of B vitamins.

The public is becoming increasingly interested in the vitamin content of food. More information is needed relative to the vitamin content of different foods, to help determine the nutritive value of various diets. The importance of dairy products as a source of vitamins of the B-complex is discussed by Chapman et al. (4) and Sullivan et al. (20). Wokes et al. (21) have reported that vegetarians whose diet contained dairy products showed no apparent ill-effects; whereas, another group of vegetarians who ingested no dairy products developed several illnesses which were alleviated by B_{12}.

Cheese occupies an important place in the American diet. The per capita yearly consumption of ripened and unripened cheeses has risen to more than 14 lb. The literature contains relatively limited information on the vitamin content of cheese. Many of these studies were concerned with the vitamins A, D, ascorbic acid, thiamine, and riboflavin content of cheese (5, 7, 8, 10, 12) and little attention has been given to the other vitamins of the B group (15, 19, 20).

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[3] Deceased.

Aykroyd and Roscoe (2) were perhaps the first to assay cheese for vitamin B_2 content. In 1936, Day and Darby (6) reported that American cheese contained 80 μg of riboflavin per 100 g, Swiss cheese 60 μg, and cream cheese 14 μg. Most of the work on cheese was done during the 1940's (5, 7, 8, 14, 20), because of the meat shortage at that time and the greater importance of cheese from a nutritional point of view. Several of these studies, however, were made on only a few samples and their results differ widely, possibly because of the different assay methods employed. The present study was conducted, therefore, because of the lack of information concerning the content of some of the newer B vitamins in several varieties of cheese commonly used in this country. A report on the riboflavin content of cheese has been published by Hathaway and Davis (14), and in this paper are presented results of assays of several cheese varieties for other B-complex vitamins.

EXPERIMENTAL PROCEDURE

Market samples of various cheeses were obtained from the University of Nebraska dairy and local food stores, and from Omaha and Milwaukee. The vitamin content was determined immediately, or the samples were held frozen until the assay could be made. The cheeses were classed as follows: Soft-unripened varieties, like Cottage and cream cheese; semihard varieties, like brick and Muenster; hard varieties, like Swiss or Cheddar cheese; very

hard varieties, like Parmesan and Romano; high proteolytic varieties, like Blue; and unclassified varieties. The B vitamins determined were: niacin, pantothenic acid, pyridoxine, biotin, and folic acid. In the initial stages of the study, the microbiological methods as described by the Association of Vitamin Chemists (1) and Chapman et al. (4) were tried. However, because of the differences in the pH, and in the solids and protein contents of different cheese varieties, a vitamin assay method for any vitamin could not be applied in exactly the same way to all the different varieties of cheese. Lawrence et al. (18) also observed that an assay method for a vitamin in milk may not be applicable to other products. Therefore, the microbiological assay technique for each vitamin was modified slightly for application to different cheese varieties. The methods in brief are described below.

Niacin. A weighed sample of cheese, ranging between 1.0 to 10.0 g, depending upon the kind of cheese, was blended with approximately 20 times its volume with 1.0 N sulfuric acid. The blend was autoclaved at 15 psi for 30 min, cooled, brought to pH 6.8 with 20% sodium hydroxide, and filtered. The niacin content of the filtrate was determined microbiologically, using *Lactobacillus arabinosus* ATCC 8014 as the assay organism.

Pyridoxine. A weighed sample of cheese, 0.3 to 0.4 g, was mixed thoroughly with 0.005 N hydrochloric acid and autoclaved at 15 psi for 4 hr. The sample was cooled, adjusted to pH

TABLE 1

Niacin content of a variety of cheeses

Cheese variety	No. of samples	Average solids	Niacin content		
			Range	Mean [a]	Std. [b] dev.
		(%)	(μg/100 g)	(μg/100 g)	(μg)
Highly proteolytic					
Blue	33	61	68-2,346	1,247	577.0
Roquefort	8	63	84-1,044	629	313.0
Camembert	10	51	46-1,124	586	414.1
Liederkranz	8	36	60- 767	428	284.4
Limburger	10	50	35- 390	158	114.1
Beer Kaese	10	65	34- 213	85	27.5
Port Salut	9	52	30- 120	59	28.0
			Avg	664	
Very hard					
Parmesan	10	75	44- 500	290	171.5
Romano	10	70	25- 173	77	53.3
Edam	10	72	19- 64	38	13.2
			Avg	135	
Hard					
Provolone	10	60	50- 314	191	103.0
Swiss	10	59	35- 233	120	219.1
Gruyère	9	57	62- 145	98	35.7
Gouda	10	61	24- 185	63	47.8
Cheddar	30	65	13- 212	58	46.0
			Avg	93	
Semihard					
Husker	10	62	30- 287	99	72.6
Brick	13	60	12- 163	89	45.3
Muenster	11	64	21- 125	67	34.5
Mozzarella	9	51	15- 126	62	33.0
			Avg	80	
Soft					
Cottage-creamed	10	25	22- 233	120	66.0
Cottage-plain [c]	10	26	51- 325	112	90.0
Neufchatel	8	40	29- 158	86	42.6
Cream	20	51	42- 175	83	35.0
			Avg	98	
Total	278		Grand avg	277	

[a] On wet or fresh weight basis.
[b] Standard deviation.
[c] Commercially known as dry Cottage cheese.

TABLE 2

Vitamin B_6 content of a variety of cheeses

Cheese variety	No. of samples	Average solids	Vitamin B_6 content		
			Range	Mean [a]	Std. [b] dev.
		(%)	(μg/100 g)	(μg/100 g)	(μg)
Highly proteolytic					
Camembert	22	51	142-367	248	189.5
Blue	22	61	101-239	200	56.0
Liederkranz	13	36	124-333	189	63.1
Roquefort	20	63	61-210	104	34.8
Limburger	22	50	39-163	89	31.1
Beer Kaese	19	65	42-139	83	22.2
Port Salut	8	52	47- 85	59	12.8
			Avg	146	
Very hard					
Parmesan	15	75	64-114	96	13.2
Edam	20	72	61-111	84	14.9
			Avg	89	
Hard					
Swiss	32	59	51-154	91	30.4
Cheddar	63	65	49-147	84	23.0
Provolone	21	60	58-122	83	18.0
Gruyère	46	57	55-116	81	13.2
Gouda	20	61	49-124	80	20.4
			Avg	84	
Semihard					
Husker	9	62	63- 96	79	11.9
Muenster	10	64	54-100	76	15.3
Brick	28	60	49-105	73	17.0
Mozzarella	10	51	51- 79	64	10.8
			Avg	73	
Soft					
Cottage-plain [c]	14	26	21- 90	56	25.3
Cottage-creamed	32	25	28- 83	53	18.1
Cream	32	51	32- 85	53	12.8
			Avg	53	
Total	478		Grand avg	94	

[a] On wet or fresh weight basis.
[b] Standard deviation.
[c] Commercially known as dry Cottage cheese.

4.5 with sodium hydroxide, and filtered. The filtrate was subjected to the vitamin assay, using *Saccharomyces carlsbergensis* ATCC 9080 as the test organism.

Pantothenic acid. A weighed sample of cheese (0.5 to 1.0 g) was mixed with 50 ml of water, adjusted to pH 6.8-7.0, and autoclaved at 15 psi for 15 min. After cooling, the bound vitamin in the sample was released by incubating it 24 hr either with mylase P or the double enzyme system (alkaline phosphatase and pigeon liver extract). After incubation, the mixture was filtered and made to volume, and pantothenic acid was determined, using *L. plantarum* (formerly *L. arabinosus* 17-5) as the assay organism.

Biotin. A 20- to 30-g sample of cheese was blended with 60 ml of distilled water, and 10 ml of the blend was mixed with 25 ml of 2.0 N sulfuric acid and autoclaved 1 hr at 15 psi. The autoclaved sample was diluted to 100 ml and extracted twice with ethyl ether. Following the extraction, a 10-ml aliquot of the sample was diluted to about 80 ml with distilled water in a 100-ml volumetric flask, its pH adjusted to 6.8, made up to volume with water, and used for the vitamin assay. The assay organism used was *L. arabinosus* ATCC 8014.

Folic acid. One-half gram of the cheese sample was mixed with 5.0 ml of 0.2 M phosphate buffer, pH 7.0, and heated 5 min at 100 C. The sample was cooled, mixed with 20 mg acetone-extracted chicken pancreas, and incubated at 37 C 24 hr. Following the incubation, the sample was placed in a boiling water-bath for 5 min, cooled, extracted with ethyl ether, and filtered. The vitamin content was determined in the filtrate, using *Strepto-*

coccus faecalis ATCC 8043 as the assay organism.

RESULTS

Niacin content of several varieties of cheese. A total of 278 samples of 23 cheese varieties were assayed for niacin, and the results are presented (Table 1). The average niacin content for all the cheese samples was 277 μg per 100 g. There were wide variations in the vitamin content of different samples of cheese of the same variety, as well as of different varieties and groups. The proteolytic cheeses contained the highest concentration, with very hard, soft-unripened, hard, and semihard being next in decreasing order. The standard deviations of the vitamin content of the different varieties of cheese varied widely, ranging from 13 for Edam to 577 for Blue cheese.

When the data were recalculated on the dry or moisture-free solids basis, essentially the same relationship was observed between the niacin content and types of cheese.

Pyridoxine (B_6) content of cheese. A total of 478 samples of 21 varieties of cheese were assayed for B_6, and the results are presented (Table 2). The grand average of the vitamin B_6 content of all the cheeses assayed was 94 μg per 100 g. In general, the vitamin concentration was of a decreasing order in the proteolytic, very hard, hard, semihard, and soft-unripened groups.

As observed for niacin, there were wide variations in the vitamin B_6 content of different samples of the same variety of cheese. However, the differences in the B_6 content of several groups of cheese were not as wide as in niacin. The standard deviations of all the varieties ranged between 11 and 190.

When the data were calculated on a dry solids basis, it became evident, however, that the soft-unripened group was the highest in the concentration of B_6, with the proteolytic group being very close to it. The hard, semihard, and very hard were the next, in decreasing order.

Pantothenic acid content of cheese. As pre-

TABLE 3

Pantothenic acid content of a variety of cheeses

Cheese variety	No. of samples	Average solids	Pantothenic acid content		
			Range	Mean [a]	Std. [b] dev.
		(%)	(μg/100 g)	(μg/100 g)	(μg)
Highly proteolytic					
Blue	13	61	1,004-3,416	2,046	234.0
Liederkranz	8	36	1,252-2,622	1,985	467.8
Roquefort	15	63	1,193-3,634	1,953	646.1
Camembert	15	51	464-6,578	1,398	2,037.0
Limburger	14	50	196-2,781	1,277	647.9
Beer Kaese	12	65	224-1,683	695	386.9
			Avg	1,523	
Very hard					
Edam	11	72	102-1,255	282	327.0
Hard					
Gruyère	5	57	544- 680	614	54.2
Provolone	6	60	29-1,025	476	377.3
Swiss	14	59	175-1,673	441	62.9
Gouda	15	61	65- 740	340	201.9
Cheddar	29	65	111- 711	331	140.0
			Avg	388	
Semihard					
Brick	16	60	108- 643	293	166.4
Husker	6	62	37- 349	240	108.6
			Avg	279	
Soft					
Cream	35	52	95- 645	271	96.7
Cottage-creamed	22	25	79- 416	223	92.4
Cottage-plain [c]	8	26	21- 873	144	290.3
			Avg	239	
Total	244		Grand avg	691	

[a] On wet or fresh weight basis.
[b] Standard deviation.
[c] Commercially known as dry Cottage cheese.

TABLE 4

Biotin content of a variety of cheeses

Cheese variety	No. of samples	Average solids	Biotin content		
			Range	Mean [a]	Std.[b] dev.
		(%)	(μg/100 g)	(μg/100 g)	(μg)
Highly proteolytic					
Camembert	20	51.1	1.92-17.84	5.70	4.00
Limburger	19	50.4	1.30- 6.59	2.26	2.20
Beer Kaese	9	64.7	1.06- 5.61	2.09	1.40
Liederkranz	11	36.3	0.63- 5.51	1.96	1.45
Blue	17	61.4	0.99- 3.65	1.64	0.80
Roquefort	15	62.9	1.02- 2.81	1.49	0.56
Port Salut	14	52.3	0.53- 3.61	1.18	0.74
			Avg	2.51	
Very hard					
Parmesan	10	75.2	0.90- 3.78	1.70	6.20
Edam	16	72.4	0.75- 5.14	1.52	1.11
Romano	15	70.2	0.55- 4.75	1.34	1.10
			Avg	1.50	
Hard					
Provolone	9	59.6	1.11- 2.19	1.79	0.47
Cheddar	57	65.0	0.98- 2.95	1.73	0.53
Gouda	20	61.4	0.65- 2.50	1.68	5.20
Swiss	25	59.0	0.35- 2.30	0.94	0.56
Gruyère	20	56.7	0.37- 1.92	0.84	0.30
			Avg	1.44	
Semihard					
Mozzarella	13	50.9	0.61- 2.33	1.62	0.49
Brick	30	59.9	0.87- 2.45	1.59	0.49
Muenster	11	64.0	0.96- 3.24	1.39	0.86
Husker	14	62.4	0.49- 1.49	1.18	0.41
			Avg	1.48	
Soft					
Cottage-plain [c]	9	26.3	1.44- 2.94	1.99	0.88
Cottage-creamed	21	25.4	0.48- 2.48	1.96	0.67
Neufchatel	11	39.8	0.79- 2.30	1.93	0.72
Cream	20	51.8	0.99- 3.72	1.64	0.77
			Avg	1.85	
Total	406		Grand avg	1.79	

[a] On wet or fresh weight basis.
[b] Standard deviation.
[c] Commercially known as dry Cottage cheese.

sented in Table 3, the pantothenic acid content in 244 samples of 17 varieties ranged between 144 and 2,046 μg per 100 g for plain Cottage and Blue cheese, respectively. The grand average of all the samples was 691 μg of pantothenic acid per 100 g of cheese. Here again, there were wide variations in the pantothenic acid content of individual samples of the same variety, as well as in different varieties of cheese. The vitamin content was highest in the proteolytic group and the lowest in the soft group. However, on the dry solids basis, the vitamin content of the soft-unripened group of cheese was higher than that of the hard groups.

Biotin content of different cheeses. As presented (Table 4), the biotin content of 406 samples of 23 varieties of cheese ranged from 0.84 μg per 100 g for Gruyère to 5.70 for Camembert cheese. The grand average biotin content of all the samples was 1.79 μg per 100 g of cheese. The proteolytic cheeses as a group were the highest in biotin content, and soft, very hard, semihard, and hard were next, in that order. However, when the vitamin content was calculated on a dry solids basis, the soft-unripened group was found to be the highest, with the proteolytic group being next to it. The semihard, hard, and very hard were the next, in decreasing order.

Folic acid content of cheese. A total of 716 samples of 23 varieties of cheese were assayed and the data are presented (Table 5). The folic acid content ranged between 6.4 and 120.6 μg

per 100 g for Swiss and Liederkranz, respectively, and the grand average for all cheeses was 22.3 μg per 100 g. The proteolytic group as a whole was the most potent source of the vitamin, and the hard cheese group was the lowest. Essentially the same relationship was observed when the vitamin content was calculated on a moisture-free basis.

Vitamin content of processed cheese, spreads, and special cheeses. Recently, new varieties of processed cheese, cheese spreads, and special cheeses have appeared on the market, and information concerning the vitamin content of such cheese varieties is not available. Therefore, several processed cheeses and cheese spreads were assayed for the various B vitamins, and the results are tabulated (Table 6). Also, although not presented in the table, ten samples of Velveeta and 31 samples of Kaukauna Klub were assayed for pantothenic acid, and they contained 786 and 985 μg of pantothenic acid per 100 g, respectively. In general, the water-soluble vitamins content in these types of processed cheese and spreads was comparable to that observed in natural hard cheeses. This may be because mostly natural cheeses are used in the manufacture of processed cheese and since most of these vitamins are heat stable no great loss occurs during processing. Also while there were some variations in the individual vitamin contents of different types of cheese, there was little variation between different samples of the same type of cheese. This should be expected, since the methods for the

TABLE 5

Folic acid content of a variety of cheeses

Cheese variety	No. of samples	Average solids	Folic acid content		
			Range	Mean [a]	Std. [b] dev.
		(%)	(μg/100 g)	(μg/100 g)	(μg)
Highly proteolytic					
Liederkranz	21	36	75-248	120.6	49.5
Camembert	18	51	34- 96	62.2	37.5
Limburger	25	50	19- 97	57.5	26.5
Roquefort	24	63	20- 82	49.0	38.0
Blue	34	61	21- 45	36.4	5.2
Beer Kaese	20	65	8- 33	22.2	13.0
Port Salut	19	52	8- 34	18.2	8.0
			Avg	51.5	
Very hard					
Edam	41	72	6- 35	16.2	9.4
Parmesan	20	75	2- 15	7.3	3.5
Romano	24	70	2- 20	6.8	4.9
			Avg	11.4	
Hard					
Gouda	20	61	8- 36	20.9	11.6
Gruyère	51	57	5- 14	10.4	8.7
Provolone	15	60	4- 18	10.4	4.3
Cheddar	112	65	4- 21	10.4	4.5
Swiss	26	59	3- 15	6.4	1.3
			Avg	10.9	
Semihard					
Brick	30	60	5- 39	20.3	17.0
Muenster	25	64	3- 30	12.1	8.4
Husker	34	62	5- 19	10.4	5.1
Mozzarella	21	51	5- 17	9.9	5.1
			Avg	13.4	
Soft					
Cottage-plain [c]	27	26	21-105	42.9	29.0
Cottage-creamed	40	25	9- 36	23.6	12.0
Cream	56	51	5- 27	13.6	5.0
Neufchatel	13	40	7- 16	11.3	2.8
			Avg	22.1	
Total	716		Grand avg	22.3	

[a] On wet or fresh weight basis.
[b] Standard deviation.
[c] Commercially known as dry Cottage cheese.

TABLE 6

Vitamin content of a variety of processed and special cheeses

Processed cheese or spread	Average solids	Niacin			Pyridoxine			Biotin			Folic acid		
		No. of samples	Mean	Std. dev.	No. of samples	Mean	Std. dev.	No. of samples	Mean	Std. dev.	No. of samples	Mean	Std. dev.
	(%)		(μg/100 g)	(μg)		(μg/100 g)	(μg)		(μg/100 g)	(μg)		(μg/100 g)	(μg)
Hot processed													
American	61.5	12	81.1	41.1	15	82.4	11.5	15	1.7	0.46	26	7.8	1.2
Cheese-bacon spread	47.9	3	316.3	84.8	8	83.1	32.5	10	2.7	0.81	14	6.4	1.6
Garlic	53.3	8	116.6	60.5	..	.....	.....	14	4.0	0.99	18	12.5	9.2
Jalapenos	54.6	7	114.6	53.0	6	114.3	12.5	14	3.9	0.61	16	9.1	3.1
Velveeta	53.2	14	148.3	70.0	20	122.7	12.5	15	6.2	1.34	18	8.2	2.9
Cold processed													
Kaukauna Klub	57.3	15	51.8	23.5	26	141.3	25.5	27	4.9	1.10	21	5.4	1.6
Old English	65.5	10	41.5	16.5	7	76.0	13.2	13	2.6	1.15	22	16.0	6.8
Unclassified													
Ski Queen	87.4	5	813.4	247.8	..	.....	.....	25	16.6	1.52	8	4.6	2.4

manufacture of these types of cheese are highly standardized.

In general, the hot processed cheese varieties did not differ too greatly in vitamin content from the cold processed varieties, except in the case of niacin content, which was somewhat lower in the cold-processed varieties. Ski Queen, a whey cheese which constitutes the third group of unclassified or special cheese, was unusually high in the niacin, biotin, and folic acid content.

DISCUSSION

The average values of several vitamins in a large number of cheese varieties assayed were fairly typical of the few reported in the literature. Sullivan et al. (20) assayed 12 varieties of cheese and noted their niacin, pantothenic acid, and biotin contents ranged from 30 to 1,600, 130 to 960, and 1.1 to 7.6 μg per 100 g of cheese, respectively. Hardinge and Crooks (11) have reported that in Cheddar, Cottage, and processed cheeses, the pantothenic acid content ranged from 280 to 480; the biotin ranged from 3.6 to 4.6; folic acid ranged from 11 to 31 μg per 100 g; and Cheddar cheese contained 66 μg of B_6 per 100 g. The results of the present study revealed that the average niacin, B_6, pantothenic acid, biotin, and folic acid contents in a large number of cheeses were 277, 94, 691, 1.79, and 22.3 μg per 100 g, respectively.

In general, there were wide variations in vitamin content not only in different varieties and classes but also in different samples of the same variety. This might be due partly to the differences in the vitamin content of the milk used in the manufacture of the cheese. In addition, as pointed out by Burkholder et al. (3), the vitamin content of cheese depends in large measure on the organisms and methods used in its manufacture and the subsequent curing period.

The proteolytic types contained the highest concentrations of the vitamins. Perhaps the microorganisms present in these types synthesize vitamins during curing, as was observed by others (3, 19, 20). Pilot studies were carried out in this laboratory to determine the rate of synthesis of vitamin B_6 during the curing of certain types of cheese. Using the same lot of milk, Coulommier, Romadur, and Camembert cheeses were made and cured at 55 to 60 F. At the end of 5 days' curing period, Coulommier, Romadur, and Camembert contained 57.2, 55.0, and 65.7 μg of B_6 per 100 g of cheese, respectively. At the end of 64 days' curing, the vitamin content of cheeses increased to 131.6, 123.0,

and 215.0 μg per 100 g, respectively, indicating that during curing the organisms synthesize vitamin B_6. The organisms in Camembert cheese were able to synthesize the vitamin more rapidly than the organisms of the two other varieties.

Except for the biotin and folic acid content, the very hard or hard varieties of cheese were the next highest in vitamin content, followed by the semihard or soft-unripened types. The low folic acid content of the hard cheeses might result from the high processing temperature used in their manufacture, since folic acid is more heat-labile than B vitamins in general (1), particularly in an acid medium.

Ski Queen cheese variety was very high in niacin, biotin, and folic acid. It is made from cow's or goat's milk whey and contains more than 87% solids. Also, Velveeta cheese, in the manufacture of which whey solids are included, was relatively high in the B vitamins. The high concentration of the vitamins present in these cheeses might be because the whey solids contain a major part of the water-soluble vitamins present in the original milk. These observations are in harmony with the results of Sullivan et al. (20).

Since most of the cheese samples assayed were purchased from the market, no information was available regarding the vitamin content of the milk used in their manufacture. Therefore, the values for the B vitamins content of milk as reported in the literature were taken to calculate theoretically the retention of the vitamins in cheese from milk. The literature records average values for the niacin, B_6, pantothenic acid, biotin, and folic acid content of milk as 910, 700, 3,100, 4.7, and 60 μg per 100 g, respectively (4, 9, 11, 13, 17, 18). On the basis of the average values for these vitamins found in the cheese samples, it was observed that the cheeses contained 9 to 55% of the vitamin content of milk.

The significance of B vitamins in nutrition is well established. While they have been shown to be essential in the metabolism of carbohydrates, proteins, and fats in the human diet, the daily requirements for an adult have been established for only a few of the B vitamins. Day and Darby (6) have reported that a 4-oz portion of American cheese would provide approximately one-third of the daily requirement of riboflavin. The present data reveal that cheese is fairly rich in most of the B vitamins.

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ABSENCE OF FATTY ACID SPECIFICITY DURING LIPOLYSIS OF SOME SYNTHETIC TRIGLYCERIDES BY B-ESTERASE PREPARATIONS FROM MILK[1]

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SUMMARY

The specificity of B-esterase preparations from bovine milk for short-chain fatty acids was studied by using as substrates glyceryl 1-oleate 2,3-dicaproate (OCC) and glyceryl 1-palmitate 2,3-dibutyrate (PBB). These triglycerides were emulsified into skimmilk, incubated with the B-esterase preparations for varying lengths of time at 38 C, and the products of lipolysis isolated by column and thin-layer chromatography. The fatty acid compositions of the isolated free fatty acids and mono- and diglycerides were determined by gas-liquid chromatography. Absence of specificity for short-chain acids was indicated by the release of equimolar quantities of caproate and oleate or butyrate and palmitate during lipolysis periods of up to 1 hr. When PBB was incubated at 4 C for 24 hr, there was apparent specificity for butyrate which was probably caused by the comparatively rapid lipolysis of dibutyrin in contrast to glyceryl 1-palmitate 2-butyrate at the diglyceride stage.

One of the vexing problems faced by investigators studying lipases has been the apparent specificity for short-chain acids shown by some of these enzymes (19). For example, when tributyrin was compared to tripalmitin and to milk fat as substrates, the relatively rapid rates of hydrolysis obtained with tributyrin lent superficial support to the belief that milk and other lipases showed specificity for short-chain acids (7, 19). Because of the extreme difficulty in preparation of comparable emulsions with two triglycerides differing widely in molecular weight, melting point, and solubility, the validity of these comparisons may be questionable. There have been very few reports describing the use of known triglycerides with long- and short-chain acids on the same molecule as substrates for lipases. Entressangles et al. (8) found that purified pancreatic lipase preferentially released 4:0 from the diglyceride, glyceryl 1-palmitate-3-butyrate. Similar investigations of milk lipase have not been reported. Harper (12) analyzed the short-chain acids liberated from milk fat by milk and other lipases and found that substantial amounts of 4:0 were released. However, recent studies employing pancreatic lipase (20) and milk lipase (16) have suggested that most of the 4:0 in milk fat is esterified at the primary ester positions of the triglycerides. Both pancreatic and milk lipases are known to preferentially hydrolyze the primary ester linkages of triglycerides (9, 10, 16, 17, 23). Does this positional specificity extend to mixed triglycerides containing short-chain acids? If it does, then the apparent specificity of milk lipase for 4:0 and other short-chain acids may be explained by positional specificity. If not, then short-chain fatty acid specificity may exist.

The difficulties involving determinations of fatty acid specifications mentioned above can be eliminated if both fatty acids are esterified at the primary positions of the same triglyceride as in glyceryl 1-palmitate 2,3-dibutyrate. We have prepared two mixed triglycerides and lipolyzed them with B-esterase preparations from milk. B-esterase preparations have many similarities to milk lipases (17). The employment of B-esterase preparations in lipolysis studies aids reproducibility and permits attain-

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ment of short reaction times. This paper describes the results obtained from lipolyses of synthetic mixed triglycerides with milk B-esterase preparations in a study of fatty acid specificities.

EXPERIMENTAL PROCEDURES

B-esterase preparations. These were prepared by a modification of the method of Montgomery and Forster (24), sent to the University of Connecticut by air mail–special delivery, and stored on arrival at −20 C. Three different preparations were used. These were prepared from gravity skimmilk from (1) cows 2201 and 3203, (2) cow 2201, and (3) cow 2218. Three milligrams were equivalent to 1 ml of milk in all preparations.

Synthetic triglycerides. (a) The monoglycerides were made from isopropylidene glycerol[2] (distilled through a 28 plate spinning band column) and palmitoyl (Matheson, Coleman, and Bell) (2) or oleoyl chloride (6). Oleic acid was halogenated with oxalyl chloride to provide oleoyl chloride (3). Some of the monoglycerides were obtained by washing and recrystallizing commercial 1-monopalmitin and 1-monoolein[3] of high purity. The 1-monopalmitin was crystallized from petroleum ether (30-60 C) at room temperature and 1-monoolein from mixtures of petroleum ether (30-60 C) and ethyl ether at −20 C. These materials and the synthetic monoglycerides contained at least 98% 1-monoglyceride when tested by periodate oxidation (15) and were free from di- and triglycerides when checked with thin-layer chromatography (TLC) (18, 25). The purified 1-monopalmitin contained in excess of 97% 16:0, whereas the 1-monoolein contained at least 92% 18:1.

(b) Glyceryl-1-palmitate-2,3-dibutyrate (PBB) and glyceryl-1-oleate-2,3-dicaproate (OCC) were prepared by direct acylation with butyryl or caproyl chloride of the appropriate 1-monoglyceride (26). The triglycerides were purified by passage through neutral alumina (activity grade 1) in petroleum ether (30-60 C) (21) and crystallization from acetone at −40 C for PBB, and −96 C for OCC. These purifications were repeated until the triglycerides were free from mono- and diglycerides when examined by TLC. Gas-liquid chromatography (11) and, in the case of PBB, column chromatography (1) were used to determine the fatty acid compositions of the triglycerides. OCC and PBB contained the appropriate fatty acids in the correct quantities when analyzed.

Lipolyses. The substrates were emulsified into pasteurized skimmilk with a Waring Blendor in the amount of 200 mg per 10 ml. Sixty milligrams of B-esterase preparation was added for each 10 ml of skimmilk and mixed in, also with a Waring Blendor. The enzyme in the control was inactivated by boiling. The mixtures for each trial, consisting of 15-, 30-, and 60-min periods of lipolysis and a control, were prepared in one batch. The mixtures were distributed in the amount of 10 ml each into digestion flasks and incubated with shaking in a 38 C water bath for the specified times, except for a 24-hr sample (with PBB) which was placed in a refrigerator at 4 C for 24 hr. Timing started when the B-esterase preparations were added to the emulsions. All three batches of B-esterase were used with OCC and Preparations 1 and 2 with PBB. The controls were devoid of FFA, MG, and DG.

Recovery and analysis of the products of lipolysis. At the termination of the lipolysis periods, the samples were acidified and extracted by the silica-gel method with 200 ml ethyl ether (13). Preliminary trials comparing ethyl ether and ethyl ether: ethyl alcohol (90:10) as extractants indicated that ethyl ether satisfactorily extracted all of the products of lipolysis. The material extracted in the ether was then separated into neutral lipids and free fatty acids (FFA), as described by McCarthy and Duthie (22), by passing the ether from the silica-gel extractions directly through an alkaline silicic acid column. The neutral lipids were separated into mono- (MG), di- (DG), and triglycerides (TG) by TLC (18, 25). The neutral lipids were dissolved in a minimal amount of ether and applied in a continuous band to the base of one or more thin-layer plates. The plates were developed with solvent systems of ethyl ether: petroleum ether (30-60 C) (1:1). The separated glycerides were made visible with a brom thymol blue spray (14), scraped off, and extracted with ethyl ether. A photograph of a developed and sprayed plate is shown in Figure 1. Preliminary identifications were obtained by comparisons of known compounds with unknowns on thin-layer plates, infrared spectrophotometry, and periodate oxidation for the monoglyceride (15, 18). After preliminary identifications were established by these methods, further identifications were made by position on the thin-layer plates.

[2] Aldrich Chemical Co., 2369 N. 29th Street, Milwaukee 10, Wisconsin.

[3] Distillation Products Industries, Rochester, New York.

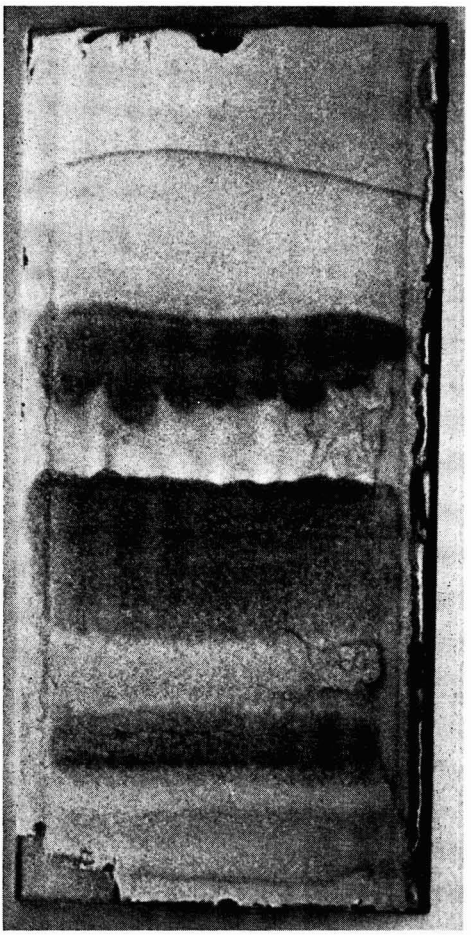
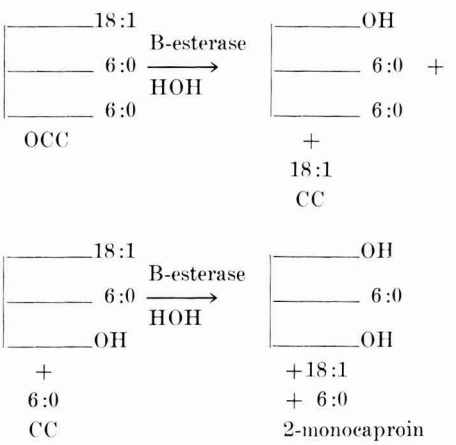


FIG. 1. Thin-layer plate illustrating separation of mono- (bottom band), di- (middle band), and triglycerides (top band). Plate developed with ethyl ether–petroleum ether (30-60C) (1:1) and sprayed with aqueous brom thymol blue.

The FFA were eluted from the separation column into an evaporation flask containing 22 ml of the isopropanol-KOH (1) and a magnetic spinner, which provided thorough mixing for conversion of the FFA to K salts. The solvents were removed on a flash evaporator and the K salts transferred to a 100-ml pear-shaped flask with methanol. The methanol was removed by evaporation and the K salts of the FFA's were acidified, converted to butyl esters, and identified by GLC employing temperature programing (11). When the intact, pure triglycerides were converted to butyl esters, the correction factors allowing for incomplete recovery of 4:0 and 6:0 developed in our earlier study on milk fatty acids (11) were found to be applicable.

RESULTS AND DISCUSSION

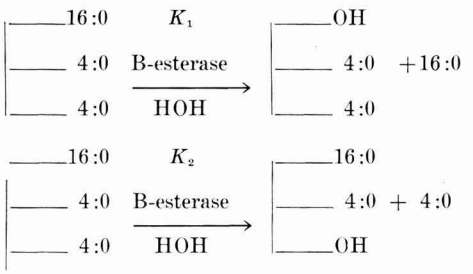
All of the fractions obtained from lipolysis of OCC contained fatty acids in about the proportions predicted by positional specificity for the primary ester positions and lack of fatty acid specificity (Table 1). These relationships are shown in the equation below, in which the fatty acid compositions are, as M%: FFA, 6:0 — 50, 18:1 — 50; DG, 6:0 — 75, 18:1 — 25; and MG, 6:0 — 100; 18:1 — 0.



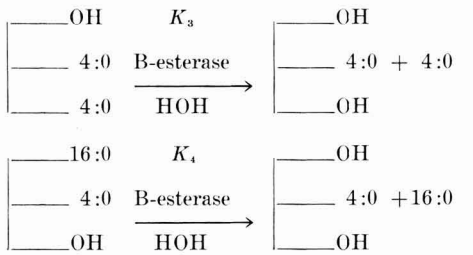
If specificity for 6:0 existed, then there would be more 6:0 than 18:1 in the FFA, the 6:0 content of the DG would decrease, and the composition of the MG would remain largely 6:0. Since the results in Table 1 were in close agreement with the theoretical fatty acid compositions, B-esterase preparations did not release 6:0 more rapidly than 18:1, when both were esterified at the primary positions of triglycerides.

When PBB was lipolyzed with B-esterase preparations for periods of up to 60 min, the results indicated that 4:0 was not favored over 16:0 (Table 2). The theoretical relationships for primary-position specificity and lack of fatty-acid specificity are the same for PBB as for OCC, and are illustrated above. In addition, with PBB we ran a 24-hr lipolysis at 4 C to show how the results from long periods of incubation can be misinterpreted as indicating specificity for 4:0. When these samples were analyzed there was more 4:0 (M%) than 16:0 in the FFA, superficially indicating specificity for 4:0. We wished to determine whether

reaction constants K_1 or K_2 (below) were equal or different.



However, as the diglycerides appear, reactions (below) represented by reaction constants K_3 and K_4 become possible.



Because of the differences in molecular size and solubility between these diglycerides K_3 would be expected to be greater than K_4. That is to say, the dibutyrin would be expected to be lipolyzed more rapidly. The data will be biased if this occurs. Reactions represented by K_3 and K_4 must be kept at a minimum. This can be done experimentally only under those conditions where the original triglyceride concentration is large in proportion to the concentrations of the two diglycerides formed. Such is the case during short incubation periods. That such conditions can occur during long periods of incubation is borne out by the data in Table 2, where most of the diglyceride in the 24-hr sample was accounted for as glyceryl 1-palmitate 2- (or 3) butyrate (PB). The dibutyrin was, therefore, lipolyzed more rapidly than the PB and the results were biased toward apparent specificity for 4:0. Short periods of incubation are generally recommended for studies of specificity and fatty acid distribution in triglycerides, because lipolysis of the di- and monoglycerides leads to faulty interpretation of the results (4). The discussion above also illustrates the value of determining the composition of the partial glycerides in studies of fatty acid specificity. The 24-hr results cannot be explained by random lipolysis, because all of the fractions would contain as M%: 4:0, 67 and 16:0, 33. Nor can they be explained by the effects of temperature on the substrate, since PBB was liquid at 4 C.

With both OCC and PBB, the monoglycerides formed were largely monocaproin and monobutyrin (Tables 1 and 2). Therefore, B-esterase preferentially attacked primary ester linkages of triglycerides with short-chain acids on the 2-positions. The presence of the small quantities of monopalmitin and monoolein in the monoglycerides may possibly be explained by isomerization of 6:0 and 4:0 to the 1-position during the diglyceride stage and the release of these acids, leaving 1-monoolein and 1-monopalmitin.

We were not overly concerned about losses of 4:0 and 6:0 during our manipulations, because the procedures used quantitatively recover these acids (13, 22) and the fatty acid compositions of the mono- and diglycerides agreed closely with the theoretical values. Therefore, lack of recognition of short-chain specificity could not have been due to losses of

TABLE 1

Fatty acid composition of the FFA and mono- and diglycerides formed during the lipolysis of glyceryl 1-oleate 2,3-dicaproate by milk B-esterase concentrates [a]

Incubation time	FFA		Diglycerides		Monoglycerides	
	6:0	18:1	6:0	18:1	6:0	18:1
	(M%)					
15 min	52.6 [b]	47.4	78.3	21.7	97.9	2.1
30 min	46.9	53.1	74.2	25.8	93.5	6.5
60 min	58.2 [b]	41.8	71.3	28.7	96.5	3.5
Theoretical [c]	50.0	50.0	75.0	25.0	100	....

[a] Lipolysis conditions: 200 mg substrate, 60 mg B-esterase concentrate, and 10 ml of pasteurized skimmilk incubated with shaking at 38 C.

[b] Averages of trials with two different batches of B-esterase concentrate. The 30-min figures are averages of trials with three different batches.

[c] Assuming specificity for the primary ester linkages, but not fatty acid specificity.

TABLE 2

Fatty acid composition of the FFA and mono- and diglycerides formed during the lipolysis of glyceryl 1-palmitate 2,3-dibutyrate by milk B-esterase concentrates [a]

Incubation time	FFA		Diglycerides		Monoglycerides	
	4:0	16:0	4:0	16:0	4:0	16:0
	(M%)					
15 min	42.9 [b]	57.1	70.8	29.2	Not recovered	
30 min	53.5	46.5	75.7	24.3	100	....
60 min	48.9	51.1	78.2	21.8	100	....
24 hr	65.7	34.3	57.3	42.7	97.7	2.3
Theoretical [c]	50.0	50.0	75.0	25.0	100	....

[a] Lipolysis conditions: 200 mg substrate, 60 mg B-esterase concentrate, and 10 ml skimmilk incubated with shaking at 38 C except for 24-hr sample, which was stored at 4 C.
[b] Averages of trials with two different batches of B-esterase concentrate.
[c] Assuming specificity for the primary ester linkages, but not fatty acid specificity.

4:0 and 6:0 or the compositions of the mono- and diglycerides would have differed from the actual values.

The conditions employed in our studies differ somewhat from the conditions in natural milk. Our "milk" contained more enzyme(s) and less substrate than normally present in milk. The skimmilk used as a dispersion medium for the substrate was pasteurized. It is not known if triglycerides of the type employed above are present in the natural milk fat. The B-esterase preparation used, while possessing many of the attributes of milk lipases (18), may not actually be milk lipase and may contain several lipases. Nevertheless, we believe that fatty acid specificity of lipases is best studied with relatively simple systems, and substrates of known structure. Although it is possible to prepare even simpler systems by using buffers and some suspending agent such as gum arabic, we preferred to use a system somewhat more similar to milk. If the fatty acids being compared are placed on the 1 and 3 positions of the triglyceride molecule, rate studies can be made without interference from wide differences in melting point and solubility, as would be the case if simple triglycerides were used.

CONCLUSIONS

When glyceryl 1-oleate 2,3-dicaproate (OCC) and glyceryl 1-palmitate 2,3-dibutyrate (PBB) were lipolyzed for 15-, 30-, and 60-min periods with milk B-esterase preparations, there was no evidence for preferential release of 6:0 and 4:0 from the triglycerides. When PBB was lipolyzed for 24 hr at 4 C there was evidence which could be misinterpreted as specificity for 4:0, probably due to relatively rapid lipolysis of dibutyrin.

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EFFECT OF ANTIOXIDANTS ON THE FLAVOR OF COLD STORAGE BUTTER [1]

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SUMMARY

Fresh salted sweet cream butter containing 0.01% by weight of nordihydroguaiaretic acid (NDGA), butylated hydroxytoluene (BHT), and BHT + *iso*-propylcitrate (IPC), respectively, was stored at −18 and 38 F. The autoxidation of the butter was followed organoleptically and by the thiobarbituric acid (TBA) test and peroxide value. None of the antioxidants retarded the development of oxidized flavors in butter kept at −18 and 38 F. BHT + IPC acted as a pro-oxidant in butter kept at 38 F. Oxidized flavor development was retarded by NDGA in butter stored for several months at −18 F and then transferred to 38 F, but the NDGA gave an objectionable bitter flavor to the butter during storage. The TBA test and peroxide value were found to have little value for predicting the flavor score under these storage conditions.

Antioxidants are widely used in many foods to prevent the autoxidation of fats, but their value in butter is uncertain. Some investigators report that antioxidants will retard the onset of autoxidative deterioration, whereas others find them of little value and report that the antioxidants may contribute new undesirable flavors to butter (2, 5, 6, 9, 11-17). There has been some discussion recently of the desirability of changing the standards of identity of butter to permit the inclusion of antioxidants and other additives (1). In other fats the particular antioxidant and its level are quite important in determining the optimum storage life under a particular set of conditions (10). The present experiments were designed to test a few antioxidants under butter storage conditions typical in the United States.

MATERIALS AND METHODS

Four lots of butter with 2.5% salt were prepared from one lot of fresh sweet cream obtained in March. The cream was pasteurized at 164 F for 30 min. One lot of butter contained no additives and served as a control. The other three lots contained 0.01% by weight of butylated hydroxytoluene (BHT), 0.01% nordihydroguaiaretic acid (NDGA), and 0.01% BHT + 0.01% *iso*-propylcitrate (IPC), respectively. The antioxidants were dissolved or suspended in the butter color and added after churning with the salt. Uniform dispersion of the butter color during working indicated uniform dispersion of the antioxidant.

The butter was stored in 12 boxes of 5 lb each. Nine of the boxes were stored at −18 F and the remainder at 38 F. Every two months one of the boxes was transferred from −18 F storage to 38 F storage. The butter was judged for flavor every month by a panel of experienced judges and was graded on a scale of 0 to 10, as shown in Table 1. The butter was also tested bimonthly for autoxidation by the thiobarbituric acid (TBA) test and peroxide value.

The TBA test, based on the method of Dunkley and Jennings (3), was conducted as follows: A 10.00-g sample of butter was weighed

TABLE 1

Definition of the flavor score

0—No oxidation	6—Slightly metallic
1—Flat	7—Metallic
2—Slightly oxidized	8—Strongly metallic
3—Oxidized	9—Oily
4—Strongly oxidized	10—Fishy
5—Tallowy	

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in a glass-stoppered 15-ml centrifuge tube. The tube was immersed in boiling water for 1 min to melt the butter and 1 ml of 1 N HCl was added. The tube was shaken vigorously and centrifuged for 5 min. The fat layer was removed with a medicine dropper and discarded. Tests showed that no more of the material which reacts with TBA could be extracted from the fat. The aqueous layer was washed with 5 ml of petroleum ether and centrifuged for 5 min. The petroleum ether layer was drawn off with a medicine dropper and discarded. Next, 1.5 ml of TBA reagent (0.025 M thiobarbituric acid in 1 M phosphoric acid) was mixed with the aqueous layer, three drops of n-hexyl alcohol were added as a foam depressant, and the tubes were heated in a boiling water bath for 15 min. The red color which developed was extracted with 4 ml of a mixture of iso-amyl alcohol:pyridine (2:1, v:v). The tubes were centrifuged for 5 min and the absorbancy of the upper layer was determined at 490, 535, and 580 mμ with a Beckman DU spectrophotometer. The absorbancy at 535 minus the mean of the readings at 490 and 580 mμ was taken as a measure of autoxidation. The determinations were done in duplicate and the absorbancies were usually found to agree within 0.01.

The peroxide number was determined according to Hills and Thiel (8).

RESULTS

Figure 1 presents the average flavor scores for butter stored at 38 F. The flavor scores of the samples with antioxidants are not much different from the control. The lot of butter containing NDGA developed an objectionable bitter taste after a few months' storage. One of the judges could not detect the bitter taste. The flavor scores of the individual judges were analyzed statistically by Friedman's test (4), and the scores for the four lots of butter were inhomogeneous at the 0.01 significance level. When the scores for the butter with BHT + IPC were deleted, the scores for the other lots of butter were inhomogeneous only at the 0.1 significance level. Thus, the butter with BHT + IPC differed from the other three lots considerably more than they differed from each other. However, the butter with BHT + IPC was in a more advanced stage of autoxidative deterioration than the other lots, and the BHT + IPC is evidently a pro-oxidant.

Figure 2 presents the average flavor scores for butter stored at −18 F. It is apparent from the figure that there is not much difference in the lots containing antioxidants and the control. The scores for the four treatments were inhomogeneous only at the 0.5 significance level. The judges tended to score the butter stored at −18 F more autoxidized than that stored at 38 F during the first six months of storage. Beyond six months, the −18 F butter did not progress much beyond the tallowy stage, whereas the 38 F butter became progressively worse during the entire storage period. Actually, the designation tallowy covered a broad range of intensity, and some of the

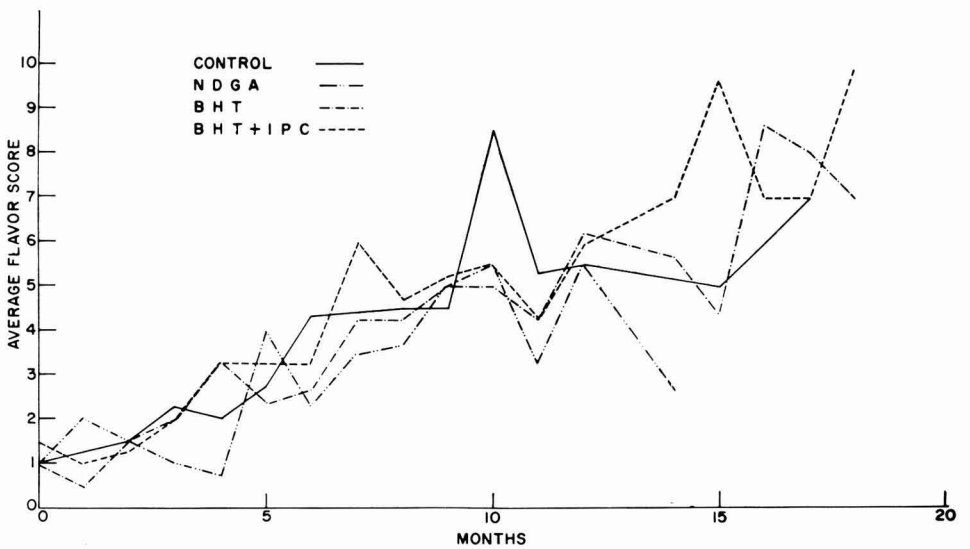

FIG. 1. Average flavor scores of butter stored at 38 F.

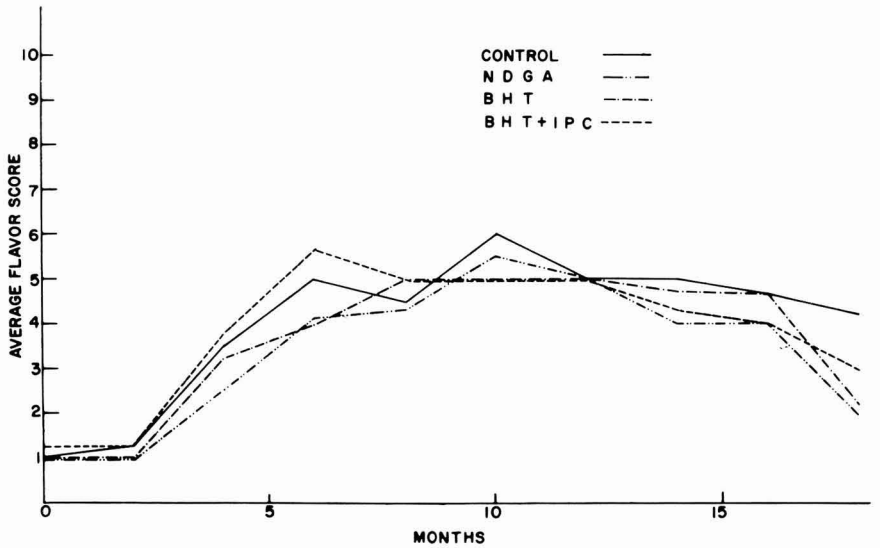

FIG. 2. Average flavor scores of butter stored at −18 F.

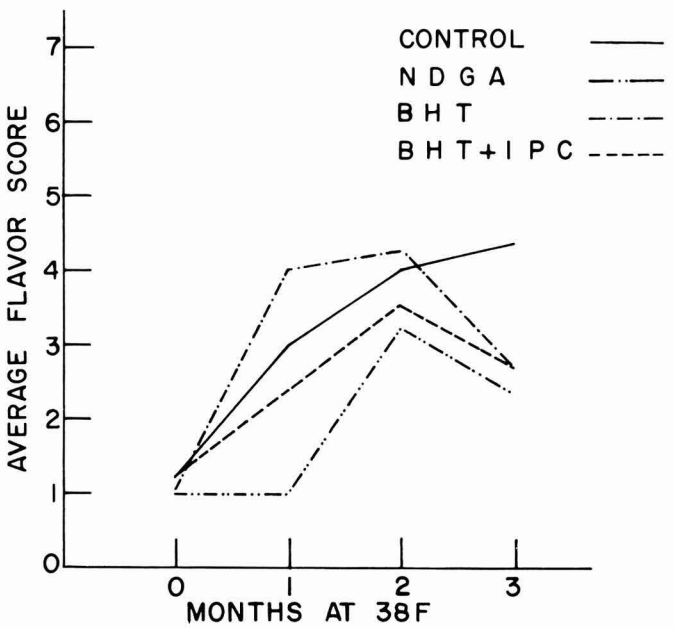

FIG. 3. Average flavor scores of butter stored two months at −18 F, then transferred to 38 F storage.

samples were considerably more objectionable than others.

Figures 3 and 4 show the flavor scores of butter stored for two and four months at −18 F and then transferred to 38 F for three months. The flavor score of these samples continued to increase at about the same rate as those kept at −18 F. As noted above, this is somewhat faster than those stored at 38 F. Samples stored longer than four months at −18 F were generally judged tallowy and the score did not increase during three months of storage at 38 F. A statistical analysis of the butter stored at −18 F for various periods and then transferred to 38 F showed the scores were inhomogeneous at better than the 0.005 significance level. When the scores for the butter containing NDGA were deleted, the scores for the three other lots were still inhomogeneous at the 0.025 significance level. When the scores for the butters with NDGA and BHT + IPC were deleted, the scores for the control and BHT butter were homogeneous. In the transferred butter both NDGA and BHT + IPC acted as antioxidants and improved the oxidative stability significantly, but the effect of the NDGA was by far the larger.

Some TBA analyses of the butter stored at −18 and 38 F are shown in Figures 5 and 6, respectively. In both cases, the presence of NDGA gave a considerably lower TBA absorbancy. The butter containing BHT gave lower TBA tests at 38 F. There was considerable variation in the TBA from month to month. These large differences in TBA results stand in contrast to the small differences in

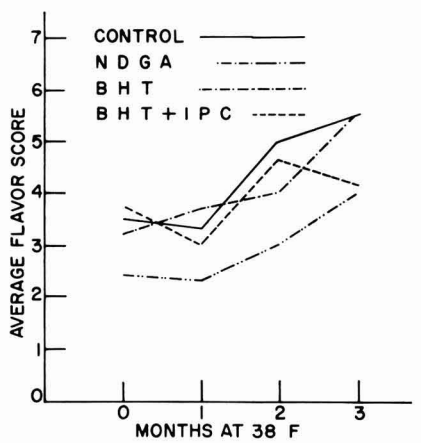

FIG. 4. Average flavor score of butter stored four months at −18 F, then transferred to 38 F storage.

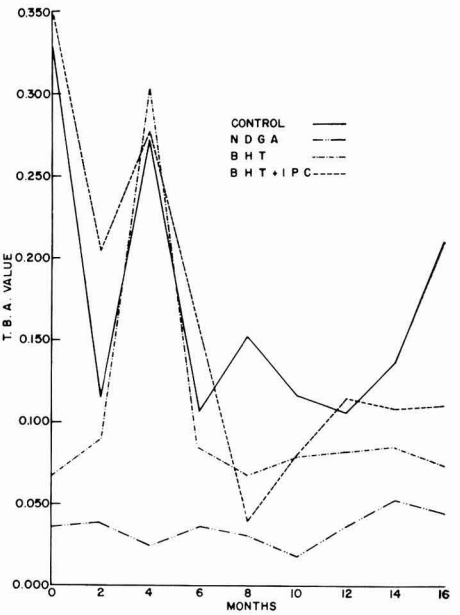

FIG. 5. TBA values of butter stored at −18 F.

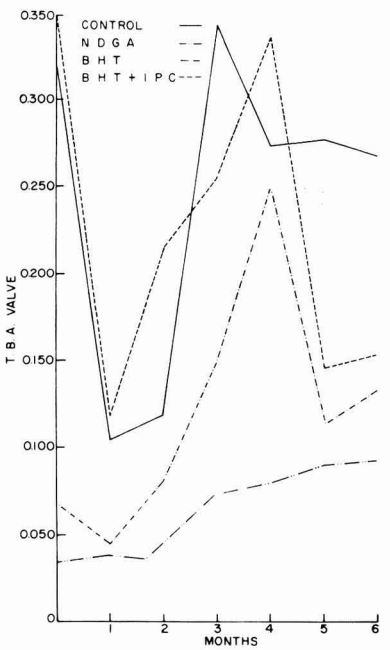

FIG. 6. TBA values of butter stored at 38 F.

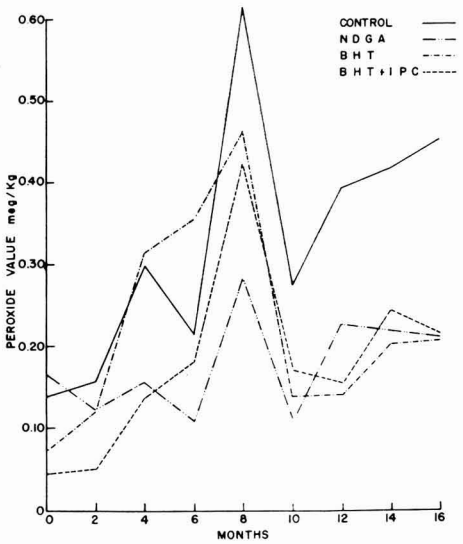

FIG. 7. Peroxide values of butter stored at −18 F.

flavor scores for the same butter. Obviously, the TBA test is not a reliable index of the organoleptic score under these conditions.

Figures 7 and 8 show some peroxide values for butter stored at −18 and 38 F, respectively. The peroxide values at 38 F agree with the TBA results in showing less oxidation for the lots of butter containing NDGA and BHT. But there are large variations in the peroxide values which do not correspond to similar variations in TBA test. Like the TBA values, peroxide values do not reliably predict the organoleptic score.

DISCUSSION

The peroxide value and TBA test are often used as indices of autoxidation in fats, but the autoxidation of fats is a complex set of reactions. The peroxide value and TBA test measure the extent of certain of these reactions under a given set of conditions, but there is no reason to expect that they will correspond closely under all conditions with the degree of oxidation judged organoleptically. In the data presented, the relatively lower peroxide values and TBA values of butter containing NDGA and BHT at 38 F did not correspond to better flavor scores. This demonstrates the hazard of basing autoxidation studies on these chemical tests when the organoleptic score is the important variable in question.

In general, the results demonstrate no benefits from the use of antioxidants. Although NDGA gave a better flavor score in the samples transferred from −18 to 38 F, it also imparted an objectionable bitter flavor to the butter. We have not been able to rationalize the results obtained completely with any of the theories of the behavior of antioxidants. It is hard to explain why NDGA was significantly effective in butter transferred from −18 to 38 F, when it is not effective with butter kept at either temperature.

The greater rate of oxidation of butter at −18 F than at 38 F during the first six months of storage may be related to the pro-oxidant effect of the natural and added antioxidants as expounded by Heimann and von Pezold (7). They found that antioxidants might accelerate or retard autoxidation, depending on the temperature and concentration. Lowering the temperature was found to increase pro-oxidant more than antioxidant action. If this were so, one would expect the butter with added antioxidants to be worse than the controls at −18 F, which was not the case. Also, the butter transferred from −18 to 38 F continued to oxidize more rapidly than that kept at 38 F. It is also difficult to explain on the basis of this theory why BHT + IPC had a pro-oxidant effect at 38 F but not at −18 F. A more extensive knowledge of the compounds responsible for the organoleptic sensations and of the mechanism of their production will probably be required to explain these results.

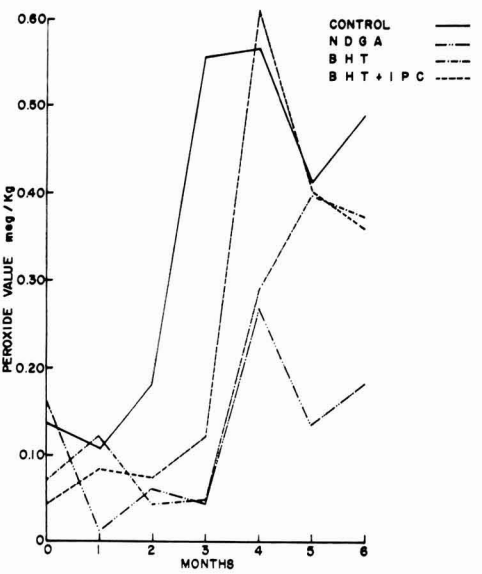

FIG. 8. Peroxide values of butter stored at 38 F.

ACKNOWLEDGMENT

We thank Dr. E. W. Bird and Dr. V. H. Nielsen for their advice about the design of these experiments, and Professor Herbert David for aid in the statistical analysis.

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RELATIVE MILK PRODUCTION VALUE OF BARLEY, DRIED BEET PULP, MOLASSES DRIED BEET PULP, AND CONCENTRATED STEFFEN FILTRATE DRIED BEET PULP [1]

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SUMMARY

Concentrated Steffen filtrate (CSF) is a by-product of beet sugar manufacture which may be added to beet pulp. A study was conducted to compare the feeding value of CSF dried beet pulp with plain and molasses dried beet pulp. Each of the three types of beet pulp was fully equal to barley in the replacement of approximately 25% of the energy of a basal ration consisting of 70% alfalfa hay and 30% barley.

Concentrated Steffen filtrate (CSF)[2] is a by-product of the Steffen process for the desugarization of beet molasses. It is a concentrated solution remaining after the removal of sucrose, and contains all of the other extractable solids of the sugar beet.

This material has been used for some time in a modified form (condensed beet solubles product) as a livestock feed. Washington workers (1) found beet pulp containing a combination of CSF and molasses to be equal to conventional molasses beet pulp as a part of the concentrate mix for milking cows.

Since considerable CSF is produced in beet growing areas, there was interest in studying further its nutritional value for the feeding of dairy cattle. Of particular interest was the value of dried beet pulp which contained approximately 28% CSF solids. In addition, it was desired to compare this material with plain and molasses dried beet pulp, and to evaluate all three beet pulp preparations with reference to barley.

EXPERIMENTAL PROCEDURE

The study was made in two feeding trials, each with eight first-lactation, grade Holstein cows allotted to four treatments according to the extra-period, Latin-square, change-over design proposed by Lucas (8). The animals had all calved within a period of 25 days and were started on trial at an average of 62 days postcalving. The trials were of 175 days' duration with five 28-day periods, each preceded by a period of seven days to allow cows to adjust to changes in feeding regimes.

Prior to the trials the cows were all handled alike and fed alfalfa hay free-choice, with a high level of ground barley. A rationing plan was established for each cow for the entire trial on the basis of milk production predicted from a ten-day indexing period immediately preceding the initiation of the trials.

A basal ration consisting of 70% alfalfa hay and 30% barley was fed at full and at restricted levels of intake, which accounted for Treatments 1 and 2 in each of the feeding trials. The full-fed basal ration was estimated to meet the maintenance and production requirements of the cows and the restricted basal was fed to furnish 75% of the estimated net energy requirements. With this proportion of alfalfa hay in the ration, protein intake was adequate, even at the restricted level of intake.

The four test feeds were added in turn to the restricted basal ration in amounts estimated to support levels of production comparable to that on the full-fed basal ration. In Trial A, barley and dried beet pulp (BP) were added to the restricted basal in Treatments 3 and 4, respectively; and in Trial B, molasses dried beet pulp (MBP) and concentrated Steffen filtrate beet pulp (CSFBP) were involved.

The addition of the test feeds to the restricted basal resulted in rations which contained approximately 45% concentrates. In turn, the increments of test feeds accounted for about 45% of the concentrate portion and about 20% by weight of the total rations.

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[1] Supported in part by Amalgamated Sugar Company, Ogden, Utah; Holly Sugar Corporation, Colorado Springs, Colorado; Spreckels Sugar Company, San Francisco, California; Utah-Idaho Sugar Company, Salt Lake City, Utah.

[2] Information on CSF furnished by S. L. Stovall, Spreckels Sugar Company.

TABLE 1

Composition of dry matter of feeds

Feed	C.P.	E.E.	C.F.	NFE	Ash
			(%)		
Alfalfa hay	19.9	1.8	27.6	41.9	8.8
Barley	11.2	1.7	6.1	77.8	3.2
D.B.P.	7.9	0.4	22.9	65.2	3.6
M.B.P.	10.3	0.2	17.4	66.1	6.0
C.S.F.B.P.	14.7	0.4	17.7	54.4	12.8

The alfalfa hay was all from a single lot of one cutting from one field. The barley was all from one large procurement and averaged 47 lb per bushel, but varied from 40.7 to 50.1 lb among weights obtained at various times during the trials. The dried beet pulp preparations were all made from a single batch of wet pulp.

Weighed amounts of hay were hand-fed twice daily after milking in individual stanchions, with 2 hr being allowed for eating at each time. The barley and the beet pulps were fed twice daily in the milking barn. A representative sample of each feed was obtained at each feeding and composited for weekly dry matter determinations and for proximate analyses for each period. The proximate analyses of the feedstuffs are listed in Table 1.

The milk produced was weighed at each milking and a representative sample withdrawn and composited for weekly determination of milk fat by the Babcock procedure and of solids-not-fat by the Golding bead method.

A single sample was obtained at random from one day's production of each cow during each period for determination of heat of combustion of the milk solids. From these data the regression of energy value of milk solids on milk fat content was determined for use in calculating the energy production of the cows on the basis of the weekly milk analyses. Results of this procedure were virtually identical with those obtained using the following prediction equation devised by Lofgreen and Otagaki (7): $Y = 4.516 + 0.321X$, Y being the combustion value of milk solids expressed as kcal per gram and X being the milk fat per cent.

The cows were weighed immediately after milking, before being fed hay or allowed water, three days in succession each week during the entire trial. The average daily liveweight change of each cow was calculated for each period from the regression of weight on time.

RESULTS AND DISCUSSION

The pertinent data regarding feed intake, milk production, and liveweight changes in Trials A and B are summarized in Tables 2 and 3, respectively.

The response to treatments in the two trials were comparable. In each case daily milk production was reduced by 5.0 lb when the basal ration was restricted, but there were no significant differences among the full-fed rations. The per cent milk fat was not affected, while solids-not-fat were decreased significantly on the restricted basal rations, as would be expected with the restricted energy intake.

The loss of weight on restricted basal was

TABLE 2

Feed intakes, amount and composition of milk produced and liveweight changes, Trial A

	Restricted basal	Full basal	Restricted basal + barley	Restricted basal + B.P.	$S_{\bar{x}}$
Feed intake, dry matter *(pounds/day)*					
Alfalfa hay	15.8	20.6	15.8	15.8	.....
Barley	6.8	9.1	12.6	6.8	.....
Beet pulp	.....	.....	.....	5.8	.....
Total	22.6	29.7	28.4	28.4	.....
Increment	.....	7.1	5.8	5.8	.....
Milk production *(pounds/day)*	32.0[b]	37.0[a]	36.4[a]	35.9[a]	0.498
Butterfat *(avg %)*	3.50[a]	3.45[a]	3.42[a]	3.43[a]	0.064
Solids-not-fat *(avg %)*	8.42[b]	8.79[a]	8.80[a]	8.75[a]	0.065
Milk energy *(mcal/day)*	9.8	11.6	11.3	11.1	.....
Liveweight change *(pounds/day)*	−0.84[b]	0.10[a]	0.22[a]	0.51[a]	0.154

[a, b] Values with different superscripts are significantly different.

TABLE 3

Feed intakes, amount and composition of milk produced and liveweight changes in Trial B

	Restricted basal	Full basal	Restricted basal + M.B.P.	Restricted basal + CSFBP	$S_{\bar{x}}$
Feed intake, dry matter *(pounds/day)*					
Alfalfa hay	15.0	19.8	15.0	15.0	.....
Barley	6.5	8.6	6.5	6.5	.....
Molasses beet pulp	.....	.....	5.3	.....	.....
C.S.F. beet pulp	.....	.....	.....	5.3	.....
Total	21.5	28.4	26.8	26.8	.....
Increment	.....	6.9	5.3	5.3	.....
Milk production *(pounds/day)*	29.9[b]	34.9[a]	34.1[a]	33.7[a]	0.519
Butterfat *(avg %)*	3.42[a]	3.38[a]	3.29[a]	3.39[a]	0.046
Solids-not-fat *(avg %)*	8.47[b]	8.71[a]	8.62[a, b]	8.71[a]	0.069
Milk energy *(mcal/day)*	9.1	10.7	10.3	10.4	.....
Liveweight change *(pounds/day)*	−0.78[b]	−0.02[a]	0.49[a]	0.30[a]	0.188

[a, b] Values with different superscripts are significantly different.

comparable in both trials. Liveweight gains on the full-fed rations, although rather variable, were not significantly different from one another within trials, and averaged 0.28 and 0.26 lb per day in Trials A and B, respectively.

It is quite clear from these data that the barley and each of the three types of beet pulp, when added to the restricted basal ration, were equally efficient in restoring milk production and liveweight gains. While increments of 5.8 and 5.3 lb of dry matter, respectively, in Trials A and B restored 5.0 lb of milk, the energy content of the milk in Trial B was slightly lower. In Trial A the average difference in daily milk energy between the restricted basal and full-fed rations was 1.5 mcal, which is equivalent to 259 kcal per pound of barley and beet pulp increment. In Trial B the average difference between the restricted and full-fed rations was 1.4 mcal, or 264 kcal per pound of MBP and CSFBP increment, which is essentially of the same magnitude as that observed in the first trial.

These observations are in agreement with other work, both with lactating cows and fattening meat animals. Billings (2) demonstrated that plain and molasses beet pulps were of equal value in milk production rations. Other workers (3, 5) have shown that both types of beet pulp have been comparable to grains in milk production value when fed up to 60% of the concentrate mixture.

Holden (4) and Maynard and co-workers (9) found no effect upon the performance of fattening lambs when one-half of the corn was replaced with either plain or molasses dried beet pulp. Singleton et al. (10) found molasses beet pulp to be worth 97 to 111% of barley when fed at various levels up to 50% of fattening rations for cattle.

Recently, Lofgreen et al. (6) reported that for steers CSF beet pulp had a lower net energy value than molasses beet pulp but, in this case, palatability problems were encountered. In the present study with lactating cows and lower levels of feeding CSF dried beet pulp, there were no problems with palatability.

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DIGESTIBILITY OF ALFALFA HAY AND REED CANARY GRASS HAY MEASURED BY TWO PROCEDURES [1]

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SUMMARY

A feeding trial with four mature, rumen-fistulated cows was conducted using alfalfa and reed canary hay as the sole ration. Digestibility was determined by the total feces collection procedure and by the dacron bag technique.

Results of the fodder analysis plus cellulose, lignin, pentosans, and gross energy showed that the only significant differences in composition between the two hays were in lignin and pentosans.

Reed canary hay proved more digestible than alfalfa in all entities determined except protein, nitrogen-free extract, and lignin. But only the differences of crude fiber and cellulose digestibility were significant. This confirms the results of a previous study with alfalfa and timothy hay (1).

There was no significant differences between individual cows in their ability to digest the two hays. The only significant difference between breeds was in the digestion of fiber; average for the Ayrshire cows was 76.0%, for the Holsteins 70.2% ($P < .05$).

Considerably greater digestibility of crude protein, N-free extract, ether extract, energy, and lignin, and a lesser digestibility of crude fiber with the dacron bag technique, led to the tentative conclusion that the conventional procedure of a total collection digestion trial is the more reliable method for determining the digestibility of forages. A significant inverse relationship between lignin content of hay and its digestibility, and a possible direct relationship between pentosan content and digestibility, suggest that more rapid evaluation of forages may possibly be achieved through research to simplify and shorten present methods for determination of these two constituents.

A second study has been completed on the evaluation of hays by two procedures described in detail in a recent publication (1). The object of the study has been to investigate some of the differences in composition and digestibility between alfalfa and certain grasses when fed as hays to dairy cows. The previous study concerned alfalfa and timothy hay. This year the comparison has been between alfalfa hay and reed canary grass hay (Phalaris arundinacea).

EXPERIMENTAL PROCEDURE

Four mature, farrow, rumen-fistulated cows (two Ayrshires and two Holsteins) have been the subjects. The cows were divided into pairs, one of each breed to a pair, and were fed alfalfa and reed canary grass hays by a repeated switchback system for four 5-wk periods beginning October 16 and ending March 10. The last three weeks of each 5-wk period constituted the data collection period. The alfalfa hay was predominantly third-cutting and was grown on the University farm, and the reed canary hay was mostly second-cutting, raised on heavily fertilized river bottom land on a farm in a neighboring township on plots established and supervised by the Agronomy Department [3] of the experiment station. Hay fed at a daily rate of 2% of the animal's body weight and a salt–dicalcium phosphate mixture constituted the sole ration.

Conventional digestion trials involving total feces collection were conducted during the last

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[3] Grateful acknowledgment is made of the cooperation of Professor M. L. Blaisdell, Farm Superintendent B. L. Hilton, Professor R. A. Southwick, and Dr. W. G. Colby, who furnished the forage for these trials.

TABLE 1

Average composition of hays fed

Kind of hay	Dry matter	Crude protein	Crude fiber	N-free extract	Ether extract	Ash	Sugar	Cellulose	Lignin	Pentosans	Gross energy
	(%)										*(therms per 100 lb)*
Alfalfa	89.6	19.0	29.7	39.7	2.1	9.5	4.2	19.2	9.5	15.3	199.2
Reed canary grass	89.2	21.3	29.1	37.2	2.3	10.1	4.0	20.3	5.6	19.0	198.7

The difference in lignin content is very highly significant: $P < .001$.
The difference in pentosan content is significant at the 5% level.

ten days of each 5-wk period. Inflatable rubber catheters were used to separate urine and feces. Procedures and analytical methods were identical with those described and listed in an earlier report (1).

During the first ten days of each collection period four dacron bags each containing 30 g of the hay being fed to each cow at the time were placed in each cow's rumen and removed after 48 hr. This procedure was described previously (1).

RESULTS AND DISCUSSION

Average composition of the hays is given in Table 1. The only significant differences were in the content of lignin and pentosans. Worthy of mention is the very high protein content of the canary grass, due presumably to liberal application of nitrogen fertilizer. The true protein was determined and found to be 15.8%, or 74.1% of the total.

Values for digestibility of nine different entities in the hays by both procedures are summarized in Table 2. By the conventional procedure, the canary grass hay was more digestible than the alfalfa hay in all entities except protein, nitrogen-free extract, and lignin (the lignin values are highly questionable). Results from the rumen technique (dacron bags) show the same general trend. The whole picture is very similar to the results previously obtained with alfalfa and timothy hays. There is the same striking difference in digestibility of fiber and cellulose in favor of the grass hay, accompanied by somewhat smaller but still very significant differences in digestibility of energy and pentosans.

Statistical analysis of the results shows that there was no significant difference between breeds for any of the entities determined by the rumen digestion (dacron bag) technique, and only one instance of significance in the results from the conventional procedure. This was for fiber in canary grass, of which the Ayrshire cows digested on the average 76.0%, whereas the Holsteins digested 70.2%, a difference significant at the 5% level. Since only two animals of each breed have been used, this statement is not qualified.

Mention was made in an earlier report (1)

TABLE 2

Digestibility of alfalfa and Reed canary grass hay by total collection and dacron bag technique

Entity	Total feces collection procedure			Dacron bag technique		
	Alfalfa	Canary grass	Difference	Alfalfa	Canary grass	Difference
Dry matter	67.5 ± 1.7	68.3 ± 2.0	+ 0.8	73.0 ± 0.5	76.7 ± 0.3	+ 3.7***
Crude protein	77.7 ± 0.9	76.9 ± 1.2	− 0.8	90.6 ± 0.3	89.5 ± 0.7	− 1.1
Crude fiber	55.4 ± 2.1	73.5 ± 1.6	+18.1***	44.9 ± 1.8	67.6 ± 1.2	+22.7***
N-free extract	74.9 ± 1.5	64.7 ± 2.8	−10.2**	79.7 ± 1.1	72.9 ± 0.9	− 6.8***
Ether extract	38.9 ± 3.1	39.8 ± 4.8	+ 0.9	77.9 ± 3.8	81.9 ± 3.0	+ 4.0
Energy	64.4 ± 1.9	64.8 ± 2.6	+ 0.4	70.8 ± 1.2	75.3 ± 1.0	+ 4.5***
Cellulose	57.4 ± 2.7	74.1 ± 1.8	+16.7***	51.4 ± 1.1	67.6 ± 0.9	+16.2***
Lignin	12.2 ± 3.6	6.3 ± 4.1	− 5.9	30.5 ± 3.4	33.5 ± 5.1	− 3.0
Pentosans	64.5 ± 2.1	71.0 ± 1.9	+ 6.5*	61.6 ± 1.3	66.7 ± 1.0	+ 5.1***

* Significant—$P < .05$.
** Highly significant—$P < .01$.
*** Very highly significant—$P < .005$ and in some cases $< .001$.

of the considerable differences in results between the two methods employed for measuring digestibility, and explanations for the differences were suggested. Very similar differences are noted again (see Table 2). Since the conventional (total feces collection) procedure is more truly representative of the entire process of digestion, it appears that results from it may be of more value in practice than those obtained by the rumen digestion technique. This would be especially true of the marked increase in protein digestibility and the very considerable decrease in digestibility of the structural carbohydrates (fiber, cellulose, and pentosans), noted with the dacron bag technique. The drawback with the conventional procedure is, of course, the much greater cost in time and equipment. Such a situation spurs the search for simpler means of correctly evaluating forages.

In this connection some correlation coefficients were calculated between content of certain constituents of the hays and their dry matter digestibility. The only highly significant relationship was between lignin content of hay and its dry matter digestibility ($r = -.54$, df 31, $P < .01$). Unfortunately, the determination of lignin is so time-consuming that it does not lend itself to routine evaluation of large numbers of samples. Sullivan's method (2), which has been employed in our work for the past 2 yr, shows some improvement in this respect over older methods. The inverse relationship between lignin and digestible dry matter when this method was used was of slightly higher magnitude ($r = -.60$, df 15, $P < .01$) than when all our results for the past six seasons were pooled.

There is a suggestion from some of our recent work (1959-1961) that pentosan content of hay may be related to dry matter digestibility. The correlation coefficient for the 1959-1960 lots of hay was $r = 0.86$, (df 7, $P < .01$). For the two seasons combined (1959-1961) it was only $r = 0.34$, (df 15, $P < .05$). Research to simplify and shorten present methods for determination of lignin and pentosans should prove fruitful.

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EFFECTS OF LEVEL OF HERD ENVIRONMENT. I. RELATIONSHIP BETWEEN YIELD AND AGE [1]

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SUMMARY

Milk records in herds producing at high, medium, and low levels were analyzed for biases in adjustments for age at freshening. A highly significant relationship between age of cow and yield remained in the age-corrected records at all levels of environment. The bias appeared to be less marked in the high-environment herds for milk production but not for fat production. However, this apparent change in the yield-age relationship with increasing herd environment was not statistically significant. A method for predicting first-lactation from herd yield at older ages to minimize this bias and, at the same time, to provide a more efficient use of first-lactation records for sire evaluation in small herds is described.

The usefulness of records from all cows for sire evaluation depends in large part on the possibility of removing the intraherd effects of (1) age and (2) selection on level of production. Many studies have been reported on age-correction procedures (3, 6, 7-9, 12, 14), but probably the most important is that of Lush and Shrode (8), which indicates that under practical conditions, the relationship between yield and age cannot be estimated without bias. Culling removes the lower producers at young ages, and production figures available for estimating age effects are higher than they should be for the older animals. If only repeated records are used, the production at young ages is higher than it should be in relation to subsequent production. Hickman and Henderson (6) found a negative correlation between the level of first-lactation production and increase from first to second lactation, using records adjusted for age by the mature-equivalent method. Searle (13), using actual records, found a positive correlation for the same variables. This reversal of results can be explained by the biases introduced with the process of applying age-correction, because records in high-producing herds are over-corrected and those in low-producing herds are under-corrected. This produces a relationship between yield and age for corrected records which is reverse in sign to that for uncorrected records. These relationships are indications of an interaction between level of herd production and relationship between yield and age.

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The division of data by levels of herd production introduces statistical problems, if the data to be analyzed are used for the establishment of levels. The objective should be to sort the herds on some criterion referring to management or feeding levels. Various such factors were studied by Bayley and Heizer (1, 2). Of the 20 management influences they studied, 12 had no prediction value for level of herd production. Level of feeding was significantly correlated with herd yield, but the correlations were small for all influences considered. It is known that the average genetic differences among herds is small (11). Apparently, the cause of difference in herd production is not easily identified and may be explained largely by herdmanship which cannot be measured objectively.

Division of herds by level of environment. Canadian Record of Performance (R.O.P.) first-lactation age-corrected milk records started during 1958 were used to calculate averages of production for all herds. These were multiplied by the inverse of the intraherd variance, to stabilize the variance for differences in herd size. The adjusted herd averages were ranked in order of magnitude and the rank order was recorded on the herd summary cards. These cards were then merged by herd number with all records reported in the previous year (1957), and the rank order was transposed to these records, making possible division into equally sized groups of high, medium, and low environmental levels. Thus, the first-lactation records used to establish herd level were by cows that could not appear in the data to be analyzed and, therefore, the two sets of records are independent, except for whatever is common in the

TABLE 1

Performance in three levels of environment

	High	Medium	Low	Over-all
Age-corrected milk production (B.C.A.)	104.0	100.0	96.0	101.2
Age-corrected fat production (B.C.A.)	105.0	100.0	96.0	101.8
Average age at freshening (10 days)	168.0	168.0	170.0	168.8
Variance of milk production	409.5	392.5	404.6	414.6
Variance of fat production	468.6	453.9	463.6	462.3
Variance of age (100 days)	6,724.5	6,290.5	6,388.3	6,513.9
Number of records	9,195	6,300	4,572	20,063

two periods of time that contributes to difference in herd level of production.

Averages and variances of the data studied in terms of age-corrected Breed Class Average Indexes (10) for milk and fat production in the three environments are given in Table 1. This age-correction procedure expresses corrected records as a coefficient which ideally averages 100. These coefficients are obtained by dividing actual yields by production standards established nationally for all ages at calving. It is obvious from Table 1 that the method applied was successful in bringing about three different production levels, although the records analyzed were not used to establish the differences. The levels of age-corrected production in the directly sorted first-lactation milk records were 102.3, 95.6, and 90.9, or a range of 11.4 units, in contrast to a range of 8.0 units in the previous year's production in the same herds.

There is a regression towards the population mean in herd level at 1 yr, with sorting on herd level in an adjacent year. As would be expected, the differences among the levels in the data studied are not as large as was accomplished by directly sorting production from the same herds in the succeeding year into groups of high, medium, and low level. They are considered to be as large as might occur among progeny of bulls used over several herds in artificial insemination and, therefore, for practical purposes would relate to extreme biases in bull tests. The variances in Table 2 show a slightly smaller variability of production and age for the medium environment than for the high and low groups. This may be expected because, in dividing a normally distributed variable into equal-numbered, high-medium, and low groups, the medium group has the smallest range of variation. However, overlapping occurred among the high, medium, and low groups studied because they were not directly sorted but were, instead, grouped by ranking of performance in a succeeding year of performance in the same herds. Therefore, the differences among the variances would be less without such overlapping and the variances are more realistic than when no overlapping exists, as in the case with the direct-sort method. The present method does not completely solve the problem of the method of sorting affecting the results, but it is probably the most suitable method available for using herd data to char-

TABLE 2

Tests of significance for heterogeneity of individual regressions of age-corrected milk and fat yield on age at freshening for three levels of environment

	Source of variance	Degree of freedom	Mean squares		F	
			Fat	Milk	Fat	Milk
1	Total	20,062	477	415		
2	Reduction from estimating three regressions	6	3,454	6,675		
3	Residual or error variance	20,056	476	412		
4	Reduction from estimating one regression	2	9,926	18,717	20.85**	45.43**
5	Residual (1-4)	20,060	476	413		
6	Difference between the individual and single regressions	4	219	653	0.46	1.58

** Highly significant mean square.

acterize herds by level of management and nutrition.

Regression of yield on age. Since all records available had already been adjusted for age, these age-corrected records, rather than the actual records, were analyzed. This made possible examining biases in the present ace-correction method, as well as studying the level of environment effects. The age-correction method used is outlined by the Canada Department of Agriculture (10) and, of course, was applied in a similar manner regardless of level of production.

A polynominal equation was fitted separately to data of the three levels of environment and the heterogeneity of the equations tested by analysis of variance. The regression equation was of the following form

$$Y_i = \mu + b_1 a_i + b_2 a^2{}_i$$

where Y_i is an age-corrected record produced at age a_i; μ is common to all records and simply positions the equation in the normal range of production; b_1 is the linear component of the relationship between yield and age, and b_2 is the curvilinear component. These were estimated by least-squares analysis and the resulting three curves for milk yield and milk fat yield in the different environments are shown in Figures 1 and 2, respectively. The pooled within-level variances and covariances for yield and age were used to establish a fourth regression for the whole population.

At face value, it appears that for milk yield the differences between age-corrected production at young and old ages is smaller in the high environment than in both the medium and low environments, but for fat yield the three curves are remarkably parallel. The lack of

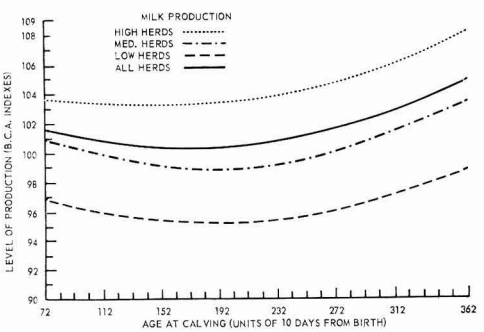

FIG. 1. Relationship between age-corrected milk yield and age at freshening in three levels of environment.

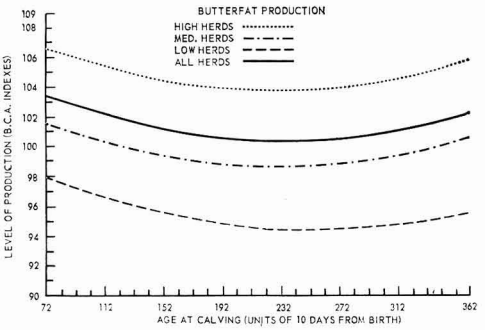

FIG. 2. Relationship between age-corrected milk fat yield and age at freshening in three levels of environment.

parallelism for milk yield conforms with evidence by Hickman and Henderson (6), that the difference between age-corrected first- and second-lactation production decreases with increasing level of production. The importance of the interaction is difficult to assess in practical terms, but an analysis of variance was conducted to determine if the differences in the shapes of the curves should be regarded as real or due to sampling error. Table 2 shows a summary of the covariance analysis and an insignificant heterogeneity of individual regressions for both milk and fat yield. The F values of 1.58 for milk and 0.46 for milk fat are decidedly insignificant. This particular test may not be the most suitable for detecting the only departure of parallelism which occurs under 4 yr of age. For the whole range of ages, the error mean square for milk yield of 413 is reduced to 412 by using a single regression vs. an individual regression for each environment. For fat yield the error mean square of 476 is the same after correction for either the single or triple regression. Therefore, there should be no gain in statistical efficiency expected from applying separate regressions for environments differing by the magnitude indicated in Table 1.

It is obvious from the significant single regression that the over-all bias due to differences in age at freshening in these age-corrected records is far more important than the heterogeneity of yield-age relationships in the present differences of environment. The highly significant reduction in sums of squares, due to fitting the average within regression, may be considered of minor practical importance; it indicates clearly that age-correction procedures used cannot be expected to eliminate completely the effect of age.

DISCUSSION

The general bias of the age-correction procedure used on these data overestimates the production of very young and very old animals or progeny groups of below or above average age. Since the proportion of old animals is very small, it is bias at the young ages which is most important. Statistical adjustment of yield data for differences in age requires the application of factors which cannot be unbiased over extended periods of time or in every feeding and management circumstances. As an alternative to using age-correction procedures, there is a temptation to use only first-lactation record for estimating breeding value of bulls and for studies on breeding records. The main danger here lies in the small number of first-lactation animals per herd. The herd size of only first-lactation animals would be seriously low for sire evaluation if, before eliminating all but first-lactation records, the herd size is in the neighborhood of 20. Despite using only first-lactation records, it is evident from Figures 1 and 2 that if age still varies from 2 to 3 yr among the first-lactation animals a general advantage would remain for age-corrected records from the young first-lactation animals. This would be particularly so for the young animals in medium and low environments, if the interaction indicated in this and a previous study using New York State data is actually real. First-lactation data are now being analyzed to determine whether selection differentials calculated in one environment are the same in other environments.

Probably the most advanced attempts at age-correction are those routinely applied in New Zealand (3) and in Sweden (4). Because of the indication of bias in age-corrected records, these methods of calculating expectancy of production for each herd gain importance. Such an expectancy could be calculated from a regression of first-lactation production on the herd average of all but first-lactation production. A prediction of this sort would be related to herd production and, therefore, would give an expected first-lactation level according to the yield-age relationship appropriate for different herd levels. In addition, the prediction method provides a comparison where all first-lactation animals in a herd have the same sire; whereas, the present contemporary comparison procedure applied regularly to these data uses only first-lactation records and is unable to make use of these single-sire, first-lactation herd records.

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SYNTHESIS OF OPTIMUM FORAGE HANDLING SYSTEMS FOR A ONE-MAN DAIRY FARM [1]

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SUMMARY

Linear programming techniques were used to select the forage-handling practices that would yield maximum family income from a dairy farm operation under corn belt conditions and to consider their effect on the entire farm organization. A high level of management and dairy production was assumed. Most phases of the dairy farm organization that could affect or be affected by the forage-handling practices were considered. Optimum farm organizations were developed restricted by the family labor available and selected amounts of capital. The total acreage in the farm was found to be primarily a function of the available capital. Maximum family income was realized when the number of cows was at the maximum permitted by the labor available on the farm. Generally, cows contributed more to net farm income than production of grain or hay for sale. A ration with a high proportion of grain was found to provide the most economical source of nutrients on farms with large areas of land. About the same number of cows was kept on the small as on the large farms but the small farm operators used more forage in the dairy ration. A rotational grazing program was found to be economically desirable. Further improvement in income could be obtained by adding first a hay conditioner and second a mow dryer. With a good yield of high-quality hay, silage was economically feasible only if a silo was already on the farm.

Few dairymen know which of the available forage-handling systems and practices should be used to build the most efficient farm organization for a particular situation. Until recently, only a few alternatives needed consideration in making a sound selection. Today, a farm operator is confronted with many alternative forage handling techniques such as: hay conditioning, mow drying, baling, chopping, pelleting, green-chop pasture, rotational grazing, etc. Less waste, greater ease of operation, lower cost, and higher feeding values have been claimed as advantages for each new practice and technique.

Narrowing profit margins requires a dairy farm operator to strive for and use the most efficient farm organization and production practices possible. Forage production, harvest, storage, and feeding methods are a very important segment of the total dairy farm operation. New technologies are being developed each year. The adoption of some of these new technologies requires major revisions in the building, equipment, and labor needs. Often changes in production methods call for major capital investments that can be recovered only gradually, over a period of years. Mistakes are costly and may tie up capital in unprofitable facilities and equipment that may be rendered obsolete by more advanced technology. Both the farm operator and the research worker find it difficult to develop and maintain the optimum farm organization when forage-handling systems and practices are constantly changing. To maximize income, a dairy farm operator needs to know what his optimum farm organization is and to keep his farm developing in a desirable manner toward that optimum organization. This requires that forage-handling systems and practices that will best meet the needs of his farm be identified and that his farm organization be modified, from time to time, to benefit from these new technologies as the returns from using them exceed the costs.

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This study is primarily concerned with the income-producing ability of the total farm organization as affected by selected forage-handling systems and feeding programs. It is not the intent of this study to dwell on the problems of the hay-concentrate ratios or on the comparative advantage of one forage over another. Despite the extensive research involving forage-concentrate combinations (6, 9, 15), the evaluations of forages and forage production (2, 7, 8), and the economies of least-cost rations (5), little attention has been given to how these relationships fit into a total farm organization.

Heady et al. (3) and Westcott (14), in studying optimum farm organizations and the adjustments due to price change and the adoption of alternative practices, did observe changes in the crop rotation, feeding programs, and herd size. Westcott found that buying hay and pasturing meadows was more profitable than restricting the size of the dairy herd to the forage-producing capacity of the farm.

The effect of the forage/grain substitution rate on milk production is a matter of considerable controversy. Several of the studies mentioned show the effects of various forage/grain ratios on milk production and the profitability of high concentrate ratios when fed to high-producing cows. Putnam and Loosli (9) reported finding no significant difference in milk production when selected forage/grain ratios were fed. Available data from studies reporting differences in milk production for variable forage/grain ratios were not adequate for use in this study.

Level of milk production per cow, size of farm operation, land use capability, labor available, capital available, and many other factors affect the desirability of any given production system or practice. In the past, numerous people have looked on the dairy herd primarily as a heavy consumer of forage crops. However, in some areas many changes are taking place. In Ohio, for example, the dairy enterprise is prominent in the corn belt section of the state. Under corn belt conditions the most desirable dairy farm organization and the production of feed nutrients may be quite different from that in other areas. The goal of this study was to develop the dairy farm organization, with emphasis on forage-handling systems and practices that would maximize net income.

EXPERIMENTAL PROCEDURE

Production coefficients were obtained from studies of existing farm operations, using a variety of forage-handling practices (see Table 1). A synthesized farm organization was developed using these coefficients for the purpose of measuring the effects of selected forage-handling systems and practices. This approach

TABLE 1

Production coefficients and crop cost data used in the linear program matrix, Western Ohio, dairy farm operation, 1960

Crop	Unit	Yield [a]	Price [b]	Annual costs per acre [c]	Man hours per acre
Corn	Bu	80	$ 1.10	$55.00	7.0
Wheat	Bu	30	1.75	39.79	6.1
Oats	Bu	60	.65	43.48	6.4
Hay, 1 cutting [d]	Ton	2.0	20.00	18.65	4.2
Hay, 2 cutting [d]	Ton	3.0	20.00	27.05	6.7
Hay, 3 cutting [d]	Ton	3.8	20.00	34.58	8.9
Grass silage (1st cut only)	Ton	6.0	.....	27.25	7.9
Corn silage	Ton	16	.....	84.13	10.0
Pasture (Conv.)	AUGD [e]	167	.....	7.09	1.6
Pasture (Rotation-grazed)	AUGD [e]	208	.....	10.88	8.2
Pasture (Green-chopped)	AUGD [e]	233	.....	21.52	4.7

[a] Crop yields were 30% above county average.

[b] Selling price: buying price was 15% higher than selling price.

[c] Annual costs as presented do not include land or labor costs.

[d] Annual costs for harvesting hay with a field chopper. The cost per ton of harvesting hay with a baler was $1.69 higher, a field conditioner $1.08 higher, and a mow dryer $2.02 higher than for harvesting hay with a field chopper. A ton of field-conditioned hay was equivalent to 1.1 T and a ton of conditioned-mow dryed hay was equivalent to 1.3 T of field-cured hay.

[e] Animal unit grazing day.

Source: Blosser (1) and studies related to the Ohio Agricultural Experiment Station Study, An Analysis and Comparison of the Economics of Forage Management Systems.

enabled the researcher to eliminate the effect of such factors as variation in the amount of capital available, existing buildings, facilities and institutions (habit or precedent), diversification, and varied levels of production efficiency. The elimination of these kinds of variations and restrictions permitted an optimum farm organization to be considered and one that would be most desirable from its ability to produce family income. This method permitted a farm to be organized by starting with bare land and adding the most efficient improvements and facilities presently available.

Land use capability, crop yields, and land values used in this study were based on those found in eight west-central Ohio corn belt counties.[3] In this area dairying accounted for 21% of cash farm receipts in 1961 and was one of the more important livestock enterprises.

Optimum farm organizations were developed for selected production possibilities and resources available by the use of linear programing. The optimum organization was defined as the one yielding the maximum farm family income. More than 80 different possible farm production activities and price relationships were considered, using the IBM 704 computer.

Linear programing is basically a system of comparing many repetitive budgets. It embodies solving a series of simultaneous equations to allocate limited resources among given activities or uses. The most profitable activities are used to the maximum extent permitted by the most limiting factor needed for production. After the limiting factors are fully consumed, substitution of other available inputs will be made until the most profitable resource allocation is achieved. The allocation of each unit of a resource used is made by determining the production activity that yields the highest possible net return.

Forage-handling systems were developed with a given availability of labor and capital. Starting with land (without buildings or fence improvement) the entire farm organization was developed until the limiting factors of labor and capital were exhausted. Each production activity included the resources required, such as labor, capital, buildings, fence, and equipment for the operation of the farm.

Although primary emphasis was on the forage program, other phases of the farm organizations were considered in developing a workable farm operation. The following assumptions and restrictions were imposed:

(a) The farm operator was assumed willing to use the forage system that would yield maximum family income.

(b) The ability of the operator was reflected in the production coefficients used. The coefficients were typical for the upper one-sixth of the western Ohio (corn belt) dairymen.

(c) Productive family and operator labor available was limited to 3,600 man-hours each year (2,600 operator, 600 family, 400 hired). Hired labor was available only from June 1 to September 1.

(d) Land was valued at $180 per acre for commercial farm land without buildings or fence improvements. Improvements would add $125-$175 per acre. Buildings and improvements were excluded to eliminate the prejudice existing facilities would have on the optimum organization.

(e) Seventy-seven per cent of the land area was cropped and 10% was in permanent pasture. The remaining 13% was used for farmstead, roads, lanes, and woods.

(f) Rotational limits were established based on agronomic recommendations which set the lower limit for meadow at 20% (C-C-C-SG-M) and an upper limit of 60% (C-SG-M-M-M).

(g) The purchase of top-quality hay (equivalent to conditioned–mow dried hay) was limited to 20 T per year. Undoubtedly, more hay could be purchased, but it was considered unrealistic to assume that more than 20 T of the quality of hay needed for high-producing cows would be available.

(h) One ton of conditioned hay was considered equivalent to 1.1 T of field-cured hay, and conditioned–mow dried hay was considered equivalent to 1.3 T of field-cured hay (11, 13).

(i) At least 75 T of silage had to be produced before it was economically feasible to provide silage storage facilities.

(j) Wheat was planted to the extent of the allotment and sold as a cash crop. Oats were planted to complete the small grain in the rotation.

(k) Dairying was the only livestock enterprise considered. Grain and hay could be purchased or sold.

(l) Each dairy unit consisted of one cow, producing 12,500 lb of 3.5% milk plus

[3] The counties were: Auglaize, Champaign, Clark, Darke, Logan, Mercer, Miami, and Shelby.

replacements. A cow would remain in the milking herd 4 yr. A parlor-pen system, bulk-handling, Grade A fluid milk was assumed. The blend price, minus handling, for milk sold in the Dayton-Springfield market was used.

(m) Daily feed intake was assumed to be 26.8 lb of total digestible nutrients per cow and replacement. (This is 115% of Morrison's feeding standards.) The ration was determined within the following limits: a) nutritive requirement, b) stomach capacity (25 lb hay, 75 lb silage, 30 lb grain, or 250 lb green chop per day), c) a minimum of 6-8 lb of concentrates per cow per day, d) maximum grain ration is 102 bu of corn, minimum grain ration is 65 bu of corn; within these limits grain could substitute at a constant rate (based on TDN) for forage (9); e) 400 lb protein supplement per cow annually; and f) a minimum of 6-8 lb of dry matter from roughage per cow per day.

RESULTS AND DISCUSSION

Three levels of available capital were considered: $120,000, $95,000, and $85,000 (See Table 2). Capital included money needed to acquire and operate the entire farm business for 1 yr (see appendix).

$120,000 of capital. Available labor was found to be the only limiting factor at the $120,000 level. As long as the return to capital exceeded the interest rate (6%), additional capital was employed until all of the available labor (3,600 hr) was used. The resulting farm organization included 204 acres of land and 35 cows in milk. A considerable amount of grain was produced and sold on this farm. The grain sold included all of the wheat that could be produced under the allotment, 4,938 bu of corn and 294 bu of oats. Family income received was $7,527 annually. The optimum ration at this level of available capital included 82 bu of corn per cow and replacement annually. Hay was fed as the only harvested forage and conventional grazing of pasture was selected.

$95,000 of capital. At the $95,000 level, capital was the limiting factor. The farm size was reduced to 133 acres and 37 cows in milk were kept. Family income earned was $5,977, or $1,550 less than at the $120,000 capital level and 3,384 hr of labor were productively employed. Three bushels less corn but more forage in the form of hay and silage were fed per cow and cropland pasture was rotationally grazed.

$85,000 of capital. When available capital was restricted to $85,000 the acreage in the farm was reduced further to 104 acres, and 33 cows in milk were maintained. Three thousand eighteen hours of labor were productively employed and $5,331 of family income was earned annually. At this level of capital, 65 bu of corn were fed per cow but more hay, silage,

TABLE 2

Optimum forage system and herd size for a one-man family dairy operation for selected levels of capital available, Western Ohio, 1960

Item	Unit	Capital [a]		
		$120,000	$95,000	$85,000
Cows in milk	Cow	35	37	33
Size of farm	Acre	204	133	104
Basic crop rotation	.....	C-C-C-SG-M	C-C-SG-M-M	C-SG-M-M-M
Corn per cow	Bu	82	79	65
Hay per cow [b]	Ton	3.2	2.7	2.0
Pasture day per cow [c]	AUGD	142	160	180
Corn silage per cow [d]	Ton	....	2.1	3.8
Supplement per cow	Pounds	400	400	400
Straw bought	Ton	14	28	25
Corn sold	Bu	4,938	....	....
Oats sold	Bu	294	192	170
Labor used	Hour	3,600 [e]	3,384	3,018
Family labor and management income	Dollar	$7,527.02	$5,977.58	$5,331.75

[a] Total capital outlay needed to implement system at new prices.
[b] Conditioned and mow-dried.
[c] Pasture conventionally grazed with $120,000 of capital and rotationally grazed at other levels of capital available.
[d] Stored in bunker silos.
[e] Total hours of labor available.

and pasture were fed. The pasture was rotation-grazed.

The number of cows kept in the herd was affected both by the amount of labor and by capital available. Generally, it was most profitable to maintain as many cows as could be financed and cared for during periods of critical labor demand. At both the $85,000 and $95,000 levels, capital availability rather than labor, determined cow numbers. More labor was available than could be used productively and was not the most limiting factor at these levels of available capital. Cow numbers were increased from 33 head at the $85,000 capital level to 37 head at the $95,000 level. At the $95,000 level 216 hours of available labor still remained unemployed on the farm and could have been used to increase family income if more capital had been available.

Cows kept for milk were reduced to 35 head at the $120,000 level of available capital. All of the available labor (3,600 hr) was employed at this level, but cow numbers were reduced by two head and a cash grain enterprise was added to the farm organization. More cows could have been financed. However, this would have utilized all of the available labor with something less than $120,000 of capital and would have yielded less family income. When labor rather than capital became the limiting resource, capital was combined with labor as long as the earnings on the added capital used exceeded its market cost of 6%.

The maximum family income was earned at the $120,000 level of capital availability when a cash grain enterprise was combined with the dairy under corn belt conditions.

High-producing cows were indeed a worthy competitor for the cash grain enterprise. This was demonstrated by the slight reduction in cow numbers that took place as the available capital decreased. If additional labor could have been obtained during the periods of critical labor demand, it would have been economically desirable to have increased cow numbers. Although a production activity can be handled easily throughout the entire year except for one period it is, in fact, this critical period that governs the total amount of production that can be carried on.

Farm acreage was primarily a function of available capital. The rotation and, consequently, the dairy ration became a function of the relative availability of labor and capital. A rotation of C-C-C-SG-M was used at the $120,000 capital level and a rotation of C-SG-M-M-M at the $85,000 capital level. More nutrients could be produced in the form of grain than in the form of forage with the relatively limited labor available on the large well-financed farm. A small farm operator had more labor available relative to capital and land, so more of the available labor was used for forage production (a heavy labor consuming enterprise) than for grain on a limited acreage. A heavy grain rotation would have employed fewer hours of labor on the small farm than the heavy forage rotation. Conversely, the available labor on a large farm could not have handled the work load of a heavy forage rotation.

Comparison of forage systems. Six alternative forage systems were compared at the $95,000 capital level, to determine the effect that various forage systems would have on herd size, farm size, and family income. Farm size, number of cows in the herd, and family income were similar for all six systems.

Chopping hay was selected by the computer in preference to baling, when no restrictions were put on the harvesting system. Yields were identical in each case, but baling costs and labor requirements were slightly higher than for chopping.

The improved quality of hay from the use of the hay conditioner and mow dryer more than offset the added costs. Income was increased by the addition of these practices. Another very real advantage of this equipment is that risk of weather damage is reduced.

Additional hay was purchased in three of the forage systems considered (see Table 3). It was assumed that hay of comparable quality to that made on the farm could be purchased. Hay prices were varied with method of harvesting as follows: field-cured, $23 per ton; conditioned hay, $25.50; and conditioned and dried hay, $30. Hay would not be profitable to purchase at a cost above $25.50 per ton.

Except when the farm size was reduced to 110 acres, and the possibility of field grazing was withdrawn, rotational grazing of pasture was selected by the computer for each of these six forage systems.

The improved quality of hay obtained by using a hay conditioner and mow dryer more than offset the added cost. It was found that a higher farm income could be obtained when high-quality hay was produced than when silage was produced and fed. Based on these findings, it would be difficult to recommend building a silo on a one-man farm. When a silo was assumed to be on the farm, corn and not grass legume silage was selected. Corn silage

TABLE 3

Optimum organization of a one-man dairy operation under selected forage systems, Western Ohio, 1960

(With $95,000 of capital available)

Item	Unit	Field-cured, baled hay	Conditioned baled hay	Conditioned, mow-dried, baled hay	Silage and baled hay [a]	Silage and chopped hay [b]	Dry lot system [c]
Cows in milk	Cow	37	38	38	39	37	39
Size of farm	Acre	135	131	130	130	133	110 [d]
Corn [e]	**Bu**	**90**	**91**	**89**	**74**	**80**	**93**
Hay [e]	**Ton**	**2.4**	**2.4**	**2.4**	**1.9**	**2.2**	**2.5**
Pasture [e, f]	AUGD	160	157	163	157	152	148
Corn silage [e]	Ton	....	....	....	3.7	2.1	....
Hay purchased	Ton	20	16	....	20	....	....
Straw purchased	Ton	28	29	29	30	28	34
Corn purchased	Bu	....	....	....	....	....	1,295
Corn sold	Bu	274	....	....	....	....	....
Oats sold	Bu	193	189	188	188	192	158
Labor used	Hour	3,397	3,415	3,402	3,521	3,384	3,391
Family labor and management income		$5,995.53	$6,048.02	$6,334.13	$6,051.30	$5,997.56	$5,747.10

[a] Hay not conditioned or mow-dried.

[b] Hay was conditioned and mow-dried.

[c] Included green-chopped pasture, conditioned-chopped, and mow-dried hay (no field grazing).

[d] Limited to 110 acres.

[e] Fed per cow and replacement annually.

[f] Pasture rotationally grazed.

Note: The basic crop rotation was C-C-SG-M-M, 400 lb of supplement were fed per cow and replacement annually under all forage programs.

was selected over grass primarily because of the relative availability of labor in September compared to labor available and needed for critical production in June. Although the cost of producing, harvesting, storing, and feeding nutrients in the form of silage was higher than for hay, the production of silage was more profitable than purchasing feeds. In a brief investigation of two-man farms it was found to be more profitable to lower hay feeding and increase the feeding of silage. This was due to the greater availability of labor.

Differences in family income for the six forage handling systems selected were small. This indicates that the other organizational factors had more effect on incomes than the choice of forage-handling plans. Ease of handling and other considerations associated with a system may be more important to a farmer than the small difference in family income.

The results do offer some guides and lead to some conclusions with respect to the effect availability of capital, size of farm, type of crop production, forage-handling methods, and the supply of labor available have on income. Farms with limited land areas and capital maximized income by marketing most of their available family labor by producing and feeding a heavy forage moderate grain ration. When more capital was available it was found profitable to produce and feed more of the needed nutrients as grain and less as forage than on corn belt farms with limited capital. A large, well-financed farm could maximize income by maintaining a fair-sized dairy herd (35 cows) with a cash grain enterprise.

Some forage-handling practices were found to be more profitable than others. Supported by this, and based on other studies, returns from pasture improvement and use of controlled grazing were economically desirable. The next most desirable forage-handling practices were found in the hay-harvesting operation. Either a hay conditioner or mow dryer was profitable. Improved hay quality resulting from the use of a hay conditioner was considerably greater than its added cost, particularly for the first cutting of hay. The use of a mow dryer was found profitable but offered a smaller advantage than the use of a hay conditioner.

Silage was one of the last alternatives considered under corn belt conditions and was selected on small farms only after the other forage-handling practices had been incorporated

into the farm organization. On a large, well-financed farm a silo could not be recommended as the needed nutrients could be produced at a lower cost in other ways. When a silo was already on the farm, it was used and was filled with corn silage.

APPENDIX

TYPE OF CAPITAL NEEDED AT THE $95,000 LEVEL

The total capital required was divided into $45,000 for land and buildings, $22,000 for machinery and equipment (new prices), $18,000 for dairy animals, and $10,000 operating capital. Assuming that 30-35% equity is needed in land and buildings and 60% for chattels, $45,000 to $50,000 could be borrowed.

With a dairy operation, receipts are stable and furnish a constant flow of income. Thus, only about one-fourth, or $2,500 of the total $10,000 operating capital needed annually, would be required at any one time during the year.

This means that a minimum of $40,000 of equity capital would be needed to establish and operate this farm.

Equity in land and buildings	$13,500
Equity in machinery	13,000
Equity in livestock	11,000
Operating capital	10,000
Total equity	$47,500
Borrowed capital	$47,500
Total capital needed, plus living expense	$95,000

If used or partially depreciated machinery were included or custom operators were employed, machinery investment might be reduced considerably.

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INFLUX OF SODIUM THIOCYANATE INTO CEREBROSPINAL FLUID IN NORMAL AND VITAMIN A DEFICIENT CALVES

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SUMMARY

The influx of thiocyanate ion (SCN) from the blood to the cerebrospinal fluid (CSF) was studied in normal and vitamin A deficient Holstein calves to provide information on the mechanism whereby the CSF pressure is increased in vitamin A deficiency. Mean CSF pressures in the normal group during the experiment were 97 mm, whereas the deficient animals exhibited mean pressures of 173 mm. SCN concentrations were determined in CSF and blood samples taken hourly during the first 9 hr and at 24, 48, 72, and 96 hr after injecting NaSCN intravenously. While the SCN concentrations in CSF and serum were slightly higher in the deficient calves, CSF thiocyanate values expressed as per cent of the serum level were very similar in both normal and deficient groups. The kinetics of penetration indicated that SCN moves from the blood stream to the CSF at rates quantitatively similar in normal and vitamin A deficient calves.

One of the first measurable effects of vitamin A deficiency is an increase in cerebrospinal fluid (CSF) pressure. This was first demonstrated in calves by Moore and Sykes (12) and has since been confirmed by numerous workers in a variety of species (9, 15, 19). The average CSF pressure for calves was found to be about 105 mm of saline, and all values ranged between 80 and 150 mm (16). With progressive avitaminosis the CSF pressure increased and pressures of 150-300 mm were measured.

The mechanism whereby the CSF pressure is increased in vitamin A deficiency is not known. Possible circumstances that could explain these differences include variations in (a) the anatomical space which contains the CSF, or (b) the biochemical constituents in the fluid, or (c) formation and absorption of the CSF. Bone growth with consequent constriction of the spinal canal has been considered an unlikely explanation of the increased pressure (11). Changes in the concentration of blood and CSF constituents could produce the increased pressure, due to alteration in osmotic relationships. Several workers have examined this aspect and no abnormalities in osmotic pressure measurements or in constituents sufficient to account for the pressure differences have been detected (7, 8, 10, 13).

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Many recent studies have demonstrated that the CSF is a secretion of the choroid plexus and ventricles of the central nervous system (5, 14). Constancy of the CSF pressure requires maintenance of a balance between the formation and removal of the fluid. Overproduction or underabsorption, therefore, could cause a greater volume of fluid in the relatively restricted subarachnoid space and ventricles, with a resultant increase in CSF pressure. Studies of the constituents of the CSF in normal and vitamin A deficient animals have not yielded information indicative of differences in the rate of formation of the CSF. These investigations were carried out under relatively static conditions, i.e., samples of fluid were removed and the concentrations of some normal substances present in the blood and CSF were determined (7, 8, 10, 13).

Experiments have been conducted in which the kinetics of penetration of a foreign substance from the blood into the cerebrospinal fluid has been examined. Thiocyanate (SCN) has been widely used as a marker and its volume of distribution (thiocyanate space) in fluids and tissues determined. The ready penetration of SCN into extracellular spaces, its ease of determination, and its relatively slow excretion favor its use for this purpose. The steady-state distribution of SCN in CSF has been found to be markedly different, however, from that of other extracellular constituents. Davson (3) and Wallace and Brodie (17) injected NaSCN into the blood of rabbits and

dogs, and compared the subsequent distribution in the blood and CSF.

To provide information concerning the rate of formation of the fluid, a study of the dynamics of the influx of a substance into the CSF from the blood was undertaken in this laboratory. According to modern views of CSF formation (4), a substance maintained at a high concentration in the blood will appear in the CSF by one or more of three main processes: (a) passage into the secretory cells of the choroid plexuses from the vascular bed and thence into the fluid formed, (b) passage by direct diffusion across the walls of the ventricles and subarachnoid spaces, and (c) passage between the secretory cells through intercellular holes. This paper presents a study of the influx of SCN into CSF in normal and vitamin A deficient calves.

MATERIALS AND METHODS

Two separate groups of Holstein bull calves were used in these studies:

Group 1. Eight Holstein bull calves 60 days of age were subjected to a 90-day depletion period to decrease body reserves of vitamin A and carotene. The vitamin A depletion ration consisted of timothy hay, low vitamin A grain, and skimmilk. The feed constituents and the blood serum of the calves were analyzed for vitamin A and carotene. When vitamin A values decreased to 5.0 μg/100 ml serum, the animals were considered to be depleted. At this point, the calves were divided into a normal control group and a vitamin A deficient group. The normal control group ration was thereafter supplemented with alfalfa leaf meal, so that the calves received 45 μg of carotene per pound body weight per day. The ration of the vitamin A deficient group was also supplemented with alfalfa leaf meal, but at a level of 15 μg carotene per pound body weight per day. Both groups received supplemental sources of vitamin D (12,500 USP units per week) and vitamin E (300 mg D-α-tocopherol acetate per week).

Sampling of CSF was found to be more difficult in the normal control than in the deficient group and resulted in loss of some of the animals before all of the trials could be completed. Because of this, eight Holstein heifers raised on diets not designed at any time to deplete their body reserves of vitamin A were also used as normal animals. Their average age at the time of use was six and one-half months. The results of the SCN distribution in these normal controls were grouped with those of the vitamin A controls.

Group 2. Six Holstein bull calves 60 days of age were subjected to a depletion period, and a similar group of six was retained as controls. When the serum vitamin A values of the deficient group were at a level of 5.0 μg/100 ml serum, both groups were supplemented with alfalfa leaf meal at a rate of 10 μg carotene per pound body weight per day for the deficient, and 75 μg carotene per pound body weight per day for the control, group.

To determine the rate of entrance from the blood to the CSF, 300 ml of a solution containing 10 g NaSCN per 100 lb body weight were injected into the jugular vein. Due to its relatively slow excretion rate, a single injection of SCN results in a fairly constant level of SCN in the blood for several hours; continuous infusion is, therefore, not necessary to maintain a steady plasma SCN concentration. CSF samples were withdrawn by cisternal puncture according to the method of Sykes and Moore (16). It was found advantageous to leave the 16-gauge spinal needles, mounted with adjustable needle stops, in place during the course of a trial. This avoided repeated punctures, with attendant injury and trauma. Between sampling intervals, a Teflon or metal plug was inserted into the hub of the spinal needle to prevent loss of fluid.

For the first 9 hr after the injection of NaSCN, hourly samples of 4-5 ml CSF were withdrawn, using a hypodermic syringe. Additional samples were taken at 24, 48, 72, and 96 hr post-injection. At the same time intervals, samples of blood were withdrawn from the jugular on the side opposite to that of the SCN infusion. CSF pressures, when obtained, were determined by measuring the height above the cisterna magna to which the fluid rose in a capillary tube attached to the needle (needle length + height of fluid in glass capillary tube).

Carotene in the diet was estimated spectrophotometrically after chromatography by the method of Wiseman, Irvin, and Moore (18). Vitamin A in serum was estimated by a modification of the Carr-Price method (2). Carotene in serum was extracted with Skellysolve B and the concentration estimated at 453 mμ against appropriate standards.

SCN was determined by the method of Bowler (1) on trichloroacetic acid filtrates of serum and CSF. The SCN concentration was estimated by colorimetric measurement at 460 mμ of the color complex developed by ferric nitrate treatment.

Statistical comparisons were made using Student's "t" test.

TABLE 1
Serum vitamin A and carotene depletion of calves (Group 1)

Feeding period (days)	Serum vitamin A (μg/100 ml)		Serum carotene (μg/100 ml)		Body weight (lb)	
Depletion period	Control	Deficient	Control	Deficient	Control	Deficient
1-29	8.1	7.0	87	79	171	179
30-61	8.2	6.8	59	61	225	254
62-68	10.2	6.6	37	30	242	273
69-75	8.9	7.7	36	36	255	286
76-82	7.6	7.6	23	23	272	304
83-89	6.5	5.2	19	22	284	319
Depleted 90	4.6	2.9	15	11	312	330
Supplemental period						
1-30	9.8	5.8	67	26	323	355
31-60	10.6	4.2	60	25	346	396
61-90	8.1	3.3	52	28	398	469
91-120	12.0	8.5	72	46	449	524

RESULTS

Table 1 shows the serum vitamin A and carotene levels and body weights of the control and vitamin A deficient calves of Group 1 during the depletion and supplemental periods. During the depletion period, vitamin A and carotene decreased in both control and deficient animals. Growth of the calves in both groups was similar. At the end of the 90-day depletion period, serum vitamin A was 4.6 μg/100 ml and 2.9 μg/100 ml in the control and deficient groups, respectively. Serum carotene values were also very similar, being 15 and 11 μg/100 ml for the normal and deficient animals. During the carotene supplementation period, the serum levels were restored to 9.8 μg vitamin A/100 ml and 67 μg carotene/100 ml in the control group, whereas the deficient group (low carotene supplementation) showed only minor increases to 5.8 μg vitamin A/100 ml and 26 μg carotene/100 ml. Serum vitamin A and carotene values were maintained at approximately these levels during most of the supplementation period. During this time body weights increased and were very similar in both groups, with one exception. One deficient calf was considerably larger (*ca.* 33%) than any of the others comprising this group and was mainly responsible for the larger mean weight of the deficient animals. All except one of the trials were conducted during the first 90 days of the supplemental period.

The Holstein heifers which had not been depleted had serum vitamin A and carotene concentrations of 26.0 μg/100 ml and 282.6 μg/100 ml, respectively. These animals weighed *ca.* 362 lb at the time of use, and were thus similar to other controls of Group 1. However, due to differences in diet, their serum vitamin A and carotene levels greatly exceeded the values shown by the bulls of Group 1 (Table 1).

Table 2 presents serum vitamin A and carotene levels of the second group of control and vitamin A deficient bull calves (Group 2) during the depletion and supplemental periods. The feeding regime was not designed to deplete the control group to the extent obtained previously, and the serum values reflect this. Thus, the deficient calves exhibited average vitamin A and carotene values during the depletion period which were about half those of the normal calves. During the supplemental period in which the SCN influx experiments were conducted, the deficient calves maintained low serum concentrations, mean values for vitamin A and carotene being on-half to one-third those of the control animals.

CSF pressures measured just before administration of SCN (Table 3) indicated significantly higher pressures in the deficient groups than in the normal animals. Measurements during the 24-96-hr post-injection period also demonstrated much higher CSF pressures in the deficient calves.

The serum concentration of SCN in both normal and deficient groups remained at a fairly constant level during the first 9 hr of the experiment (Table 4). The deficient animals, however, consistently exhibited SCN concentrations which were about 20% higher than the controls. Daily samples taken for four days following the injection showed a steady decline in SCN concentration in both groups. The deficient animals in the 24-96-hr period still maintained higher serum concentrations. The differences between the normal and deficient calves at most of the time intervals were statistically significant.

TABLE 2

Serum vitamin A and carotene depletion of calves (Group 2)

Feeding period (days)	Serum vitamin A (μg/100 ml)		Serum carotene (μg/100 ml)		Body weight (lb)	
Depletion period	Control	Deficient	Control	Deficient	Control	Deficient
1-30	6.8	5.9	24	24	102	108
31-60	7.5	4.0	12	8	148	152
61-90	12.2	3.6	30	7	212	179
Mean	9.1	4.3	22	11		
Supplemental period						
1-30	9.1	4.3	42	10	289	279
31-60	13.1	5.6	54	13	351	350
61-90	12.9	7.8	54	16	414	417
91-120	12.4	8.8	60	20	468	476
121-150	12.3	9.3	49	18	522	523
151-180	15.6	6.7	50	16	558	574
181-210	15.3	7.9	53	18	598	615
211-240	15.4	5.0	40	11	646	668
241-270	14.1	2.7	41	3	692	725
Mean	13.2	6.8	49	15		

TABLE 3

CSF pressure in normal and vitamin A deficient calves

Time after SCN injection	Pressure in mm CSF		Significance between normal and deficient
	Normal	Deficient	
(hr)			
0	97 (19)[a]	173 (15)	$p < .001$
24–96	111 (8)	195 (8)	$p < .05$

[a] Numbers in parentheses represent number of observations.

TABLE 4

Passage of SCN from blood to SCF after intravenous injection of NaSCN into calves

Number of determinations		Hours after injection	Concentration of NaSCN (mg/liter)				Per cent of serum level	
			Serum (C_b)		CSF (C_{csf})		($C_{csf}/C_b \times 100$)[a]	
Normal	Deficient		Normal	Deficient	Normal	Deficient	Normal	Deficient
16	11	1	532	600	70	85	12.6	14.1
12	11	2	508	592	96	109	18.4	18.0
12	11	3	518	579	118	130	22.8	22.2
11	11	4	507	605	122	150	23.6	22.3
10	12	5	524	572	126	154	23.9	26.3
10	12	6	531	589	156	168	28.9	28.3
10	12	7	522	592	143	181	29.9	30.1
7	9	8	534	610	177	193	33.0	31.5
6	5	9	556	596	173	181	30.8	30.0
13	9	24	461	554	132	183	27.2	35.5
8	9	48	311	391	88	111	29.0	27.9
7	9	72	200	276	31	43	18.9	14.2
9	9	96	72	133	11	15	27.6	10.3

[a] These values are the means of the ratios for individual determinations.

The concentration of SCN in the CSF rose steadily during the 9 hr after the intravenous administration of NaSCN. The CSF concentrations reflect the blood content, the deficient animals showing a higher SCN level than the control calves. Clearance from the CSF from the 9-hr level to the 96-hr period was similar in the deficient and control calves, and no significant differences exist in SCN content at either 48, 72, or 96 hr.

The rate of entrance of SCN into the CSF, when expressed as per cent of the serum level (Table 4), was very similar in the normal and deficient animals during the period immediately following the injection. Thus, initial CSF–SCN values of about 15% of the plasma level increased to about 30% during the first 8 hr. Clearance in the subsequent period, however, was somewhat different, SCN being maintained at a higher ratio in the normal animals. As previously indicated, the CSF concentration of SCN in the deficient animals was not significantly higher than in the controls (48-96 hr). This higher ratio, therefore, was due primarily to a difference in removal from the blood stream, the deficient animals clearing the SCN at a less rapid rate than normal calves (normal serum contains smaller per cent of original SCN concentration than does deficient serum).

DISCUSSION

Measurements of the rate of entrance of SCN into CSF from the blood have been made previously by Wallace and Brodie (17) in the dog and by Davson (3) in the rabbit. In the experiments reported here, SCN was administered to the calves on a body weight basis at the same levels employed by Wallace and Brodie for dogs. It is interesting to note that the blood SCN levels attained in the dogs, 7.0/meq/liter or 567 mg/liter (expressed as NaSCN) are similar to those observed in the vitamin A deficient calves and slightly higher than the level attained in normal calves. Although the blood concentrations were quite similar in the two species, the concentration of SCN in the CSF in dogs was one and one-half to two times that observed in the calves. Initial levels (1-2 hr) in calves were of the order of 100 mg/liter and at 6-8 hr were 150-200 mg/liter; comparable concentrations in the dogs were 150 mg/liter and 300 mg/liter, respectively. These differences are reflected in the distribution ratio for SCN in our data ($r = \frac{C_{csf}}{C_b} = 0.30$) as compared to the data of Wallace and Brodie ($r = 0.52$). Davson (4) observed distribution ratios for SCN in rabbits of 0.095 at 2 hr, which is a ratio only half that observed in calves.

The purpose of the experiments reported here on calves was to study the rates and amount of SCN passing from the serum into the CSF. Following intravenous injection SCN begins to pass promptly into the CSF. After the first 5 hr, SCN concentration levels off and remains relatively constant, being about 30% of the serum level.

Wallace and Brodie (17) found that the CSF–SCN concentration in dogs attained a level of approximately 50% of the serum level in about 4 hr. In contrast, CSF concentration in the calves attained only about 30% of serum levels, and this occurred somewhat later (Table 4). As noted by Wallace and Brodie (17) and Davson (5), the passage of SCN into the CSF differs from the passage of SCN into other body fluids, in that the CSF concentration never reaches that of serum and the rate of net entrance (balance between entrance and removal) is slower.

When clearance from the CSF and serum was considered by examining the rate of decline of SCN on a logarithmic basis during the 24-96 hr period, the normal and vitamin A deficient calves showed similar slopes.

The kinetics of SCN penetration are complex and are undoubtedly complicated by a metabolic toxicity factor (4) and the extent of plasma protein binding of SCN which effectively removes it from the permeability process (6). The steady-state kinetics used by Davson and Matchett (6) for studying penetration of the blood-aqueous barrier have been employed with these data, however, to determine values for the penetration constant, k. The steady-state distribution ratio, r, was taken from the observed values of C_{csf}/C_b during the first 9 hr after the injection of SCN. This value was estimated to be 0.30 for SCN in both the normal and deficient animals, and was derived by averaging the mean values for the 6, 7, 8, and 9 hr periods, when the CSF concentration, as a per cent of serum level, appeared to reach an equilibrium. The graph of $1n\left(1 - \frac{C_{csf}}{r\,C_b}\right)$ was then plotted against time for both the normal and vitamin A deficient groups (Figure 1). From the slope of these plots, a value of $k = 0.0069$ min^{-1} was obtained for the deficient and $k = 0.0074$ min^{-1} for the normal animals, indicating a very similar rate of penetration of SCN into the CSF in vitamin A deficient

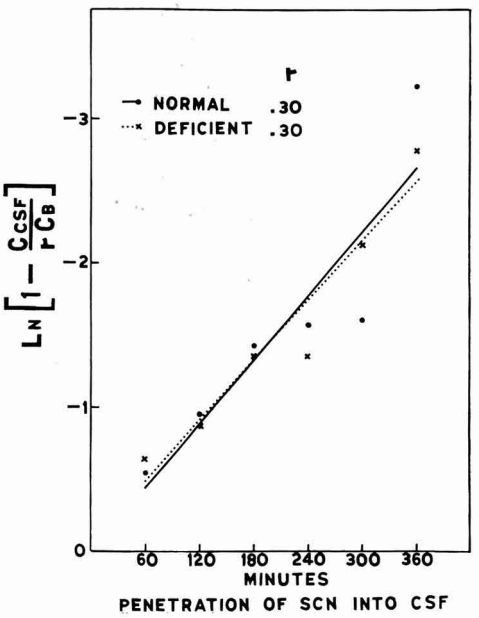

FIG. 1. Penetration of sodium thiocyanate into the cerebrospinal fluid of normal and vitamin A deficient calves.

calves. A tendency for SCN to adopt a temporary specious steady-state, particularly in normal calves, was noted at the 3-5-hr intervals (Figure 1 and Table 4). The problem of an apparent steady state has been discussed by Davson (4).

Interpretation of these findings is difficult, due to the complexity of the numerous factors involved. The extent to which the differences in CSF pressure affect rates of penetration and SCN concentrations in blood and CSF, the extent and rate of simple diffusion exchange, the extent of metabolic oxidation of SCN as a detoxification process by the nervous tissue, the relative volumes of the compartments, the rate of replacement of the fluid, the rate of reabsorption and removal of the fluid, are some of the factors contributing to CSF dynamics.

The CSF pressure differences which exist between normal and vitamin A deficient calves cannot be accounted for by a difference in the rate of penetration of a substance into the CSF from the blood, based on these studies utilizing SCN as a marker. If the rate of influx is taken to be an approximate and indirect measure of rate of formation of CSF, then the results presented here indicate a somewhat similar rate of CSF formation in normal and vitamin A deficient calves. It should be pointed out, however, that these conclusions are limited by certain of the experimental conditions:

(1) The vitamin A deficiency state studied was a mild one. While elevated CSF pressures were continually observed, none of the usual clinical manifestations and symptoms of vitamin A deficiency could be noted in the animals.

(2) Growth of the valves during the period of investigation. The experiments, of necessity, were conducted on growing immature animals, since major mechanical handling problems are associated with work on mature cattle. Changes in body size and weight are undoubtedly mirrored in changes in CSF volumes, spinal and brain membranes, with consequent differences in permeability and metabolism of the brain, choroid plexus, arachnoid villi, and other structures involved.

(3) SCN is a nonphysiological electrolyte. The results may be indicative of a very similar metabolic pathway (detoxification mechanism) for this material, even though differences in penetration, exchange, or transport of other electrolytes may exist.

From the limited studies reported here, it is difficult to state which factor or combination of factors is primarily responsible for these findings. Additional studies are indicated, to account for the CSF pressure differences between vitamin A deficient and normal calves. Two approaches which have been explored and which are the subject of further reports are (a) the determination of the rate of efflux of a substance from the CSF and (b) direct determination of the rate of CSF replacement and absorption.

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EFFECT OF VITAMIN A DEFICIENCY ON EFFLUX OF SODIUM THIOCYANATE FROM CEREBROSPINAL FLUID

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SUMMARY

Vitamin A deficient and normal Holstein calves were studied to provide information on the mechanism of the cerebrospinal fluid (CSF) pressure increase occurring in vitamin A deficiency. Thiocyanate (SCN) was injected into the cisterna magna and the concentration of this marker remaining in the CSF was estimated at 30, 60, 90, 120, 180, 240, and 300 min post-injection. Comparison of the SCN concentrations during the first 90 min showed similar values. Samples taken thereafter exhibited higher values in the deficient animals. Graphical interpretation of these data resulted in SCN disappearance rates of 3.02×10^{-3} mg/ml/min for the deficient group and 5.44×10^{-3} mg/ml/min for the control animals. This difference would indicate a lesser reabsorption of the CSF in the deficient calves and could also explain the higher CSF pressures in the deficient animals—225 mm as compared to 96 mm.

A previous study has shown that thiocyanate (SCN) moves from the blood stream to the cerebrospinal fluid (CSF) at rates quantitatively similar in vitamin A deficient and normal calves (2). This paper concerns the fate of SCN introduced directly into the CSF. These studies on CSF dynamics were undertaken to provide information on the mechanism whereby the CSF pressure is increased in vitamin A deficiency. Overproduction and/or underabsorption of the CSF has been suggested as the cause of the elevated pressures. Since substances leave the CSF by absorption through the arachnoid villi and by diffusion into central nervous system tissue and capillaries, a measure of the rate of disappearance of SCN from the CSF is related to the magnitude of these routes of absorption.

MATERIALS AND METHODS

Six Holstein bull calves 60 days of age were subjected to a depletion period to decrease body reserves of vitamin A and carotene, and a similar group of six was retained as controls. The vitamin A depletion ration consisted of timothy hay, low vitamin A grain, and dried skimmilk. The feed constituents and the blood serum of the calves were analyzed for vitamin A and carotene. When the serum vitamin A values of the deficient group were at a level of 5.0 μg/100 ml serum, both groups were supplemented daily with alfalfa leaf meal in such quantities as to provide 10 μg carotene per pound body weight for the deficient and 75 μg carotene per pound body weight for the control group.

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The deficient calves had serum vitamin A and carotene concentrations of 4.3 and 11 μg/100 ml, respectively, during the depletion period, as compared to 9.1 and 22 μg/100 ml serum for the controls. During the supplemental period the deficient calves maintained low serum concentrations, mean values being 6.8 μg vitamin A/100 ml and 15 μg carotene/100 ml compared to control levels of 13.2 μg vitamin A/100 ml and 49 μg carotene/100 ml. Serum vitamin A, carotene, and CSF thiocyanate concentrations were determined according to the procedures previously described (2).

The efflux experiments were conducted in both control and deficient calves during the supplemental period. The average age of the animals at the time when the experiments were conducted was six and one-half months. The average body weight at the time of use was 428 and 424 lb for the control and deficient calves, respectively (range 352-583 lb control and 370-655 lb deficient).

The animals were placed in a commercial cattle-restraining chute during the course of each efflux trial. A steel grid was fixed in a vertical plane 2 ft in front of the chute and the horns and nose of the calf tied firmly to the crossbars of the grid. After the punctures

TABLE 1

Efflux of SCN from CSF
Concentration of SCN in CSF after intracisternal injection

	No.	mm CSF pressure	mg NaSCN injected	SCN Concentration mg/100 ml						
				Time in minutes after injection						
				30	60	90	120	180	240	300
Control	15	96	214	164	108	70	56	28	14	9
Deficient	13	225	212	157	99	72	45	35	23	13
p Control vs. deficient		<.001		NS	NS	NS	<.05	<.05	<.005	<.05

were made, the adjustable walls of the chute were released so that the calf could stand naturally with only its head tightly restrained. The grid could be lowered or raised to insure that the cisterna magna was at the same height as the rest of the spinal column for accurate CSF pressure determinations. The use of this apparatus made it possible to conduct these experiments with only two operators.

A 16-gauge Touhy spinal needle, three and one-half inches long with Huber point, was introduced into the cisterna magna and allowed to remain in place during the 5-6 hr course of the trial. An adjustable needle-stop prevented the needle from penetrating beyond the cisterna. A two-way metal stopcock was added to the hub of the spinal needle and provided a means of closing the column and measuring pressure as well as injecting and withdrawing fluid.

After measurement of the preinjection CSF pressure, 5.0 ml of CSF were withdrawn into a syringe. Three milliliters of an aqueous solution of NaSCN, containing 50 mg NaSCN/100 lb body weight, were then injected into the cisterna magna of the calves to study disappearance from the CSF. Following this, 2.0 ml of the withdrawn CSF were injected to rinse out the stopcock and needle. Three-milliliter samples of CSF were then withdrawn at 30, 60, 90, 120, 180, 240, and 300 min after the SCN injection. Two blood samples were taken at 120 and 300 min after SCN administration, but serum concentrations of SCN were either negligible or zero.

Statistical comparisons were made using Student's "t" test.

RESULTS AND DISCUSSION

Comparison of SCN concentrations between control and deficient groups at each time interval during the first 90 min after intracisternal administration of NaSCN (Table 1) showed essentially similar values.

Samples taken after 90 min showed significantly higher concentrations in the deficient animals. Figure 1 illustrates the kinetics of the efflux of SCN from the CSF. It appears that SCN leaves the cisternal compartment by a multiple exponential process, and two single exponentials are apparent, giving a rapid and a slow rate of absorption. The rapid components are essentially the same in the vitamin A deficient and normal animals. The slow component of the deficient group shows a retardation, thereby indicating a slower rate of absorption. Extrapolation of the second slower slope to zero time indicates a decrease in SCN concentration of 3.02×10^{-3} mg/ml/

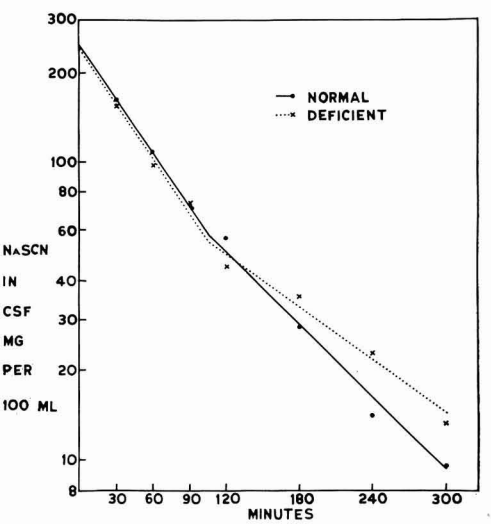

FIG. 1. Disappearance of sodium thiocyanate from the cerebrospinal fluid in normal and vitamin A deficient calves.

min for the deficient animals and 5.44×10^{-3} mg/ml/min for the control animals. This extrapolation also provided a means of estimating cerebrospinal fluid volume. Calculations utilizing these concentrations and the mean amount of NaSCN administered yielded an estimate of 124 ml for CSF volume in the normal animals and 202 ml for CSF volume in the deficient calves. Extrapolation of the slopes to yield an estimate of CSF volume, however, may be fallacious and is confounded by the different rates of absorption occurring concomitantly.

The observed slower rate of efflux of SCN in vitamin A deficient calves as compared to controls is in contrast to the results obtained in influx experiments utilizing the same marker. The rate of penetration of SCN into the CSF from the blood stream was found to be similar in a previous study (2). Bowsher (3) has discussed this possibility: "It must be remembered that, in studies of the rates of appearance in, and disappearance from, cerebrospinal fluid of various substances, times measured for rates of disappearance following introduction by substances into cerebrospinal fluid are absolute, while times of appearance are only relative to the rate of concurrent disappearance." In hydrocephalus, a condition in which the quantity and pressure of the CSF are both in excess of normal, the rate of absorption of electrolytes was found to be greatly reduced (7). Another investigator, however, studying the turnover of water, found no increase in the rate of appearance in hydrocephalus (1). The findings in this study of vitamin A deficiency are thus somewhat similar to the results obtained in hydrocephalus.

The results reported here indicate a rapid elimination of SCN from the CSF in both normal and deficient calves, although there is a slower rate of disappearance in vitamin A deficiency with its elevated CSF pressures. Other workers have demonstrated that SCN leaves the CSF at a rapid rate. Davson (4) found in rabbits that SCN leaves much more rapidly than Na^{24}, and concluded that metabolism of the foreign material by the nervous tissue is primarily responsible. Wallace and Brodie (6) injected NaSCN into the cisterna magna of a dog and found that the concentration decreased from 2.25 μg/ml at 44 min to 0.40 μg/ml at 2 hr. These concentrations are somewhat similar to those we observed. At 4.25 hr, SCN concentration was 0.033 μg/ml in the dog, a value which appears to be much lower than in calves at this time. Since the results of Wallace and Brodie were obtained in an experiment involving only one dog, comparisons with our results are necessarily limited.

Disappearance rates of other electrolytes from the cerebrospinal fluid have been investigated. Sweet and Locksley (7) studied the disappearance of K, Cl, and H_2O in humans, and found rapid rates of disappearance. Herlin (5) also found a rapid disappearance of electrolytes from the cisterna in rabbits.

Production and absorption of the CSF are concomitant physiological processes which can never be wholly separated. Disappearance of an exogenous material introduced into the CSF, however, has been assumed to yield a relative measure of the magnitude of absorption of the fluid (3). The retardation in the rate of SCN disappearance from the CSF observed in vitamin A deficient calves provides tentative evidence of reduced or defective fluid absorption in vitamin A deficiency. Underabsorption of CSF, therefore, may be the primary factor in the mechanism whereby the CSF pressure is increased in vitamin A deficiency.

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REPLACEMENT AND ABSORPTION OF CEREBROSPINAL FLUID IN NORMAL AND VITAMIN A DEFICIENT CALVES

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SUMMARY

Cerebrospinal fluid (CSF) replacement and absorption were measured in vitamin A deficient and normal calves. Replacement estimates involved removal of CSF and determination of the time necessary to replace the volume removed, and absorption estimates involved reinjection of the fluid and estimation of the time necessary to absorb this volume. Thirty-five minutes were required to replace the 3.0 ml of CSF in the normal animals, as compared to 15 min in the deficient calves. When estimating absorption capacity in the deficient calves, the CSF pressure was highly stable in 11 of the 12 experiments and the trials were terminated between 25-60 min. With normal calves the pressures returned to the original level in 17 min. The results suggest that underabsorption represents a greater factor than overproduction as a cause of increased CSF pressure in vitamin A deficient calves.

One of the initial effects of vitamin A deficiency is an increased cerebrospinal fluid (CSF) pressure (8). The mechanism whereby the increase in CSF pressure is brought about has been the subject of many previous investigations. The British investigators, Millen and Woolam (7), have reviewed their extensive studies in rabbits and chicks on the relationship of the increased CSF pressure in the hydrocephalus caused by vitamin A deficiency. They have concluded that overproduction of CSF occurred, although no measurements of the rates of appearance or departure of CSF constituents were made to support this interpretation. Indeed, underabsorption of CSF could have produced the same results. In this country, a group of workers at the Storrs, Connecticut, Agricultural Experiment Station have investigated the osmotic relationships of vitamin A deficiency and the CSF (5, 6). The biochemical constituents of serum, CSF, and aqueous humor were studied, because any changes in composition could have important osmotic effects and could bring about the increased CSF pressure. The authors concluded that the increased CSF pressure observed in hypovitaminotic A calves was due to a greater volume of fluid within the spinal fluid system. They could not distinguish between overproduction or underabsorption, however, as a causative factor, and there were only very slight changes in any of the constituents studied.

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Whether overproduction or underabsorption of the CSF is the primary factor responsible for the increase in pressure in vitamin A deficiency has been difficult to determine. In previous studies from this laboratory, no differences were observed in the rate of transfer of SCN from the blood to the CSF in normal and vitamin A deficient calves (1). However, when SCN was introduced directly into the CSF, a slower rate of disappearance was found in the deficient animals, suggesting a slower rate of CSF absorption (2).

The purpose of the present study was to estimate more directly CSF replacement and absorption by a technique described by Schaltenbrand (9, 10). This involves removal of CSF and determination of the time necessary to replace the volume removed, and secondly, reinjection of the fluid and estimation of the time necessary to absorb this volume.

MATERIALS AND METHODS

Treatment of the five normal and eight vitamin A deficient calves used in these experiments was the same as previously described (2). Use of a CSF stopcock assembly enabled CSF pressure measurements to be made continuously during withdrawal and injection of fluid (Figures 1 and 2).

Replacement of CSF. The CSF pressure was measured, a standard volume of CSF removed (3.0 ml), and the length of time necessary for the CSF pressure to return to its original level determined. The CSF pressure

FIG. 1. Measuring the dynamics of cerebrospinal replacement and absorption. The calf's head is restrained tightly against the fixed iron grid.

was measured continuously and readings taken every 3 min (deficient) or 5 min (normal). This return time was an estimate of the replacement of the volume removed.

Absorption of CSF. When stable CSF pressure was reestablished, the previously removed 3.0 ml of CSF were injected and the CSF pressure increased. The CSF pressure was measured continuously and readings were taken every 5 min (deficient) or every minute (normal). The time taken to return to the original pressure level was an estimate of the capacity of absorption.

RESULTS AND DISCUSSION

The data in Table 1 indicate marked differences in replacement and absorption of CSF between the normal and deficient animals. Thirty-five minutes were required to replace 3.0 ml of CSF in normal animals as compared to only 15 min in the deficient calves. The rate of replacement was thus two to two and one-half times more rapid in the vitamin A deficient calves.

Estimates of the absorption capacity indicated even larger differences. In 11 of the 12 experiments in deficient calves, the pressure remained elevated after reinjection of CSF and did not return to the starting pressure during the course of the trial. The CSF pressure was highly stable and, after 25-60 min, the experiments were terminated. The mean return time, therefore, exceeds 38.7 min and actually represents a mean nonreturn time. It represents an average of the time periods in which the absorption experiments on the deficient calves were terminated, the CSF pressure showing little or no tendency to fall from its elevated level. The absorption in milliliters per minute, therefore, is considerably less than 0.092, and more than 2.3 min are required to absorb 1 ml of CSF. It was our impression from the slight or negligible decreases in pressure exhibited during the experiment that absorption was very slow in vitamin A deficient calves.

Previous investigators have demonstrated that the effect of withdrawal of CSF is a sudden fall in the fluid pressure and that injection of fluid causes a rapid increase in CSF pressure (4). These changes and the initial compensatory reactions have been explained as being due to the response of the craniospinal vascular bed to withdrawal and injection of fluid from the subarachnoid space. After the initial vascular reaction, the subsequent response has usually been attributed to the rate

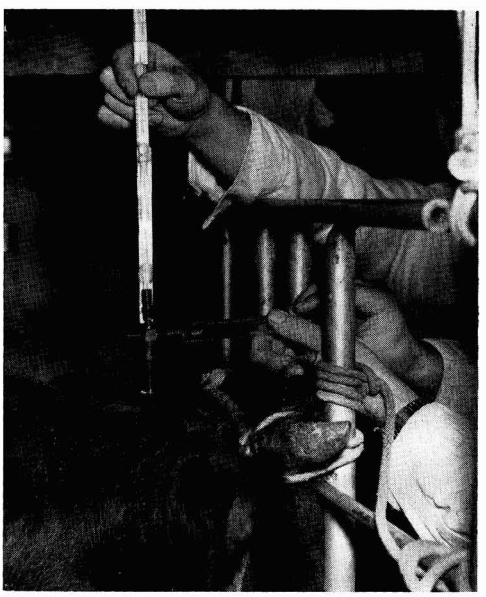

FIG. 2. Close-up illustrating stopcock and needle assembly. The CSF pressure has just been measured and a sample of fluid is being withdrawn. The adjustable needle stop near the skin surface prevents accidental penetration of the spinal cord.

TABLE 1

Replacement and absorption of CSF in normal and vitamin A deficient calves

I. Replacement

Group	No. of experiments	mm CSF pressure at start	ml CSF removed	mm CSF pressure after removal	Min to return to starting pressure	ml/min	min/ml
Normal	8	125	3	98	35.2 ± 14.0**	0.101	10.38
Deficient	15	287	3	232	15.5 ± 7.6	0.253	4.95

II. Absorption

Group	No. of experiments	mm CSF pressure at start	ml CSF injected	mm CSF pressure after injection	Min to return to starting pressure	ml/min	min/ml
Normal	8	128	3	203	17.1 ± 12.4	0.305	5.40
Deficient	12	282	3	366	>38.7*±11.8	<0.092*	>12.31*

* In 11 of 12 experiments, CSF pressure did not return to the starting pressure and remained elevated. More than 38.7 min is the mean nonreturn time and absorptions in the last two columns, therefore, are less and more than values shown.

** Standard deviation.

of production and absorption of the fluid. Thus, the Schaltenbrand technique utilized here includes the net effect of the vascular response and production and absorption of fluid. The results presented suggest that retarded or faulty physicochemical absorption of the CSF in the deficient calves was a greater factor than differences observed in re-formation of CSF. The retarded absorption observed in our study is in agreement with tracer studies, indicating that the increased fluid and pressure in hydrocephalus is due to faulty absorption (3).

It is not possible to directly compare the absorption rate for normal and vitamin A deficient calves. Such comparisons of the absorption figures of Table 1 are, therefore, misleading, because the deficient calves did not show a return to original pressures. The slower rate of absorption of about threefold $\left(\frac{.305}{.092} = 3.3\right)$ would represent only a minimum, and the rate is undoubtedly retarded to a significantly greater degree than is here indicated.

If the deficient calves had exhibited absorption rates of even 0.05 ml CSF/min, this would have resulted in some decline in CSF pressure. The results suggest that a difference in absorption capacity represents a much larger factor in increasing CSF pressure in vitamin A deficient calves than formation of fluid. This indication agrees with the previously observed slower rate of disappearance of SCN from the CSF in vitamin A deficient calves (2).

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FACTORS RELATED TO WEIGHT GAIN OF DAIRY CALVES [1]

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SUMMARY

Growth data on 659 dairy calves were used to determine the effects of breed, sex, season of birth, inbreeding, ration, and birth weight on weight gains of dairy calves to 8 wk, six months, and 1 yr of age. Breed, sex, degree of inbreeding, and ration were found to be significant sources of variation. Effects of season of birth were significant in the analysis of weight gains to 1 yr. Correlations of birth weight with weight gains were all less than .40. Early rate of gain had little or no effect on later gains, age at calving, and milk production. Although calf nutrition experiments should be designed to prevent confounding breed, sex, and inbreeding effects with ration effects, a portion of these effects can be removed by using birth weight as the independent variable in an analysis of covariance.

Body weight increase has been used as a measure of the adequacy of numerous calf rations and managerial systems. A number of factors in addition to ration have been shown to affect calf growth. Rathore (23) found sex and breed differences in growth rate of dairy calves, and Rollins and Guilbert (25) found that sex and season of birth affected growth of beef calves. There are many other similar studies on beef calves. Baker et al. (3) and Nelson and Lush (19) observed that growth rate decreased as inbreeding increased.

The relationship between birth weight and early growth rate has not been reported on large samples of dairy cattle. Forshaw et al. (13) observed a correlation of 0.46 between birth weight and weaning weight of Duroc pigs. Dawson et al. (6) and Arizona workers (1) have reported growth of beef calves to be associated postively with birth weight.

Correlations of milk production with birth weight, six-month body weight, mature body weight, and growth reported by Straus (26), Bailey and Broster (2), Plum et al. (20), Blackmore et al. (4), and Holtz et al. (15) were generally small and positive. Results reported by Reid (24) showed no significant differences in first lactation production between heifers underfed and normally fed prior to their first lactations. Since there were differences in growth rate, these results imply that there is little or no relationship between growth rate and milk production. Age at first calving, however, was later for the underfed heifers than for those fed more liberally.

Objectives of the present study were: (a) to evaluate the effects of sex, breed, season of birth, birth weight, and inbreeding on early weight gains of calves; (b) to determine the relationship of milk production to birth weight and body weight gains.

SOURCE AND CLASSIFICATION OF DATA

Data used in this study were collected from experiments in calf nutrition conducted at Iowa State University from 1945 to 1954. The rations varied, but other management practices remained relatively constant over the 9-yr period. Each animal was classified according to sex, breed (Ayrshire, Brown Swiss, Guern-

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sey, Holstein, or Jersey), season of birth (winter, December to February; spring, March to May; summer, June to August; or fall, September to November), degree of inbreeding (0-5, 6-10%, ..., 26-30%, or over 30%), and ration. Rations involved in 16 experiments were classified into five broad types: (a) rations containing either chlortetracycline or oxytetracycline; (b) liberal milk plus hay and grain; (c) limited milk (less than 300 lb milk per 100 lb birth weight) plus hay and grain; (d) milk only; and (e) rations containing unhydrogenated vegetable oil. Since there were more than 40 individual rations, the classes described above are rather arbitrary groupings and represent a gradation from the best diets to the poorest, based on growth rate and well-being of the calves.

There were 659 calves included in this study, though all calves could not be included in all of the several analyses of the data. Most of the calves were weighed at weekly intervals up to 8 wk of age and some were weighed at 12 and 16 wk. Only Holstein calves were routinely weighed at six and 12 months of age. Since only a few of the calves were weighed at all the above ages, gain from birth to 8 wk, gain from birth to six months, and gain from birth to 1 yr were treated separately. Inbreeding occurred in the Holsteins but not in the other breeds. The measure of birth weight was taken at four days of age, when the calf was removed from the dam.

ANALYSIS OF DATA

The least-squares technique described by Kempthorne (16, chapter 6) was used to evaluate simultaneously the effects of sex, breed, season of birth, and ration on 8-wk gain and birth weight. This allowed the effects of the factors to be tested for significance, both before and after adjustment of gain values for birth weight variation. The analysis is presented in Table 1. Effects of sex and breed were reduced by adjusting to a common birth weight. However, the effects of sex and breed on adjusted gains were significant sources of variation. The method of analysis assumed no interaction between the main effects, but allowed a test of the significance of pooled interactions.

Since six-month weights were available only for Holsteins, no estimates of breed effect could be obtained from analysis of six-month gain data. Constants were fitted simultaneously for birth weight, 8-wk gain, and 6-month gain, Effects of sex, season of birth, and ration on the variation of six-month gain, six-month gain adjusted for birth weight, six-month gain adjusted for 8-wk gain (essentially a test of the effects of the various variables on gain from 8 wk to six months of age), and six-month gain adjusted for both birth weight and 8-wk gain are shown in Table 2.

Most male calves left the herd between six and 12 months of age, thus reducing further the number of calves available for an analysis of factors affecting 1-yr gain. Results of the analysis of 1-yr gain data followed closely the results of the previous analyses, except that season of birth effects were significant ($P \leqslant .05$).

Constants derived from the three analyses described above are shown in Table 3. These are deviations from the general mean. An estimate of the difference between any two breeds is the difference between the constants for the two breeds compared. Comparisons of any two rations, seasons, or sexes can be made in a similar manner. The differences in birth weight associated with rations were due to

TABLE 1

Analyses of variance and covariance of birth weight and 8-wk gain

Source of variance	d.f.	M. S. birth weight	M. S. 8-wk gain	M. S. 8-wk gain adjusted for birth weight
Season	3	103	281	245
Sex	1	7,992**	5,409**	2,607**
Ration	4	158	21,886**	21,803**
Breed	4	12,570**	2,410**	814**
Interactions	122	139	288	303
Within cells	458[a]	140	249	243

** F significant at the 1% level of probability.
[a] 457 for adjusted 8-wk gain mean square.

TABLE 2

Analyses of variance and covariance of birth weight, 8-wk gain, and six-month gain (Holsteins only)

Source of variance	d.f.	M. S. six-month gain	M. S. six-month gain adjusted for birth weight	M. S. six-month gain adjusted for 8-wk gain	M. S. six-month gain adjusted for 8-wk gain and birth weight
Sex	1	63,601**	42,448**	27,519**	20,963**
Season	3	2,664	2,476	1,451	1,662
Ration	4	23,771**	19,402**	1,672	1,398
Interactions	27	1,209	1,166	971	879
Within cells	260[a]	1,393	1,304	871	845

** F significant at the 1% level of probability.

[a] 259 degrees of freedom for six-month gain mean square adjusted for either birth weight or 8-wk gain; 258 degrees of freedom for six-month gain mean square adjusted for 8-wk gain and birth weight.

the allotment procedures, since the calves did not receive the rations until after birth.

Data from two nutrition experiments were used in a preliminary study of the linear regression of body weight gain to 12 wk of age on inbreeding. One experiment was analyzed as a randomized complete block experiment with 12-wk gain of the calf as the dependent variable. Inbreeding and birth weight of the calf and inbreeding, birth weight, and 8-wk gain of the dam were used as independent variables in a covariance analysis. The portion of total variance due to regression on all five variables was .273, whereas the portion of total variance due to regression on inbreeding of calf alone was .203 ($r = -.450$). Inclusion of variables other than inbreeding of the calf added little accuracy to the prediction of 12-wk gain in this small sample. Correlation of inbreeding with 12-wk gain in the second experiment was −.275 as compared to −.450 in the first experiment. The regression coefficients for the first and second experiments, respectively, were −.98 and −.78 lb 12-wk gain per 1% inbreeding. All calves used in these two experiments were 5% or more inbred.

TABLE 3

Constants derived from least squares analysis of birth weight and gain data

Effect	Birth weight[a]	8-wk gain	Six-month gain	1-yr gain
T_1 (Male)	3.61	3.08	15.15	29.85
T_2 (Female)	−3.61	−3.08	−15.15	−29.85
B_1 (Ayrshire)	4.88	5.30		
B_2 (Brown Swiss)	12.39	4.61		
B_3 (Guernsey)	−8.24	−8.64		
B_4 (Holstein)	10.05	4.29		
B_5 (Jersey)	−18.89	−5.55		
S_1 (Winter)	.82	−.44	1.30	−10.63
S_2 (Spring)	.71	2.00	1.40	21.87
S_3 (Summer)	−.88	−.46	−9.35	−6.35
S_4 (Fall)	−.65	−1.10	6.64	−4.89
R_1 (Antibiotics)	−.78	19.27	25.59	46.49
R_2 (Lib. milk)	1.31	11.33	18.76	12.78
R_3 (Lim. milk)	.28	1.07	3.44	4.05
R_4 (Milk only)	1.26	−11.86	−7.30	.22
R_5 (Unhyd. veg. oil)	−2.06	−19.81	−44.49	−63.11

[a] All weights and gains are in pounds.

Following the above preliminary analyses, this study was expanded to include all available data. To remove more completely the ration and time trend effects, as well as sex effects, a two-way classification of the data was effected. One classification was the inbreeding classes described earlier and the second was a sex, experiment, ration class. Use of the inbreeding classes allowed an estimation of the curvilinear effects of inbreeding on weight gain.

Constants derived in the more complete analysis are plotted in Figure 1. The trend

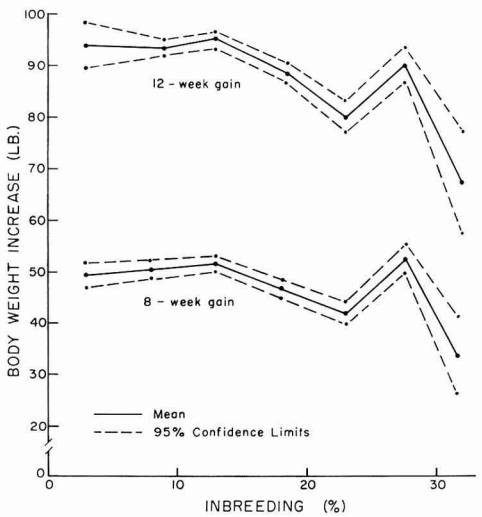

FIG. 1. Relationship between 8- and 12-wk body weight gains and coefficient of inbreeding.

shown indicates little influence of inbreeding when the coefficient is less than 15%. Beyond 15% inbreeding there was, with exception of the 26-30% class, a negative association between inbreeding and weight gain. The high value found for the 26-30% class could be due to sampling errors, since there were only six calves in that class.

In each of the analyses concerned with the effects of season of birth, sex, breed, and ration, correlations of weight gain with birth weight were computed. It is desirable to know whether weight gain in the early periods has a higher or lower correlation with birth weight than gain in the periods considered earlier in this study. Correlations of birth weight with weight gain to various ages would tend to shed some light on the differences in growth patterns of large and small calves. All correlations computed in all analyses are listed in Table 4. Very early weight gain is negatively correlated with birth weight and the value of birth weight as a predictor of weight gain increases at least to 12 wk of age. Beyond that point, the correlation tends to stabilize and sometimes even decreases.

Of the animals included in this study, 113 Holstein females had all of the following information available: birth weight, 8-wk gain, six-month gain, 1-yr gain, age at first calving, and first-lactation milk production. Correlations among these traits were computed and are shown in Table 5. Production records were 305-day, 2×, M.E., 3.5% F.C.M.; therefore, the expected correlation between calving age and production was zero. Correlations among the various measures of weight gain were part-whole relationships and were expected to be large.

DISCUSSION

As observed previously by other workers (7, 8, 12, 22, 27), sex and breed were found

TABLE 4

Correlations between birth weight and gain from birth to various ages [a]

Data analysis [b]	Dependent variable; gain to age in wk								
	d.f.	2	4	6	8	12	16	26	52
8-wk gain	457 [c]				.161**				
Six-month gain	259				.195**			.260**	
1-yr gain	181								.319**
All gains to 8 wk	349	−.100	.057	.133*	.164**				
All gains to 16 wk	149	−.051	.131	.244**	.297**	.382**	.272**		
All gains except 12 and 16 wk	161	−.159*	.001	.107	.188*			.316**	.327**

* Significant at the 5% level of probability.
** Significant at the 1% level of probability.
[a] Correlations within breed × sex × season × ration classification.
[b] Sets of data are not entirely independent, since some calves were included in two or more data analyses.
[c] All breeds represented; other analyses include Holsteins only.

TABLE 5

Correlations among birth weight, 8-wk gain, six-month gain, 1-yr gain, age at first calving, and production based on 95 degrees of freedom within ration and season (Holsteins only)

	8-wk gain	Six-month gain	1-yr gain	Age at calving	Production
Birth weight	.166	.356**	.291**	−.205*	.276**
8-wk gain		.578**	.337**	−.104	.088
Six-month gain			.559**	−.058	.300**
1-yr gain				−.067	.301**
Age at calving					.044

* Significant at the 5% level of probability.
** Significant at the 1% level of probability.

to influence birth weight of the calf. In the present study, primary attention was directed toward the relationship between weight gain and birth weight.

Relatively large differences in weight gain were found among the breeds and between the sexes. Variation due to sex and breed was reduced by adjustment for birth weight, but these effects were still important sources of variation. This would indicate that calves should be assigned to treatments in such a way as to allow treatment effects to be independent of sex and breed. A randomized block design, wherein all animals in a block are of the same sex and breed, would probably best fit this situation. If, however, the research worker is willing to accept a less accurate estimate of treatment effects, a design unbalanced with respect to sex and breed could be used and a part of the variation attributable to breed and sex removed by adjusting gains to a common birth weight by covariance. Season-of-birth effects were not a major source of variation in any of the analyses, but this does not imply that extreme heat or cold as well as other environmental factors do not affect weight gain over a short period of time. It does, however, imply that the average conditions in the four seasons in the present study were essentially equally conducive to weight increase of the young calf. Season effects were significant in the analysis of 1-yr weight gains and probably should be accounted for in experiments extending beyond six months of age. If further subdivision is possible, after all of the more important sources of variation are blocked out, season of birth should be accounted for by dividing the calves into groups as nearly contemporary as possible. The reasons for the significant season effects at 1 yr of age are not entirely clear, but the differences may be associated with seasonal variations in feeding and management practices between six months and 1 yr of age.

Inbreeding appeared to have little effect on weight change of young calves until the coefficient of inbreeding exceeded 15%. However, above 15%, inbreeding apparently depressed growth rate. This suggests that when the herd furnishing experimental animals is inbred extensively, and the range of inbreeding coefficients is great, calves should be grouped into inbreeding classes, or an analysis of covariance should be used to remove part of the variation due to inbreeding.

Correlations of birth weight with weight gains were consistently small, and less than 15% of the variation in gain could be removed by adjusting to a common birth weight. Analysis of covariance, with birth weight as the independent variable, can be justified only when there is a question regarding significance of treatment effects and a modest increase in precision will help clarify the situation, or when the research worker is unable to balance breeds and sexes in the design. The association between age and the correlation of birth weight with gain in weight indicate that large calves are slow starters, whereas small calves adjust more rapidly to extra-uterine environment.

Ration was found to affect weight gains from birth to all ages considered in this study. Analysis of covariance and correlation studies indicate that, though ration effects persist to 1 yr of age, weight gain beyond the treatment period (first 8 to 12 wk) was not affected by gain during the treatment period. The nature of the ration classifications virtually forced ration effects to be significant at 8 wk.

Reid (24), Hansson et al. (14), and Eckles and Swett (10) all reached the conclusion that growth rate and consequent age of maturity could be influenced by feeding. They did not, however, find any great influence of early rate

of growth on first-lactation milk production. The study reported herein is not in complete agreement with these findings, inasmuch as six-month and 1-yr gain were correlated ($r = .30$) with milk production. Since no correction for inbreeding was made, and inbreeding could affect both gain and milk production, this correlation could have been the result of correlated effects of inbreeding. Also, six-month and 1-yr gains represent a substantial portion of mature weight and it is possible that the observed correlation is simply that between body weight and milk production. Blackmore et al. (4) and Holtz et al. (15) concluded that the factors creating rapid early growth are independent of those causing high production. In the present study, 8-wk gain had little or no effect on later gains, age at calving, or subsequent milk production. It would seem that the calf-raising program has little effect on traits independent of and expressed after the experimental period.

If weight gain is not to be used as an index of the sufficiency of a calf ration, a health or condition index is a possible substitute. However, it has the major disadvantage of being subject to human judgment and, therefore, easily biased. Changes in skeletal measurements would identify only the most inadequate rations. The present study was not designed to test the adequacy of the two indexes suggested above, but it does clearly raise a question concerning the use of weight gain as an index of calf ration sufficiency. The unit cost of weight gains prior to the development of rumen function is considerably greater than that of gains made later. Therefore, it would seem advisable to keep calves healthy and thrifty during the early weeks of their life without great regard for weight gains. If weight gains are used as the criterion of adequacy, it should be borne in mind that the variable is a growth response which has little relationship to the ultimate lactational response of the animal.

Growth standards have been tabulated by several workers in the past. A comparison of the values reported for Holstein heifers with average growth of Holstein heifers included in this study is shown in Table 6. There was variation within the Iowa State University herd, as indicated by ration differences. The differences between standards indicate differences from one sample or herd to another. If a standard other than one for the herd in question is used, the research worker is comparing herds as well as management regimes. Obviously, a control ration should be incorporated into the design of each calf nutrition experiment designed to evaluate growth response, and standard growth curves should be used only as an additional criterion.

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TABLE 6

Growth standard values for Holstein heifers at birth, two months, six months, and 1 yr of age

Standard	Birth	Two months	Six months	1 yr
Campbell and Flux (5)	....	160	390	575
Eckles (9)	90	157	349	558
Espe et al. (11)	89	132	358	662
Morrison (18)	91	150	365	653
Ragsdale (21)	90	148	355	632
USDA (17)	96	161	396	714
Present study [a]	86	125	307	579

[a] All values based on 170 or more animals.

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ACETATE TURNOVER RATE IN THE BOVINE[1]

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SUMMARY

Data have been obtained on the rate of acetate turnover in a steer and in Holstein cows 2-4 hr after feeding. The turnover rate for mature cows was 2.1 ± 0.5 mM/hr/kg (5.5 ± 1.3 g/hr/cwt). Evidence was obtained from the calculated acetate pool size in relation to blood acetate concentration and from the calculated acetate space that the technique used is measuring the turnover rate of the acetate pool in the accessible central (blood) compartment. The data do not support the concept that the rate of acetate utilization is concentration-dependent, at least for the normal blood concentrations existing in this study. From the data in this study, in relation to various estimates of acetate absorption, it is suggested that as much as 25% of the acetate turned over may be of endogenous origin. Inferences are drawn concerning possible physiological effects on acetate metabolism and the relationship of acetate metabolism to the total metabolic rate.

Acetate, a most important endogenous metabolite in all animal species, is of importance in ruminant animals as a major exogenous metabolite as well. Much has been learned about the utilization of acetate carbon in many species; whereas, relatively few studies have attempted the measurement of the rate of metabolism of acetate in these species.

Bloch and Rittenberg in 1945 (4) utilized the acetylation of foreign amines in the rat as well as carbon-labeled acetate to estimate the acetate turnover in this species. Smyth (16), several years later, using acetate tolerance curves, estimated the turnover rate in cats as 6-7 mM/hr/kg. Harper et al., in 1953 (9), were the first to utilize the conventional isotope dilution techniques for the determination of acetate turnover. These studies in the dog indicated a turnover rate of 3-7 mM/hr/kg. The most recent work in nonruminants is that of Dayton et al. (6) with rats, using essentially the technique of Bloch and Rittenberg (4). In this study an interesting finding was the effect of changes of fat in the diet on the acetate turnover rate. On a normal diet the turnover rate was 4.5 mM/hr/kg and on a fat-free diet, 3.5 mM/hr/kg.

The first report of such studies in ruminants was a preliminary communication by Annison and Lindsey in 1958 (1). A more complete study in sheep was reported by these authors in 1961 (2). Using the single injection technique with carboxyl-labeled acetate, these workers reported a rapid turnover of acetate with a half-life ($T\frac{1}{2}$) of 3-4 min as determined from the blood acetate specific activity slope between 6 and 16 min post-injection. Using this slope, an improbably large acetate space of 70 liters, or approximately 1.5 liters/kg body weight, was estimated. Without further investigation of this technique, this group then used the constant rate infusion technique in four sheep to determine the utilization rate of acetate. In these four animals they obtained acetate turnover rates ranging from 0.7 to 5.58 mM/hr/kg.

Davis et al. (5) reported the results of a single injection experiment and a constant infusion experiment on a 160-kg steer, fasted 72 hr in the first experiment and fed in the second experiment. In the single injection experiment the specific activity data for 90, 120, and 150 min post-injection were used in the calculation of $T\frac{1}{2}$ and acetate pool size. These data gave a $T\frac{1}{2}$ of 58 min and an improbably large value for the acetate space (some 100 times larger than the animal). These results are not surprising when it is considered that the single injection technique is useful only for measurement of the accessible central com-

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partment of a mammillary system and not for the acetate pool which these workers have defined. From their constant infusion experiment an acetate turnover rate of 7.1 mM/hr/kg was calculated from acetate carbon oxidation values. Using the more conventional calculation (15), their data give a value of 4.2 mM/hr/kg for the acetate turnover rate in this steer.

Preliminary data from this laboratory, reported in 1960 (12), using the single injection technique, gave average values for a steer of 2.6 mM/hr/kg and for several cows of 1.8 mM/hr/kg. The T½ in these experiments ranged from 1.5 to 4 min, which is similar to the other reported values for acetate, except for that of Davis et al. (5). These are also similar to the T½ values reported for the long-chain fatty acids in various species (3, 8, 10, 11).

EXPERIMENTAL PROCEDURE

These experiments were conducted on a Jersey steer and on mature Holstein cows. The steer was maintained on good-quality alfalfa hay at 110% of Morrison's recommended allowance. The dairy cows were maintained at somewhat above Morrison's standards on a normal herd ration of grain and hay at about a 1:3 ratio. All experiments were conducted 2-4 hr after the AM feeding.

The definitions employed and the experimental procedures used in these single injection turnover studies have been reported in detail elsewhere (13). The only exception to the published procedure occurred in Experiments 1 and 2, where the blood sampling was at 2-min intervals. In all other experiments sampling was at 1-min intervals.

In general terms, the procedure involved rapid injection of acetate-1-C^{14} into the jugular vein, the sampling of jugular blood at 1-min intervals, except as noted, for 10-12 min, and the immediate deproteinization of these samples. Acetate was isolated from the blood filtrates chromatographically and the specific activity (cpm/mg) determined. A semilog plot of specific activity vs. time was made and the slope calculated by the method of least squares. The half-time (T½) and acetate pool size were calculated using this slope. From the T½ and pool size values the acetate turnover rate was calculated by the standard calculation for such rates.

The terms employed in this study are defined as follows:

Specific activity: Radioactivity per unit mass, e.g., cpm/mg acetate.

Half-time: The time required for the radioactivity to decline by one-half.

Turnover rate: The rate of entrance and exit to a pool in a steady state. Usually expressed as mass per unit time.

Acetate pool: The mass of acetate in the accessible (blood) compartment or in any compartment in very rapid equilibrium with this accessible compartment.

Mammillary system: The metabolic model which assumes that all peripheral compartments containing metabolite are joined through a central compartment. In these studies, the central compartment is the accessible (blood) compartment which contains the bulk of the acetate pool as defined.

Acetate space: The volume occupied by the acetate pool, expressed as a per cent of body weight, under the assumptions of an acetate concentration equal to that in blood and a body specific gravity of one.

RESULTS AND DISCUSSION

The data obtained in this series of experiments are given in Table 1. The semilog plot of the specific activity data in typical experiments is shown in the study of acetate isotope dilution techniques by Lee and Williams (13). The first point to examine in these data, since the single injection technique used here purports to measure the turnover rate of the accessible central compartment of this mammillary system, is whether the pool size bears a reasonable relationship to the blood acetate concentration. Secondly, the calculated acetate space should not be improbably large, as was reported by Davis et al. (5) and Annison and Lindsey (2). If either of these conditions is not met, considerable doubt is placed on the validity of these results. It is apparent from Table 1 that, in general, the higher the blood concentration of acetate the larger the calculated acetate pool. Although not shown in Table 1, the acetate space averaged $10.1 \pm 1.8\%$ of the body weight in these experiments. These two findings suggest that the use of this technique is valid for the measurement of turnover rate in the central (blood) compartment.

If the turnover rate obtained in each experiment and the blood acetate concentration at the time of determination is examined, it is seen that no direct relationship exists. This finding tends to cast doubt on the concept that the rate of acetate utilization is concentration-dependent, at least for the blood concentrations encountered in this study.

When these results for acetate turnover rate

TABLE 1

Acetate metabolism in dairy cattle

Expt. no.	Date	Animal	Blood acetate	T½	Pool size	Turnover rate		
			(mg %)	*(min)*	*(mg/cwt)*	*(g/hr)*	*(g/hr/cwt)*	*(mM/hr/kg)*
1	8/59	Steer J	12.9	4.0	654	53.1	6.8	2.5
2	10/59	Steer J	5.94	1.6	295	63.1	7.5	2.8
3	2/60	327 H Low prod.	5.72	1.6	175	57.2	4.6	1.7
4	2/60	287 Dry—open	4.32	1.5	181	74.9	5.0	1.9
5	6/60	187 H Dry—open	9.06	2.5	467	110.5	7.8	2.9
6	8/60	317 H Dry—open	5.90	2.3	233	55.3	4.2	1.6
7	3/61	149 H High prod.	5.70	2.1	355	81.5	7.1	2.6
8	3/61	149 H High prod.	5.00	1.6	208	64.7	5.6	2.1
9	6/61	149 H High prod.	8.46	3.8	405	54.6	4.4	1.6
Mean (steers)				2.8	475	58.1	7.2	2.7
Mean (cows)				2.2±0.8	289±108	71.2±20.4	5.5±1.3	2.1±0.5

are compared to the data on four sheep presented by Annison and Lindsey (2), it is seen that the agreement is reasonably good. These authors reported values ranging from 0.7 to 5.6 mM/hr/kg for their sheep, as compared to 2.1 ± 0.5 mM/hr/kg for the mature cows in this study. Davis et al. (5) reported a value of 7.1 mM/hr/kg for their steer, which is more than three times the mean value for the cows in this study. When just comparing steer values this is still almost three times the values reported here.

It is of interest to compare the turnover rates obtained in this study (2.1 ± 0.5 mM/hr/kg for cows) with various calculations of acetate absorption from the rumen. The turnover rates obtained in this study should result from the turnover of both endogenous acetate and exogenous acetate and, therefore, absorption rates would be expected to be somewhat less than the values reported here for turnover. Stewart et al. (17), using a combined in vitro–in vivo technique, have estimated an acetate absorption of 1.8 mM/hr/kg in a steer. From goat rumen perfusion studies, McCarthy (14) estimated a ruminal production of acetate of 1.9 mM/hr/kg. Dobson (7) has estimated the fatty acid absorption of sheep as 1.6 mM/hr/kg, as calculated from various data in the literature. Annison and Lindsey (2) have estimated absorption as 1.5-2.2 mM/hr/kg in sheep. These comparisons suggest the correctness of the assumption that the measured turnover rate includes both endogenous and exogenous acetate production. It seems reasonable to estimate that the endogenous production under these conditions may account for as much as 25% of the acetate turnover.

Although only two steer experiments were conducted, these data suggest several possible differences between growing steers and mature cows. The whole body turnover of acetate appears to be not greatly different in the growing steer and the mature cow. The acetate turnover rate per unit body weight, however, appears to be greater in the growing steers. If this difference is real, it suggests that the rate of production of acetate per unit body weight is larger in the growing animal. This is probably true of the exogenous production and may very well be true of the endogenous production as well due to the differing endocrine climate in the growing animal. These suggestions should be submitted to further experimental examination.

As noted in the introduction, only relatively few studies of the rate of acetate turnover have been carried out. Ignoring species differences, the differing techniques employed and the differing physiological states encountered, when these turnover values for rats (4), cats (16), sheep (1, 2), and the rates for cattle

determined in this study are plotted logarithmically against body weight, a straight line plot is obtained. Since only few data are available, the slope was estimated graphically as 0.8. This value is very close to the usually accepted values of 0.7 to 0.75 for the slope of resting or basal metabolism vs. body weight. That these values should be similar is not surprising, considering the position which acetate occupies in oxidative and synthetic metabolism.

The data obtained in this study indicate the ability of this technique to measure acetate metabolic rates, if the observational period is such that the changes in specific activity observed are predominantly due to turnover in the accessible central compartment and not predominantly due to reflux and carbon recycle. The contention, based on acetate oxidation studies (18), that the acetate pool must be small and the turnover of this pool rapid, is fully supported by these data. The suggestion from these data and others (2, 7, 14, 17), that exogenous acetate may account for as little as 75% of the acetate turned over, suggests that the sources of this acetate, particularly the long-chain fatty acids, merit further investigation.

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EFFECT OF PARATHYROID HORMONE ON CALCIUM AND OTHER PLASMA CONSTITUENTS OF DAIRY CATTLE NEAR PARTURITION [1]

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SUMMARY

The administration of parathyroid extract within 2 hr postpartum to first calf heifers, normal mature cows, and mature cows with a past history of milk fever did not appreciably alter the plasma levels of calcium, magnesium, or alkaline phosphatase. Plasma inorganic phosphorus was increased moderately in most of the animals by the action of parathyroid extract. At the time of parturition mature animals showed a greater decrease in calcium and inorganic phosphorus and a greater increase in magnesium than did the younger animals. Alkaline phosphatase remained relatively constant in all animals. Thirty-one parturitions were studied and six cases of milk fever observed. Data suggest that milk fever can result from either a low level of calcium or a low level of both phosphorus and calcium. Cases of milk fever which relapsed were of the latter type. Parturient cows were not responsive to parathyroid extract and its administration just after calving had no apparent effect on the occurrence or severity of milk fever.

The parathyroid deficiency theory of parturient paresis or milk fever has had wide acceptance since it was first proposed by Dryerre and Greig in 1925 (3). However, there is little experimental evidence supporting this theory, other than the observation of hypocalcemia and the successful use of calcium to relieve the symptoms of this disorder. Although parathyroid extract (PTE), when administered intramuscularly, has been shown to elevate the level of blood calcium in most species of animals (20), it has not been tested adequately in the prevention of bovine milk fever. Little and Mattick (9) administered to two cows 450 or 600 Hanson Units [3] of parathyroid activity, intramuscularly, daily for four days prepartum to one day postpartum. Data showed no definite effect of the Paroidin injections over the controls for that year, but the treated animals showed lower calcium levels at the next parturition a year later, when no parathyroid extract was given and when one of the cows developed milk fever. These workers concluded that the parathyroid extract may have been beneficial to some extent, possibly by preventing milk fever. Seekles, Sjollema, and Van Der Kaay (17) were able to relieve the symptoms of milk fever in one cow by the intravenous administration of 100 USP units of parathyroid hormone. Hibbs, Pounden, and Krauss (8) have given a brief report that parathyroid extract (3,000-5,000 USP units/animal) was of no benefit to three cows with early milk fever symptoms but was of benefit to a similar cow when injected in conjunction with oral vitamin D. Stott and Smith (19) thyroparathyroidectomized seven cows, 3-6 yr of age, approximately one month prepartum without the subsequent occurrence of symptoms of milk milk fever. The parathyroid deficiency theory of milk fever has been reviewed by Hibbs (7) and the role of the parathyroids in regulating the level of blood calcium has been reviewed by Boda and Cole (2).

Preliminary experiments have indicated that

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[3] One Hanson unit is 0.01 of the amount of parathyroid activity required to increase serum calcium by 1.0 mg % in parathyroidectomized dogs within 6 hr (20). One U.S.P. parathyroid unit is 0.01 of the amount of parathyroid activity required to increase serum calcium by 1.0 mg % in normal dogs within 16-18 hr (21).

parathyroid extract administered intramuscularly to dairy cattle elevated plasma calcium to a maximum level in 8 to 12 hr and had a duration of action of more than 30 hr (12). Since milk fever is most likely to occur within 12 to 24 hr postpartum, the administration of parathyroid extract within 2 hr postpartum could be expected to increase plasma calcium during this critical period.

This experiment was designed to study the effects of the postpartum administration of parathyroid hormone on the occurrence of milk fever, and to determine whether normal cows and cows with a past history of milk fever have a different response of plasma calcium, phosphorus, magnesium, and alkaline phosphatase to the administration of parathyroid hormone at parturition.

EXPERIMENTAL PROCEDURES

Twenty-seven dairy animals of Holstein, Guernsey, or Jersey breeding were studied in 31 parturitions during this experiment (Table 1). First calf heifers (Group I) and the normal mature cows (Group II) were from the Purdue University Dairy Herd. Animals with a history of milk fever in their last two lactations (Group III) were obtained from Indiana farmers and maintained at the School of Veterinary Science and Medicine. All of the animals were fed and managed by the practices used in the University Dairy Herd.

TABLE 1

Distribution of animals used to test the effect of parathyroid extract at parturition

Group		a (no PTE)	b (with PTE [a])
I	First calf heifers	4	4
II	Normal mature cows in their fourth or higher lactation with no milk fever history.	5	4
III	Mature animals with milk fever histories in at least the last two parturitions.	8	6

[a] 400 U.S.P. units/100 lb (3,000-6,000 units/animal).

Animals which received parathyroid extract were given 400 USP units/100 lb (3,000-6,000 units/head) intramuscularly in the cervical region of the neck within 2 hr postpartum. All of the PTE used in the experiment was of the same batch. Since the experiment required more than a year, the potency of the PTE was tested near the end of the experiment by its administration to two dry cows, to determine if it had lost its ability to elevate plasma calcium.

Blood samples were obtained in heparinized syringes from the jugular vein and were centrifuged to remove red cells. Plasma was analyzed for inorganic phosphorus by adding one volume of plasma dropwise to four volumes of 10% trichloroacetic, filtering, and determining the phosphorus in an aliquot of the filtrate by the A.O.A.C. colorimetric method (1). Phosphorus and alkaline phosphatase (18) were determined within 12 hr after collection of the blood. Magnesium was determined by the method of Grette (4). Calcium levels were initially determined by Grette's method (4), which involves oxalate precipitation and titration of the calcium with ethylenediaminetetraacetic acid (EDTA), but most of the calcium levels were determined by a new method involving the direct titration of the plasma calcium with EDTA, using Cal-Red as the indicator (13). Daily plasma levels of these constituents were determined in most cases from seven days prepartum to seven days postpartum; for 48 hr postpartum blood samples were drawn every 2 hr for the first 12 hr, every 4 hr for the next 12 hr, and every 6 hr for the next 24 hr.

RESULTS AND DISCUSSION

The mature cows in this study showed a wide variation between animals in their plasma levels of calcium, inorganic phosphorus, and magnesium. In contrast, the plasma levels of these constituents in the first calf heifers were much more uniform. Alkaline phosphatase values were quite consistent in all groups at approximately 2 to 3 mM units (18) and these values did not change at the time of parturition, with the age of the animal, or with the administration of parathyroid hormone.

The first calf heifers all calved normally and none developed milk fever. The plasma levels of calcium and inorganic phosphorus were mildly depressed at parturition, but magnesium showed no change. Administration of parathyroid extract within 2 hr after parturition had no appreciable effect in these young animals (Figure 1, Groups I a and b). Average calcium levels decreased 1.4 mg % just after parturition in control heifers and 1.3 mg % in heifers which received PTE. The lowest level of plasma calcium was observed at the time of

parturition, followed by a slow postpartal increase and a second decline in six of the eight heifers 16 to 24 hr postpartum.

The normal mature cows did not have a history of previous milk fever and, like the first calf heifers, did not show symptoms of milk fever during their current lactation (Figure 1, Groups II a and b). Furthermore, eight of the 14 cows with a past history of milk fever were unaffected during the current parturition (Figure 1, Groups III a and b). Four of these eight cows had received PTE at parturition and four had not. If milk fever did not occur, the mature animals in the several categories (with and without previous milk fever and with and without PTE) could not be distinguished from mature cows in the other categories by their post-parturient changes of plasma calcium or magnesium, even though there was variation between individual animals within a group. These cows appeared to have a modest increase in plasma inorganic phosphorus due to PTE. Together, these mature animals without milk fever showed an average decline of 2.5 mg % of plasma calcium at parturition and this decline was not substantially affected by PTE administration (Figure 1; Groups II a and b, and Groups III a and b). Other scientists (11, 15) have also observed a greater decrease in plasma calcium and in inorganic phosphorus at parturition in mature cows than in younger cows. Since the prepartal decline in the level of plasma calcium has been associated with milk secretion (15), higher-producing mature cows could be expected to show a greater decline in plasma calcium than first-calf heifers.

Preliminary experiments with EDTA infusions (12), and the experiments of Hansard, Comar, and Plumlee (6) show that young cattle metabolize calcium more efficiently than do mature cattle. In the younger animals of the current experiment the lowest level of plasma calcium was observed 1 to 2 hr after parturition and it rose rapidly. In the mature cows not showing milk fever the lowest level of plasma calcium was not observed until 6 to 12 hr postpartum and it rose more slowly. By 48 hr postpartum, the plasma calcium in both age groups had returned to a normal level of about 10 mg %. In the mature animals there was a gradual increase in plasma magnesium which reached its maximum average level (3.0 mg %) about 4 hr postpartum, then slowly returned to a normal level of approximately 2.0 mg %.

Of the 14 cows with a previous history of milk fever six developed milk fever during the experiment and two of these relapsed (Figure 2). The data suggest that two types of milk fever occur and these result from a low level of plasma calcium, or a low level of both phosphorus and calcium. Three cases of the low calcium type were observed and one of the animals received PTE at the time of parturition. When the symptoms of milk fever were observed in these cows plasma calcium levels were 4.7 to 6.4 mg %, inorganic phosphorus levels were 2.0 to 5.4 mg %, and magnesium levels were 3.2 to 4.0 mg % (Figure 2: Cows A-5, A-6, and B-4). These cases of low calcium milk fever all showed good recovery following a single treatment with a commercial calcium-phosphorus preparation.

Milk fever of the low-calcium low-phosphorus type was observed in three cows, of which one had received PTE at parturition (Figure 2: Cows A-2, A-4, and B-3). When treatment with a commercial calcium-phosphorus preparation was initiated, plasma calcium levels were 3.0 to 4.8 mg %, phosphorus levels were 0.4 to 0.8 mg %, and plasma magnesium levels were 3.1 to 4.8 mg %. Magnesium frequently varied inversely with calcium. Two of these cases relapsed and required multiple infusions of calcium-phosphorus solution. At the time of the third treatment, PTE was injected intramuscularly (400 U.S.P. units/100 lb). After this dual treatment, both inorganic phosphorus and calcium were elevated and remained at an adequate level so that milk fever symptoms did not reoccur. It is believed that PTE was beneficial in the treatment of these two cases of low-calcium, low-phosphorus milk fever. In the third low-calcium, low-phosphorus case the cow had been injected with PTE following parturition and calcium therapy was delayed to allow the hormone to exert its effect. Within 2 hr after milk fever symptoms were first observed the animal rolled on to her side and bloated. Following calcium-phosphorus therapy milk fever symptoms were alleviated and plasma levels of calcium and inorganic phosphorus increased (Figure 2, B-3). However, the animal died 4 hr after calcium-phosphorus therapy, due to inhalation of rumen ingesta as revealed by post-mortem examination.

The observations on low-calcium, low-phosphorus milk fever are similar to those reported by Marr, Moodie, and Robertson (10) and Robertson et al. (14), who found that milk fever animals which relapse or show a poor response to calcium therapy often have a very low plasma level of both phosphorus and calcium. They

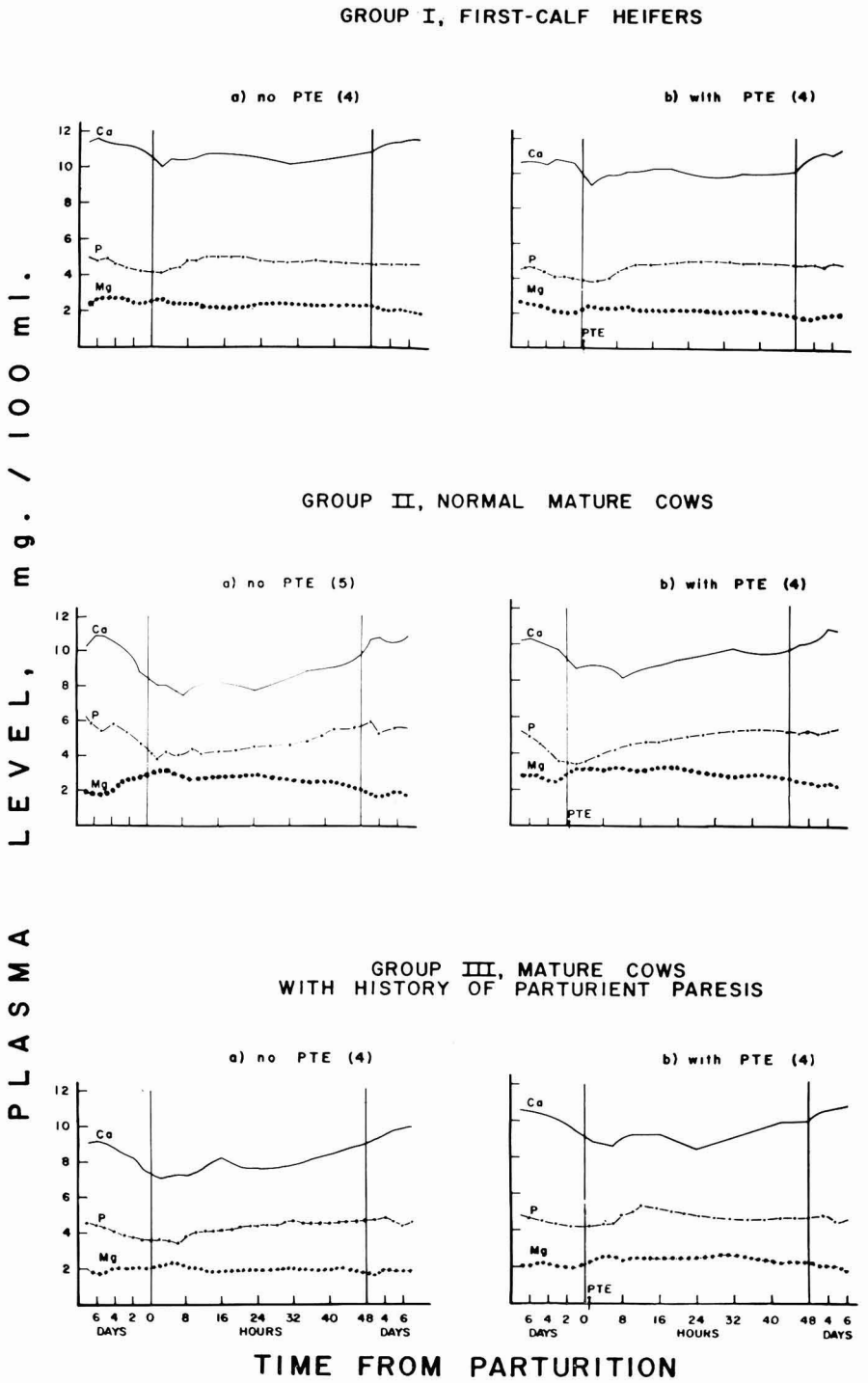

FIG. 1. Average plasma levels of calcium, inorganic phosphorus, and magnesium in cows which calved normally (the number of animals in each subgroup is shown in parentheses).

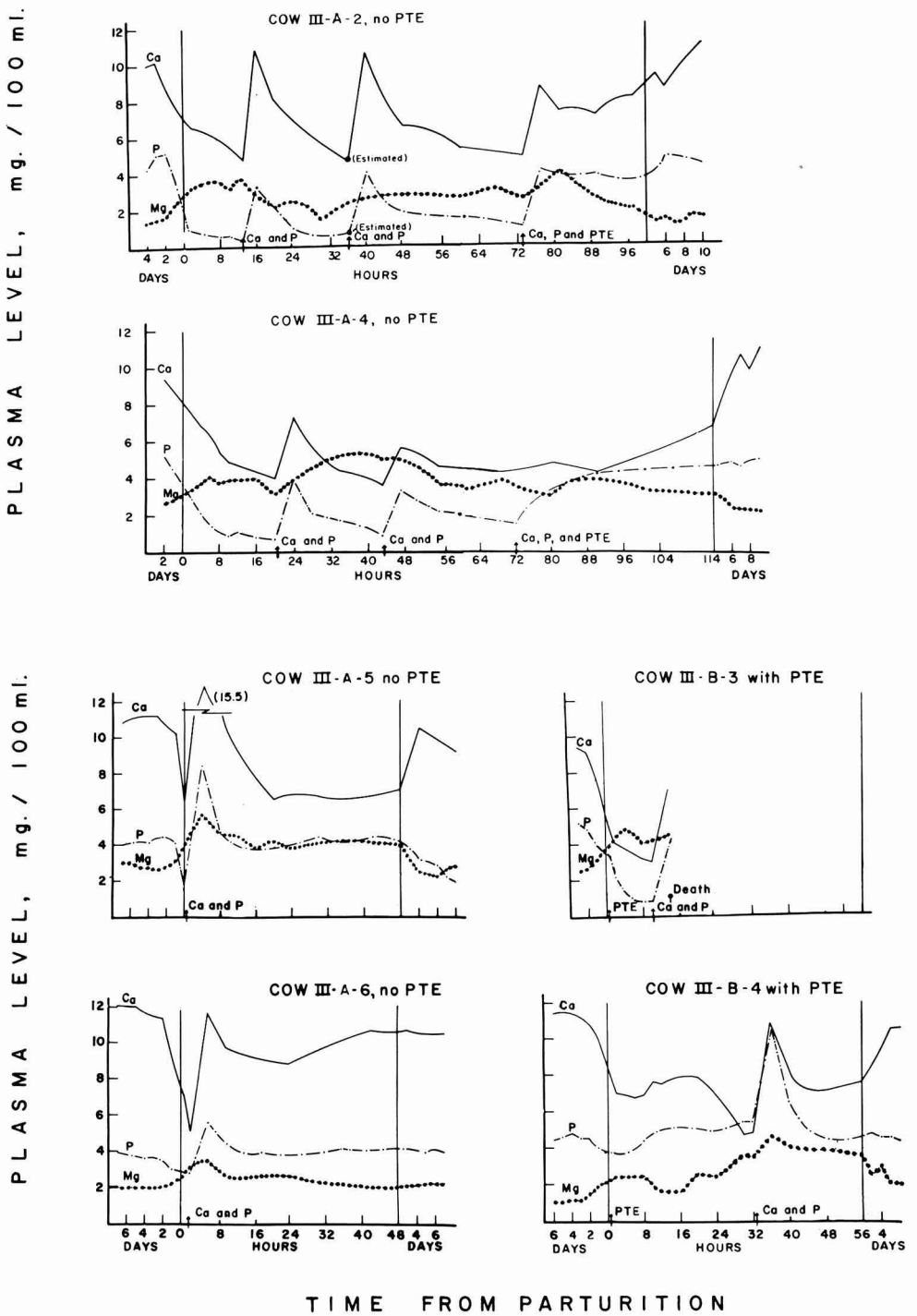

FIG. 2. Plasma levels of calcium, inorganic phosphorus, and magnesium in cows which developed parturient paresis. The arrows (↑) indicate the time of treatment with parathyroid extract and/or calcium-phosphorus solutions.

observed that the changes in magnesium in milk fever cows are no greater than those in normal parturitions and also indicated that udder inflation was beneficial in severe cases of milk fever, as it prevented milk secretion and the additional drain of calcium and phosphorus from the body. Hallgren (5) related that downers which did not respond to calcium therapy could be treated by the further injection of phosphorus alone. These reports help emphasize the role of blood phosphorus in parturient paresis.

Experiments of Robinson, Huffman, and Burt (16) with dairy calves have shown that PTE causes a marked increase in the level of plasma calcium and an increase in the excretion of urinary phosphorus. Experiments in this laboratory using EDTA infusions to depress plasma calcium (12) have shown that yearling heifers have a greater response to exogenous PTE than do mature cattle. For these reasons it is especially difficult to explain why the first calf heifers were not responsive to the administration of PTE at parturition. Near the end of the experiment a portion of the PTE was administered to two dry mature cows and their plasma calcium was increased 1.7-1.9 mg % after 16 hr and the maximum levels were maintained through 32 hr from the time of injection (Figure 3). Thus, the PTE had remained

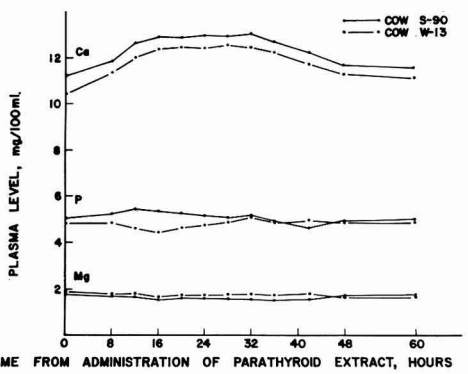

FIG. 3. Response of two dry mature cows to parathyroid extract.

potent throughout the experiment. Plasma phosphorus was increased modestly in one cow and decreased modestly in the other due to the parathyroid injection, but there was no change in the level of plasma magnesium. These data extend the observation of Hibbs et al. (8), that PTE will elevate plasma calcium in nonparturient cows. Since dry cows showed a marked increase in plasma calcium in response to PTE, and since parturient cows did not, the experiments show that exogenous parathyroid extract is ineffective in parturient cows. Either an inactivation of the hormone (8) or a temporary nonresponsiveness of the cow at parturition could explain the apparent ineffectiveness of parathyroid hormone at calving time.

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EFFECTS OF WHEAT GERM OIL ON REPRODUCTIVE EFFICIENCY IN REPEAT-BREEDER COWS [1]

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SUMMARY

Clinically normal repeat-breeder cows that had failed to maintain pregnancy after three or more services were alternately assigned to the experimental or control group. The experimental cows received 2 oz of wheat germ oil on their feed once a week for six consecutive weeks. A significantly higher percentage of the 79 treated cows maintained pregnancy after the first post-treatment service than of the 88 untreated cows. The difference in post-treatment conception between treated and untreated cows that had three unsuccessful services before treatment was not significant; however, there was a highly significant difference in favor of the treated cows that had been bred four or more times before initiation of treatment.

The effects of wheat germ oil on reproductive efficiency in cattle and swine have received considerable attention. However, most of the original work was concerned with the vitamin E content of the oil (4). Since it is difficult to obtain an animal diet deficient in vitamin E, its addition to normal, well-balanced animal diets has proved to be of no value. Some investigators have reported favorable results from adding wheat germ oil to normal diets. Unfortunately, most of those studies were not adequately controlled to permit accurate interpretation of the results.

In the field of human medicine, Watson and Tew (10) and Silbernagel and Patterson (8) reported wheat germ oil to be a valuable therapeutic agent in reducing the incidence of the complications of pregnancy. Compton (2, 3) concluded from a 2-yr study that no benefits resulted from adding wheat germ oil to the ration of pregnant sows. However, sows that received wheat germ oil farrowed larger pigs, and a greater percentage of their pigs survived. Salisbury (6) found no effects on the reproductive performance of dairy bulls resulting from feeding 1 oz of solvent process wheat germ oil daily for 1 yr. Bay and Vogt-Moller (1), Tutt (9), and Pacini (5) have reported beneficial results from treating repeat-breeder cows with wheat germ oil. Pacini (5) suggested that the favorable results might not have been entirely due to vitamin E.

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[1] Furnished by VioBin Corporation, Monticello, Ill.

EXPERIMENTAL PROCEDURES

This study was conducted during a 3-yr period at intervals to include all seasons of the year, using clinically normal, parous, repeat-breeder cows representing four dairy breeds in the Kansas State University herd. All cows that had been bred two or more times and had been determined to be clinically normal were arbitrarily assigned to either the experimental or the control group. All cows were checked for estrus twice daily and were inseminated approximately 8 hr after being detected in estrus. Semen from bulls of known reproductive capacity was used, and every effort was made to use semen from the same bulls in both the experimental and control groups, and to have all inseminations performed by one individual.

All cows of both groups were fed and managed in the same manner. During the 3-yr period that this study was conducted, the ration fed to the cows varied considerably, depending on availability of feeds. The roughage varied from alfalfa hay fed ad libitum and small quantities of silage to ad libitum feeding of sorghum silage and limited quantities of hay. Some brome pasture was available during the pasture season. A grain mixture containing approximately 16% crude protein was fed according to production. In addition to the ration received by all cows, the experimental cows received 2 oz of wheat germ oil per week for

6 wk. The oil was added to one feeding of grain each week. Alternate cows that had been bred two or more times were assigned to the experimental group and received the first feeding of wheat germ oil on the same day or the day following returning in estrus. A similar cow of the same age and breed, that represented an equal number of repeat-services, was assigned as her control. The number of cows that conceived to the service concurrent with the initiation of treatment was calculated. It is doubtful that these cows could have been influenced by the first treatment; therefore, if conception occurred at this service the cows were not included in the analyses. The number of post-treatment services required for conception by each experimental and each of the control cows was calculated. The data for both groups of cows were analyzed statistically by use of the Kolmogorov-Simirnov test (7). All cows that were determined to be clinically abnormal were excluded from the study.

RESULTS AND DISCUSSION

The data presented in Table 1 are arranged to indicate the distribution of cows among the post-treatment service groups. The Kolmogorov-Smirnov test was used to determine the significance of differences in the distribution of the cows among the service-per-conception groups. The analyses indicated no significant difference between the treated and untreated cows that had been bred three times pretreatment ($D = 0.14$, $P > .30$). A highly significant difference existed between the distribution of treated cows bred four or more times before treatment initiation and similar untreated cows ($D = 0.46$, $P < 0.001$). Pooling data for all cows on experiment gave highly significant ($D = 0.30$, $P < 0.001$) differences in distribution in favor of the wheat-germ-treated cows. A much higher percentage of the treated than untreated cows settled as a result of the first post-treatment service.

Only 22 or 22% of 101 cows that had been bred two or more times, which were to be assigned to the experimental group conceived to the service concurrent with the initiation of treatment, as compared to 19 or 18% of 107 similar cows that were to serve as controls. This low rate of conception is at least partially explainable by the fact that at this time the herd was being used for artificial insemination practice.

The percentage of cows bred that conceived to a specific post-treatment service and the number of services per conception were calculated; however, the limited number of animals in each group would not permit an accurate interpretation of the results. It is also realized that the biological significance of this study would have been improved with a considerably greater number of animals.

It has been adequately proven that vitamin E, found in high concentration in wheat-germ oil, has no favorable influence on the reproductive efficiency of cattle on normal diets (4). The results reported in this study suggest that other substances in wheat germ oil may be capable of beneficially influencing reproduction in some clinically normal repeat-breeder cattle.

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TABLE 1

Effects of wheat-germ oil on the reproductive performance of repeat-breeder dairy cows

Post-treatment service on which conception occurred	Cows bred 3× before treatment				Cows bred 4× or more before treatment				Total cows on experiment			
	Treated		Untreated		Treated		Untreated		Treated		Untreated	
	(No.)	*(% of total)*	*(No.)*	*(% of total)*	*(No.)*	*(% of total)*	*(No.)*	*(% of total)*	*(No.)*	*(% of total)*	*(No.)*	*(% of total)*
1st	28	68	23	52	30	80	15	34	58	73	38	43
2nd	9	22	15	34	2	5	13	30	11	14	28	32
3rd	3	7	0	0	1	3	10	23	4	5	10	11
4th	0	0	3	7	2	5	1	2	2	3	4	5
5th	1	3	2	5	1	3	3	7	2	3	5	6
6th	0	0	1	2	0	0	2	4	0	0	3	3
7th	0	0	0	0	2	5	0	0	2	3	0	0
	41		44		38		49		79		88	

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SURVIVAL OF BULL SPERM IN MILK AND YOLK EXTENDERS WITH ADDED CATALASE

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SUMMARY

After 12 days of storage at 5 C the percentage of motile sperm in CUE containing 0, 1, 10, 100, and 200 μg of catalase per milliliter of extender was 28, 37, 44, 44, and 45, in CU-16 was 35, 43, 47, 52, and 50, in CU-16 plus chlorpromazine hydrochloride was 29, 35, 35, 41, and 43, and in 20% yolk-citrate was 24, 34, 38, 37, and 34, respectively. All levels of catalase improved sperm survival significantly ($P < .01$), but little additional response was observed with catalase levels higher than 10 μg/ml of extender. The concentration of catalase required to produce a maximum response was much greater than that theoretically required to eliminate any hydrogen peroxide produced. No beneficial effect of adding 20 μg of catalase per ml of skimmilk or skimmilk-glycerol extender was found. The percentage of motile sperm after 12 days of storage at 5 C averaged 47 for CU-16 extender with catalase, 11 for skimmilk, and 12 for skimmilk with catalase. The reason for the differential response to catalase in egg yolk versus milk has not been elucidated.

Bull semen has been reported to contain little catalase (7). Tosic and Walton (12) showed that bull sperm produced H_2O_2, and that added catalase was helpful in preventing H_2O_2 from depressing motility of sperm. Also, catalase had been shown to partially protect sperm cells from the harmful effects of agitation, oxygen, and exposure to light (2, 5, 9, 10, 14). But VanDemark et al. (13) found no effect of catalase on fertility of semen used principally within two days of collection. Present trends of retaining some semen in the field for longer periods of time before use could result in H_2O_2, causing appreciable damage to sperm cells. The studies reported here were undertaken to establish levels of catalase optimum for sperm survival in egg yolk and milk extenders. Since these studies were completed catalase has been reported by Hafs (8) to improve fertility of bull sperm stored in homogenized whole milk containing glycerol, but not to improve fertility of sperm in egg yolk. No studies with varying levels of catalase were reported.

EXPERIMENTAL PROCEDURE

Semen obtained from bulls in the regular stud of the New York Artificial Breeders' Cooperative, Inc. was rapidly evaluated for sperm motility and concentration. Sufficient semen was added to the experimental extenders to give ten million motile sperm per milliliter of extender. On the average, the semen was extended at a rate greater than 1:100.

Composition and preparation of the CUE and CU-16 extenders used has been described previously by Foote et al. (4, 6). Skimmilk was prepared according to the method described by Almquist et al. (1). The final concentration of glycerol in the glycerolated skimmilk was 10% by volume. All extenders contained 1,000 units of penicillin and 1,000 μg of dihydrostreptomycin per milliliter.

The catalase used throughout was a twice-crystallized lyophilized beef liver catalase obtained from Nutritional Biochemicals Corporation. The standard method for assaying this product changed during the investigations reported here. The same product originally listed as containing 100 units/mg later was listed as containing 3,000 units/mg. To avoid confusion, all data here are listed in terms of micrograms (μg), rather than units of catalase.

Two milliliters of experimentally extended semen was stored at 5 C in corked 2.5-ml plastic tubes. At regular intervals these tubes were mixed, subsampled, and the percentage of motile sperm determined microscopically at 430× in a stage incubator set at 37 C. The pH's of the extenders were determined with a Beckman Model G pH meter.

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TABLE 1
Sperm survival in yolk extenders with added catalase
(Average of ten ejaculates)

Days stored at 5 C	CUE				CU-16				50% EYC [a]			
	Catalase, μg/ml				Catalase, μg/ml				Catalase, μg/ml			
	0	1.0	10	100	0	1.0	10	100	0	1.0	10	100
1	62	62	62	63	63	62	63	62	60	61	62	61
4	49	54	54	56	52	52	55	52	49	46	52	48
8	46	45	46	51	45	52	48	48	37	44	43	45
12	31	38	39	48	29	36	40	39	24	30	32	36
Average	47	50	50	54	47	50	52	50	42	45	47	48

[a] This 50% yolk-citrate extender was the type used in earlier studies (13, 14).

The statistical analyses were carried out as outlined by Steel and Torrie (11). Bulls were considered to be a random variable, and catalase levels, types of extenders, and other variables were considered to be fixed effects.

RESULTS

In the first experiment, levels of catalase were chosen which might bracket some of the levels used before purified preparations were readily available commercially (12, 14). Catalase was added to CUE and CU-16, as well as a 50% egg yolk–citrate extender used in earlier studies (13, 14), at levels of 0, 2.5, 5.0, and 10 μg/ml of extender. Catalase significantly improved survival of sperm in ten ejaculates of semen stored for 12 days at 5 C ($P < .01$). The highest motility was observed in the extenders containing the most catalase. Since the upper limit of response did not appear to have been established, the experiment was repeated, using a greater range of catalase concentrations. The results of this second experiment are shown in Table 1. Again catalase improved sperm survival at all levels ($P < .01$). Increasing the level of catalase above 10 μg/ml seemed to be beneficial in CUE but not in the other extenders. The 50% egg yolk–citrate extender gave significantly poorer results ($P < .01$), but the motility estimations were more difficult to make in this extender. The pH of all extenders was within the range from 6.6 to 6.8.

The results of a third experiment are shown in Table 2. In this experiment a higher level of catalase was added, a 20% yolk-citrate extender was included, and chlorpromazine hydrochloride was added to one extender. The latter compound was included because of the possibility of its affecting cell permeability (4).

Again, catalase improved sperm survival ($P < .01$). The highest level of catalase was not superior to the 100 μg/ml level, and most of the improvement was achieved by including 10 μg/ml. Clumping of sperm was observed occasionally in the extenders containing high levels of catalase. Sperm survival in the 20% yolk-citrate was inferior to the other extenders ($P < .01$).

Subsequent studies revealed that catalase at 20 μg/ml gave a uniformly good response in yolk-extenders without the clumping observed at higher levels. The effect of adding this level of catalase to skimmilk and to skimmilk-glycerol extenders in two experiments is shown in Tables 3 and 4. No response to catalase was observed in the skimmilk, or in the skimmilk-glycerol extender. Sperm survival in the CU-16 was significantly higher than in skimmilk extender ($P < .01$), and glycerol improved sperm survival in skimmilk ($P < .01$).

DISCUSSION

The addition of from 1 to 100 μg of catalase per milliliter of yolk extender substantially improved the survival of bull sperm stored at 5 C. Additional amounts of catalase did not improve sperm survival and tended to increase clumping of sperm. The beneficial effect of catalase in sperm survival is presumably accomplished through the decomposition of H_2O_2 by catalase. Desjardins and Hafs (3) have reported that egg yolk contains small quantities of catalase. The consistent response reported here with added catalase indicates that the amount present in egg yolk is insufficient for optimum sperm survival.

The amount of catalase required for maximum response is far more than should be required to decompose the H_2O_2 produced (12). Perhaps a high extracellular concentration is required to obtain a small quantity of intracellular catalase, where H_2O_2 production might damage the cell. However, it is not certain that catalase will penetrate the sperm cell membrane. The inclusion of chlorpromazine hydrochloride, as a possible means of altering perme-

TABLE 2

Sperm survival in 20% yolk extenders with added catalase
(Average of ten ejaculates)

Days stored at 5 C	CUE				CU-16				CU-16Th [a]				20% EYC [b]			
	Catalase, μg/ml				Catalase, μg/ml				Catalase, μg/ml				Catalase, μg/ml			
	0	1	10	100	200	0	1	10	100	200	0	1	10	100	200	0	1	10	100	200
	(% motile sperm)																			
1	60	60	60	61	61	59	59	60	60	59	60	60	59	60	60	56	56	53	54	52
4	56	54	57	57	54	57	58	58	58	57	57	58	55	57	56	50	51	51	49	47
8	49	50	57	53	53	55	57	58	57	54	47	48	48	53	52	42	46	44	45	42
12	28	37	44	44	45	35	43	47	52	50	29	35	35	41	43	24	34	38	37	34
Average	48	50	54	54	53	52	54	56	57	55	48	50	49	53	53	43	47	46	46	44
pH's	6.9	6.9	6.9	6.9	6.9	6.6	6.6	6.6	6.6	6.6	6.5	6.5	6.5	6.5	6.5	6.8	6.8	6.8	6.8	6.8

[a] Contained 200 μg/ml of chlorpromazine hydrochloride (Thorazine).
[b] Buffer composed of 2.9% sodium citrate buffer plus 0.6% sulfanilamide; extender was four parts buffer to one part egg yolk.

TABLE 3

Sperm survival in skimmilk with added catalase
(Average of ten ejaculates)

Days stored at 5 C	CU-16 plus 20 μg/ml catalase	Skimmilk	Skimmilk plus 20 μg/ml catalase
	(% motile sperm)		
1	55	55	55
4	57	48	49
8	53	31	30
12	47	11	12
Average	53	36	36

ability, provided no evidence for increased effectiveness of low concentrations of catalase.

The ineffectiveness of catalase in the skimmilk extender is difficult to explain. The necessary substrate is present in milk from which sperm cells could produce H_2O_2 in the same manner as in egg yolk. Hafs (8) has reported that catalase added to homogenized whole milk containing glycerol resulted in a sizable and statistically significant improvement in fertility, but catalase had no effect on fertility when added to yolk extender. Whether glycerol interacts in any way with catalase to improve fertility is unknown. It did not interact with glycerol to affect motility. The beneficial effects of glycerol may be because glycerol can be utilized by sperm to produce fructose, which in turn sperm can utilize by glycolysis.

The experiments reported here clearly demonstrate that catalase prolongs survival of bull sperm in yolk extenders but not in skimmilk. The reasons for this differential response, as well as the reasons for the apparent excess of catalase required for optimum response in egg yolk, have not been elucidated.

TABLE 4

Sperm survival in skimmilk-glycerol with added catalase
(Average of ten ejaculates)

Days stored at 5 C	Skimmilk		Skimmilk-glycerol	
	0 catalase	20 μg/ml catalase	0 catalase	20 μg/ml catalase
	(% motile sperm)			
1	61	60	59	59
4	54	50	56	54
8	32	33	44	49
12	12	16	32	34
Average	40	40	48	49

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DILUENTS FOR BOVINE SEMEN. XI. EFFECT OF GLYCEROL ON FERTILITY AND MOTILITY OF SPERMATOZOA IN HOMOGENIZED MILK AND SKIMMILK [1]

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SUMMARY

When 10 or 13% glycerol was incorporated in skimmilk diluent by gradual addition to partially diluted semen at 5 C, spermatozoan livability during 14 days of storage at 5 C was higher ($P < 0.01$) than in the absence of glycerol. These glycerol levels prevented the sharp decline in motility observed between the fourth and sixth days of storage for semen diluted in skimmilk without glycerol. Glycerol levels of 16 to 25% did not significantly improve livability over semen diluted without glycerol.

Four field trials involving 21,676 inseminations were conducted to compare the fertility of semen diluted in milk with that diluted in milk–10% glycerol. Homogenized milk was used in two trials and fresh, unfortified skimmilk in the other two. The combined data showed that addition of glycerol significantly ($P < 0.01$) increased fertility of semen used the second, third, and fourth days after the day of collection by 7.4, 12.2, and 19.5 percentage units, respectively. The increase of 2.3 percentage units obtained with glycerolated semen used the day after collection was not significant. In a limited split-ejaculate trial, fertility of semen diluted in homogenized milk containing 15% glycerol was not significantly different from that in 10% glycerol on any of the three days of use.

In 1952, Polge and Rowson (10) reported that incorporation of glycerol improved the fertility of liquid bovine semen. The next year, Holt (6) substantiated this report with more extensive fertility data involving a comparison of egg yolk–citrate diluent with and without 10% glycerol. Whether glycerol would prolong the time that liquid semen could be used with satisfactory fertility results was not reported.

During 1955, the feasibility of using combinations of glycine and skimmilk as a diluent for bovine semen was investigated at this laboratory (4). Addition of glycerol to skimmilk-glycine improved livability and increased the otherwise relatively low motility readings observed during the first week of storage at 5 C. Subsequent work showed that glycerol prolonged sperm livability in skimmilk diluent without glycine, and fertility trials were begun early in 1956. That year McLean (7) reported improved livability when 10% glycerol was added to either egg yolk–citrate or homogenized milk diluents. The effect of different glycerol levels on spermatozoan motility and fertility in fresh milk diluents has not been reported.

The present report concerns the effect of glycerol level on the livability of bovine spermatozoa in skimmilk and the fertility of liquid semen over a four-day period when diluted in glycerol-containing homogenized milk and skimmilk.

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GENERAL PROCEDURE

Fresh, unfortified skimmilk was used in the laboratory study and in two of the five fertility trials; homogenized milk was used in the other fertility experiments. The milk was heated at 92 to 95 C for 10 min. In both laboratory and field experiments, similar techniques were used for preparation of the diluted semen. As soon as possible after collection, the semen to be glycerolated was partially diluted in milk diluent without glycerol and cooled gradually to 5 C. Final dilution was achieved by adding an equal volume of cooled milk diluent containing twice the desired final concentration of glycerol. The glycerol frac-

tion was added at 5 C in three equal volumes at 10-min intervals. Control semen was diluted completely at room temperature just prior to cooling. In all studies, the final diluted semen contained 1,000 units of potassium penicillin G and 1,000 μg of dihydrostreptomycin sulfate per milliliter.

The split-ejaculate design was used, except in Trial 4. In Trial 4, six bulls normally collected on Saturday were divided into two groups; the ejaculates of one group were treated with milk–10% glycerol, whereas those of the other group served as controls. The treatments were alternated between bull groups on succeeding weeks. In all trials, both glycerolated and control semen was shipped in filled tubes. Variations in semen handling procedures are described under the individual experiments.

Livability of diluted semen stored at 5 C for 14 days was studied by estimating the percentages of progressively motile spermatozoa at 37 C. Fertility was recorded as the per cent 60- to 90-day nonreturns for each day of use after collection. Semen was used from two to four days, but none was used on the day of collection.

SPECIFIC PROCEDURES AND RESULTS

Livability study. Glycerol levels of 0, 10, 13, 16, 19, 22, and 25% by volume were tested. Mean percentages of progressively motile sperm for eight ejaculates from six bulls are presented (Table 1). Highest mean livability was obtained with 10% glycerol. This level supported livability superior ($P < 0.01$) to the 0, 19, 22, and 25% levels, but was not significantly better than the 13 and 16% levels when tested by Tukey's honestly significant difference among means. Mean motility with 13% glycerol was higher ($P < 0.01$) than with 0, 22, and 25% glycerol, but only significantly better ($P < 0.05$) than 19% glycerol.

Fertility trials. The 10% level of glycerol was selected for initial fertility testing because it was best in the livability study. A preliminary trial to compare milk and milk–10% glycerol diluents was started in June, 1956. Technicians were requested to use semen of each experimental bull to breed at least one first- or second-service cow per day for a period of four days after the day of collection. The number of services on Days 1, 2, 3, and 4 was 4,495, 1,937, 731, and 535. There was no difference in the average fertility of semen diluted in homogenized milk with or without 10% glycerol when used the first day after collection. However, the fertility of glycerolated semen used the second, third, and fourth days after collection was 8, 9, and 13 percentage units higher than that of untreated control semen. Since the technicians were carrying fresh semen from nonexperimental bulls of the same breed, it is possible that the results were biased by selection of the herd and female in which the older experimental semen was used.

Trials 1 and 2. Because of the indicated beneficial effect on fertility of adding glycerol, two fertility trials were initiated to gather data in which use of the semen would be less subject to technician selection. Trial 1 was conducted during October, 1956, at a different artificial breeding cooperative. Three very popular Holstein bulls collected two days per week were selected. The semen diluted in skimmilk–10% glycerol was used for four days, but the control semen was used only two days. Only enough glycerolated and control semen was shipped to technicians on the day of collection to meet the needs for the next day. The remaining semen was held in bulk storage in a walk-in cold room and portions shipped on succeeding days.

In Trial 2, conducted by the breeding cooperative which carried out the preliminary trial,

TABLE 1

Effect of glycerol level on livability of spermatozoa in skimmilk diluent (Mean percentage of motile sperm in eight ejaculates)

Glycerol level	Days of storage at 5 C									
	1	2	3	4	6	8	10	12	14	Mean
(%)										
0	70	66	64	64	58	46	30	21	11	43.0
10	70	70	66	66	64	60	51	46	34	52.7
13	70	69	68	65	64	55	49	42	31	51.2
16	70	68	68	63	60	55	40	36	24	48.2
19	69	65	61	58	56	48	35	28	14	43.2
22	69	64	60	56	52	46	32	28	18	42.5
25	65	52	51	42	36	31	22	11	6	31.9

TABLE 2

Fertility of semen in Trials 1 and 2

Age of semen	No. services [a]		Per cent nonreturns		
	Control	Glycerol	Control	Glycerol	Diff.
		Trial 1—Skimmilk			
Day 1 [b]	636	747	72.2	74.8	+2.6
Day 2	711	721	65.0	69.6	+4.6
Day 3	.....	1,328		68.7	
Day 4	.....	697		66.9	
		Trial 2—Homogenized milk			
Day 1	2,110	2,166	73.2	75.5	+2.3
Day 2	2,460	2,425	62.4	70.6	+8.2
Total	4,570	4,591			
Mean			67.4	72.9	+5.5

[a] Trial 1 based on first services; Trial 2 on first and second services.
[b] Day after semen collection.

semen from bulls collected on Saturday was routinely used for two days after collection. No semen was shipped on Sunday. The three-month trial with semen used routinely for two days was started in December, 1956, and involved semen from five Guernsey and Holstein bulls. Nonreturns were recorded for both first and second services.

The fertility results for Trials 1 and 2 (Table 2) show that the addition of 10% glycerol retarded the decline in fertility of semen with increasing age. With glycerol present, fertility decreased about 5% from Day 1 to Day 2 in both trials, whereas without glycerol the decline was about 9%. Of particular interest in Trial 1, despite the lack of control data, was the very small decrease in fertility from Day 2 to Day 4.

Trials 3 and 4. Based on these results, two trials were initiated in 1957, to compare the fertility of semen diluted in milk with and without 10% glycerol, when both were used beyond the second day after collection. In Trial 3, semen shipments were made daily for four days, as in Trial 1. However, the semen was held in the central laboratory in filled test tubes rather than in bulk. The trial included eight ejaculates from each of the two Guernsey and four Holstein bulls in heaviest service at the time. Trial 4 involved only bulls previously collected on Saturday to provide semen for use on Sunday and Monday. These six bulls were divided into two groups. Rather than being collected on Saturday, one group was collected on Thursday and the other on Friday, and this procedure was reversed on alternate weeks. The semen was held in filled test tubes at the central laboratory for either one or two days before shipment on Saturday. Thus, half of the eight ejaculates collected per bull were used for insemination on the second and third days and half on the third and fourth days after collection. In Trial 4, the milk–20% glycerol was added to the partially diluted semen in volumes of 20, 30, and 50% at 10-min intervals.

The fertility results (Table 3) substantiate the earlier data, in that the inclusion of 10% glycerol in milk diluents markedly reduced the decrease in fertility with advancing age of semen as compared to milk diluent without glycerol. In both trials, the mean fertility of semen diluted in milk–10% glycerol was superior ($P < 0.01$) to that in milk alone.

The combined fertility results for the four trials conducted at three breeding cooperatives (Table 4) show a significant ($P < 0.01$) improvement in mean fertility on Days 2, 3, and 4 when glycerol was added to the milk diluents. The difference of 2.3% in favor of milk–10% glycerol on Day 1 was insignificant.

Trial 5. The livability experiment showed no significant difference among the 10, 13, and 16% levels of glycerol, and fertility trials with levels of glycerol other than 10% had not been reported. Thus, a limited split-ejaculate field trial was conducted during November, 1959, to compare 10 and 15% glycerol. Semen from three bulls collected two days each week was used to inseminate 3,470 cows. Semen collected on Friday from these bulls was used routinely for three days after the day of collection; no semen was collected on Saturday or Sunday. While the partially diluted semen was being cooled, the glycerol fraction was added dropwise from a separatory funnel over a period of at least 30 min.

The average nonreturn rates for the 24 ejacu-

TABLE 3

Fertility of semen in Trials 3 and 4

Age of semen	No. first services		Per cent nonreturns		
	Control	Glycerol	Control	Glycerol	Diff.
		Trial 3—Skimmilk			
Day 1	743	704	68.6	70.7	+ 2.1
Day 2	941	833	67.7	74.5	+ 6.8[a]
Day 3	839	825	62.9	68.9	+ 6.0[a]
Day 4	627	714	50.1	69.7	+19.6[b]
Total	3,150	3,076			
Mean			63.2	71.1	+ 7.9[b]
		Trial 4—Homogenized milk			
Day 2	324	292	63.0	71.9	+ 8.9[a]
Day 3	916	825	51.6	69.4	+17.8[b]
Day 4	538	579	44.8	63.9	+19.1[b]
Total	1,778	1,696			
Mean			51.6	68.0	+16.4[b]

[a] Difference statistically significant at the 5% level.
[b] Difference statistically significant at the 1% level.

lates are shown in Table 5. Although the mean fertility for milk–15% glycerol was 2.4 percentage units lower than that for milk–10% glycerol, this difference and those for day of use were not statistically significant.

DISCUSSION

Although it has been shown that glycerol improves the motility of bovine spermatozoa stored in milk diluents at 5 C (1, 7), different glycerol levels were not compared. In the present study, motility in fresh, heated skimmilk was maintained at a significantly higher level with 10 or 13% glycerol than with no glycerol ($P < 0.01$). There was a gradual decline in mean livability when glycerol level increased from 10 to 25%. Glycerol levels lower than 10% were not tested. However, further studies at this laboratory (3) showed that 5% glycerol supported higher livability in fresh skimmilk than 10% glycerol ($P < 0.01$), but that fertility was not significantly different. Willett and Ohms (12) assessed livability in a nonfat milk solids-cysteine diluent with glycerol levels of 0, 1.25, 2.5, 5.0, and 10.0%. They found that motility increased with increasing glycerol and that motility with 5 or 10% glycerol was significantly greater than with no glycerol ($P < 0.05$).

Based on the livability studies, 10% glycerol was selected for use in the fertility trials. Results of the four trials clearly demonstrated that the inclusion of 10% glycerol retarded the decline in fertility associated with increasing age of liquid semen. Although inclusion of glycerol did not significantly increase fertility of semen used the day after collection, highly significant ($P < 0.01$) mean increases of 7.4, 12.2, and 19.5 percentage units were obtained for semen used the second, third, and fourth days after collection. Limited data indicated no significant difference between 10

TABLE 4

Combined fertility results for four trials[a]

Age of semen	No. services		Per cent nonreturns		
	Control	Glycerol	Control	Glycerol	Diff.
Day 1	3,489	3,617	72.1	74.4	+ 2.3
Day 2	4,436	4,271	64.0	71.4	+ 7.4[b]
Day 3	1,755	1,650	57.0	69.2	+12.2[b]
Day 4	1,165	1,293	47.6	67.1	+19.5[b]
Total	10,845	10,831			
Mean			63.7	71.5	+ 7.8[b]

[a] Excluding Days 3 and 4 of Trial 1, for which there were no control data.
[b] Difference statistically significant at the 1% level.

TABLE 5

Fertility of semen diluted with heated homogenized milk containing 10 or 15% glycerol, Trial 5

Age of semen	No. first services		Per cent nonreturns		
	10% Glycerol	15% Glycerol	10% Glycerol	15% Glycerol	Diff.
Day 1	775	771	75.6	72.4	−3.2
Day 2	548	614	72.4	73.6	+1.2
Day 3	386	376	74.4	68.1	−6.3
Total	1,709	1,761			
Mean			74.3	71.9	−2.4

and 15% glycerol when the semen was used for three days. The finding that the addition of glycerol to milk diluents reduced the decline in fertility with increasing age of semen is in agreement with other reports (9, 13). However, there is only general agreement on the extent of fertility improvement for semen of a particular age.

The only detailed report comparing fertility of bovine semen in milk and milk–10% glycerol diluents was published in England by O'Connor and Smith (9). In a study involving 14,319 first inseminations, they found a significant difference ($P < 0.01$) in favor of semen diluted in fresh skimmilk–10% glycerol only when it was used on the fourth day after collection.

Part of the disagreement may be due to differences in handling of the diluted semen. In the present trials all semen was shipped in filled test tubes, but in the study by O'Connor and Smith this was not a standard practice (8). The importance of shipping filled tubes is indicated from unpublished data of the late E. L. Willett (11). A limited trial in 1957 with four Holstein bulls compared fertility of semen diluted in homogenized milk–10% glycerol when shipped either in filled or half-filled 8-ml tubes. Technicians were requested to use about half of the semen in each tube the first day after collection and the remainder on Day 2 after collection. Based on 1,725 services, the difference of 6.7 percentage units in favor of filled tubes on Day 2 was highly significant ($P = 0.005$). The increase of 1.3 percentage units for filled tubes on Day 1 was not significant (988 services). Thus, the beneficial effect of glycerol in retarding the decline in fertility with older semen may be masked in part by oxygen damage to spermatozoa during transport and field handling. Hafs (5) recently showed that inclusion of catalase in milk-glycerol diluent further reduces the decline in fertility of semen used for four days after collection.

In the only other controlled study, Williams et al. (13) compared fertility of semen diluted in homogenized milk with and without 10% glycerol over a period of three days after collection. Contrary to the present report, and that of O'Connor and Smith (9), they found a significant ($P = 0.02$) increase with glycerolated semen used the first day after collection. Their finding of a highly significant ($P < 0.01$) increase on the second and third days of use when glycerol was added agrees with this report.

The advantages of using milk-glycerol diluent in a liquid semen program, and suggestions for the proper preparation and handling of this diluent, have been presented elsewhere (2). The importance of semen handling procedures is suggested by the differences in the fertility of glycerolated semen on Days 3 and 4 in Trials 3 and 4. On Day 3 fertility was similar in the two trials, but on Day 4 there was a substantial decrease only in Trial 4. In this trial the semen was shipped the second day before use and thus was subjected to routine field handling for one day. In Trial 3, the semen was shipped the day before use and exposed to field handling only on the day of use.

It is not known whether bulls of relatively low fertility show a greater improvement in fertility with milk-glycerol diluent than those of relatively high fertility.

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METABOLISM OF BOVINE SEMEN. XI. FACTORS AFFECTING THE TRANSPORT OF FRUCTOSE IN BOVINE SPERMATOZOA [1]

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SUMMARY

Using fructose-C^{14}, the transport of fructose across the cell membrane of washed bovine spermatozoa has been studied. It was found that fructose transport was sensitive to incubation temperature, to cold shock and to inhibitors such as cyanide, iodacetate, fluoride, and *p*-chloromercuribenzoate. Mutual interference by metabolizable sugars was observed, and transport occurred against a concentration gradient of fructose under anaerobic conditions. Anoxia stimulated the transport process.

The selectivity of living cells toward charged particles has been recognized for some time, but it has been generally assumed that cell membranes are freely permeable to uncharged molecules such as hexoses. Doudoroff et al. (4) opened a new era in 1949 with the suggestion of specific transport mechanisms for sugars. In exploiting this break-through, numerous tissues (3, 6, 7, 9-11) have been used in studying the transfer of sugars across cell membranes: that is, between the cell interior and interstitial fluid, blood plasma, or an artificial bathing medium. As recently reviewed by Bishop (1), many reports have appeared on the metabolism of sugars by spermatozoa, but there appears to be no information on the mode of sugar entry into spermatozoa.

This report presents results of a series of experiments conducted to investigate the nature of the movement of fructose across the cell membrane of bovine spermatozoa.

EXPERIMENTAL PROCEDURE

Semen was collected from Holstein bulls with the artificial vagina, examined for quality, and ejaculates from three or four bulls pooled for use in each trial. The spermatozoa were washed in Ringer solution to free them of seminal plasma, then resuspended in Ringer solution or the buffer to be used in the experiment.

To measure transport, 10^9 cells were suspended in a final volume of 1.5 ml in 6-ml Warburg flasks and incubated with uniformly labeled fructose (fructose-C^{14}). Periods of incubation were restricted to 30-60 min, to limit the metabolic conversion of fructose and release of radioactivity from the cell. After incubation, the cells were washed as rapidly and completely as possible to remove extraneous radioactivity, then plated on cupped glass planchets and dried under infrared lamps. Carbon dioxide produced during incubation was collected in potassium hydroxide in the center well of the flasks and converted to barium carbonate for radioassay (5).

The transport values reported were obtained by adding the radioactivity recovered in the cells and that recovered in CO_2, with the exception of the data in Table 6. These were obtained by measuring loss of radioactivity from the cells into the medium and the procedure will be presented in the next section.

RESULTS AND DISCUSSION

One of the problems encountered in using fructose-C^{14} to measure transport was the development of a satisfactory method of preparing sperm for radioassay. After incubation with fructose-C^{14}, three washings were required to remove extraneous activity from sperm. Since this procedure involved about 15 min, the possibility existed that much of the radioactivity might come out of the cell during wash-

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TABLE 1

Effect of washing buffer temperature and composition on retention of fructose-C^{14}

Buffer	Temperature	
	4 C	21 C
	(counts per minute)	
Ringer-phosphate	2,250	4,100
Skimmilk	1,400	1,050
Ringer-phosphate-fructose	2,900	3,640

ing. To determine possible effects of temperature and the wash medium on sperm radioassays, sperm were incubated with fructose-C^{14} at 37 C, then aliquots were washed with three media at two temperatures, as shown in Table 1.

It is noted that in Ringer-phosphate and Ringer-phosphate-fructose, retention of radioactivity by sperm was greater at 21 C than at 4 C. A possible explanation is that at the 4 C wash some temperature shock to sperm occurred which reduced the ability of the cells to retain fructose (8). The performance of skimmilk as a wash in these experiments supports this explanation, in that skimmilk provides more cold shock protection, and the 4 C:21 C ratio was much higher than in the washes with less cold shock protective action. All radioassays on the milk treatments were lower than those in chemical media; this would be expected as a result of the shielding effect of the milk solids present on the planchets.

Fructose was included in the third wash medium to determine its effectiveness in reducing loss of radioactivity by simple diffusion from sperm. It did not reduce loss from the cell, indicating that transfer of fructose from the cell involves more than simple diffusion. In subsequent trials, the standard post-incubation washing procedure consisted of three washes with Ringer-phosphate at 21 C.

Effect of temperature of incubation. Several trials were conducted to determine the effect of temperature of incubation on the rate of transport; these are summarized in Table 2.

TABLE 2

Effect of time and temperature of incubation on transport of fructose-C^{14}

Temperature	Minutes of incubation			
	10	20	40	80
(C)	*(counts per minute)*			
18	555	850	950	1,180
28	715	990	1,250	1,325
38	910	1,160	1,325	955

Spermatozoa were incubated at the three temperatures with fructose-C^{14}, aliquots removed at specified time intervals, washed with Ringer-phosphate at 21 C, dried, and radioassayed. Uptake due to simple diffusion should be relatively insensitive to temperature, whereas active transport should be temperature-dependent (6). The results in Table 2 are indicative of a transport system, in that uptake varied with temperature. However, the response to temperature was not as great as might be anticipated from strictly metabolic considerations; possibly, the data reflect a combination of diffusion and active transport.

Studies of the effect of temperature on transport are complicated by the sensitivity of sperm to temperature shock, which by changing membrane permeability would be expected to increase transfer by diffusion (8) or, alternatively by decreasing metabolic rate, would be expected to decrease transport (2). The effects of cold shock were studied by immersing flasks containing sperm in an ice bath for 15 min, warming to incubation temperature, adding fructose-C^{14}, and incubating as indicated in Table 3. In each experiment cold shock depressed the transfer of radioactivity into the cell.

TABLE 3

Effect of cold shock on transport of fructose-C^{14}

Expt. no.	Incubation conditions	Control	Cold-shocked
		(cpm)[a]	
10	Glycine buffer, 37 C	2,880	1,440
11	Ringer Tris buffer, 37 C	3,460	2,850
12	Ringer, 0.004 M iodoacetate, 37 C	1,645	1,200
13	Milk, 4 C	495	350

[a] In cells and CO_2.

Sensitivity to inhibitors. In these experiments, washed sperm were exposed to the concentration of inhibitor indicated in Table 4 for 10 to 20 min before fructose-C^{14} was added to the medium. Some of the inhibitors increased the yield of radioactivity in the cells above that in uninhibited cells, but in all cases the measured transport (activity in cells plus that in CO_2) was reduced by the inhibitor. The data are indicative of a transport system, in that passive diffusion is not influenced easily by inhibitors, and the concentrations employed should not alter membrane permeability.

Competitive entry. With a transport system, metabolizable sugars would be expected to com-

TABLE 4

Effect of inhibitors on recovery of radioactivity in cells and carbon dioxide

	Cells	CO_2
	——(*cpm*)——	
Expt. 7 & 8 (mean)		
Control	485	10,400
Sodium cyanide, 0.04 M	1,870	110
Iodoacetic acid, 0.004 M	860	12
p-Chloromercuribenzoate, 0.004 M	935	35
Sodium fluoride, 0.04 M	510	15
Expt. 9		
Control	490	4,930
Mercuric chloride, 10^{-5} M	590	4,400
Mercuric chloride, 2×10^{-4} M	295	90

pete with one another for entry; with diffusion, each should enter relatively independently of the other. In the trials reported in Table 5, uptake of fructose-C^{14} was measured with no unlabeled sugar added, and with unlabeled fructose, glucose, or arabinose added to a final concentration of 0.01 M. The reduction in recovered activity upon the addition of unlabeled fructose was the result of dilution; a fourfold increase in concentration resulted in a reduction of yield to one-fourth the previous value. Adding glucose, however, resulted in a much greater reduction in yield, indicating a greater affinity for glucose than for fructose. Arabinose is only slowly metabolized by spermatozoa and offered considerably less competition to the transport of fructose-C^{14}.

Transfer against concentration gradient. Perhaps the most stringent criterion of an active transport system is the ability to concentrate against an osmotic gradient (6). Since fructose concentration inside the cell is difficult to measure and not easily altered appreciably, this problem was studied by measuring efflux rather than influx.

Sperm were incubated with fructose-C^{14} to permit entry into the cell. After washing the cells free of extraneous radioactivity, the C^{14}-loaded cells were incubated in fresh buffer with and without 0.1 M unlabeled fructose, as shown in Table 6. After this second incubation the cells were removed by centrifugation and the supernatant dried and assayed for radioactivity which had transferred out of the cells. In the aerobic experiments, CO_2 was collected, radio-assayed, and the effluent cpm in the table is the sum of radioactivity in the supernate and in CO_2. This procedure may be questioned, in that CO_2 may not necessarily be the result of transport, since it may represent catabolism within the cell and diffusion of the gas from the cell. However, if one considers only the supernate, the relationships are the same—efflux into the sugarless buffer exceeds that into fructose.

TABLE 5

Effect of unlabeled fructose, glucose, and arabinose on the transport of fructose-C^{14}

Expt. no.	Unlabeled substrate added			
	None	Fructose	Glucose	Arabinose
	——(*cpm*)——			
14	.....	610	255	1,560
15	.....	260	160	755
16	3,820	995	300	1,650
Mean		622	238	1,322

TABLE 6

Effect of anoxia and fructose concentration in the medium on outflow of fructose-C^{14} from the cell

Mean of expt. numbers		Fructose concentration, M	
		0	0.1
		——(*effluent cpm*)——	
17, 18	Aerobic	720	560
22–26	Anaerobic	595	695

In the anaerobic experiments there was essentially no CO_2 production; thus, only the radioactivity in the supernate was considered after it was established that CO_2 radioactivity was negligible. These anaerobic trials were important in two respects. First of all, the efflux into 0.1 M fructose was greater than that into the medium without fructose, thus demonstrating concentration against an osmotic gradient. Secondly, anoxia increased the rate of transport across the cell membrane, in agreement with the glucose-muscle cell studies of Morgan et al. (9).

General discussion. The data presented show that bovine spermatozoa possess several of the characteristics of an active transport system for fructose: these include sensitivity to temperature, dependence on active metabolism, sensitivity to inhibitors, mutual interference by metabolizable sugars, and concentration against an osmotic gradient. The evidence, while extensive, perhaps should not be regarded as conclusive, for fructose is metabolized so rapidly that it is difficult to limit the study to transport without complicating the issue with the problems brought on by rapid metabolism and movement of intermediates.

In this study, radioactivity appearing in the cell and that in CO_2 was summed to give a

value for transport. While this permits comparisons to be made, it is subject to inaccuracy due to metabolites, other than CO_2, which may pass from cell to medium and thus escape measurement. More definitive information on transport may be obtained by using sugars structurally similar to fructose but metabolized slowly or not at all. Such studies are now under way and will be reported when completed.

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ISOLATION OF ENTEROVIRUSES FROM A HERD OF DAIRY CATTLE [1]

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SUMMARY

A high incidence of enteroviruses isolated by the use of bovine kidney cell cultures has been found in rectal swabs from normally reared calves. In no case were enteroviruses recovered from calves less than 4 wk of age. Only one of seven parturient cows was shown to be shedding enteroviruses.

Two ether-resistant virus types were identified which differed in serologic specificity, characteristics of plaque formation in bovine kidney cell cultures, and in vitro host range. The incidence of these types of enteroviruses could not be correlated with the appearance of clinical signs in these calves.

The isolation of viral agents from bovine feces or rectal swabbings has been reported lately (5). In some cases the presence of these agents has been associated with certain clinical signs (8, 9), while in others the animal hosts were apparently healthy (6, 10-12). Primary isolation of the agents has, as a rule, been accomplished in bovine kidney cultures (1, 6-9). Certain strains have also been found to be cytopathogenic in the cultured tissues of other species (7, 8, 10).

The present report describes the isolation, by means of bovine kidney cell cultures, of enteroviruses obtained in rectal swabs from calves and cows of a normal dairy herd. These viruses have been characterized by an application of the plaque technique in the cultured kidney cells of four species.

MATERIALS AND METHODS

Herd under investigation. The animals employed in this study belonged to The Ohio State University dairy herd. Calves born in this herd remained with their dams and nursed ad libitum during the first three days of life. They were then moved to the calf barn, where they were fed about 10% of their body weight per day of surplus herd milk or colostrum, with free access to water, grain, and hay. They remained in the calf barn until they reached four to six months of age, during which time they were exposed in varying degrees to 20 to 30 other calves.

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Cell cultures. Primary monolayer cultures were prepared by the trypsinization of kidneys from yearling or older cattle with the subsequent growth of the dispersed cells in a culture medium consisting of: 0.5% lactalbumin enzymatic hydrolysate; 5.0% bovine serum; 94.5% Hanks' balanced salt solution (BSS); and 100 μg of dihydrostreptomycin, 200 units penicillin, and 50 units of nystatin (Mycostatin [2]) per milliliter. Cultures were prepared both in 4-oz prescription bottles and in 16- by 150-mm test tubes (4). Cell cultures were also prepared from porcine, human, and monkey (Macaca cynomolgus) kidneys in a similar manner and grown in 4-oz prescription bottles.

Virus isolations. A sterile cotton swab on a six-inch spplicator was inserted at least three inches into the rectum. The swab was applied to the epithelium with a rubbing motion and withdrawn together with some fecal material, whenever this was possible. The swab and the sample were placed in a thick-walled 16- by 100-mm centrifuge tube containing 2.5 ml of BSS, to which had been added 1,000 μg of dihydrostreptomycin, 2,000 units of penicillin, and 500 units of nystatin per milliliter. Having arrived at the laboratory, the swab was swirled in the solution to dislodge the fecal material and was removed from the tube. The remaining fecal suspension was centrifuged at approximately 3,800 rpm for 40 min. The supernatant was aspirated from the sedimented fecal material and frozen for later testing.

Both tube and bottle bovine kidney cell cultures prepared as described previously were used routinely for the primary isolation of

[2] Mycostatin is produced by E. R. Squibb and Sons, New York.

TABLE 1

Proportions of calves of various ages found to be shedding enteroviruses on June 9, 1959

Age in weeks	3	6	9	10	11	12	13	14	16	18
Number positive / Number tested	0/1	1/3	1/2	1/1	0/2	0/2	1/2	0/1	0/1	0/1
Age in weeks	21	22	23	24	25	27	29	31	45	All
Number positive / Number tested	2/2	1/1	0/1	1/1	0/1	0/1	2/2	0/1	1/1	11/27

virus from rectal swabs. As a rule, two tube cultures were inoculated with 0.1 ml each of the fecal extract and maintained with a medium which differed from the growth medium only in the fact that no serum was added; and one bottle culture was inoculated with 0.2 ml of the extract and overlaid with 12 ml of a similar maintenance medium containing, in addition, 1% of agar. The tube cultures were observed microscopically for evidence of viral cytopathogenic effects, whereas the bottle cultures were observed macroscopically for characteristic areas of cell degeneration designated plaques.

Serological procedures. Four plaque-isolated strains of viruses isolated from this herd were used to immunize rabbits. Ten milliliters of the undiluted virus suspension were homogenized with a like quantity of complete Freund's adjuvant in a 20-ml syringe. Five-milliliter aliquots of this emulsion were injected intramuscularly into the right and left fore and hindquarters of the rabbits, together with a 1-ml intramuscular injection of the undiluted culture fluid into the right hindquarter. The rabbits were bled from the heart 6 wk later and the serums harvested and frozen.

Neutralization tests with these virus strains and serums were performed in bovine kidney cell cultures, using the plaque method described by Bohl et al. (2). Serums were tested for neutralizing potency at dilutions of 1:10, 1:100, and 1:1,000.

EXPERIMENTAL PROCEDURE AND RESULTS

Herd survey. To establish the presence of enteroviruses in The Ohio State University dairy herd, rectal swabs were obtained from all calves housed in the calf barn on June 9, 1959. The results of this initial survey are presented in Table 1.

Detection of enteroviruses in calves as a function of age. This portion of the study was undertaken to ascertain the age at which enteroviruses could first be detected in calves, as well as some indication of the persistence of the infection. Rectal swabs were taken from seven cows and their calves at the time of parturition. These calves and six other neonatal calves were then swabbed at weekly intervals for various periods of time.

The 13 calves which were examined weekly for rectal excretion of virus were started on the experiment at ages of 0 to 4 wk. One calf died of enterotoxemia at nine days of age and was not found to be shedding enteroviruses at birth or at death. The histories of the remaining calves are summarized in Table 2.

It will be noted that virus was not detected during the course of the observations in only one case. In the remaining 11 animals, the age at the time of the first positive sample ranged from 4 to 12 wk, with a mean of 7 wk. The appearance of plaques formed in a bottle culture used for primary isolation of virus from one of these rectal swabs is illustrated in Figure 1.

In those cases in which calves were tested at birth, rectal swabs were also taken from the dams of these animals. Of the seven cows swabbed at parturition, only one was found to be shedding virus at that time.

Characterization of the virus strains. All of the virus strains established from these rectal swabbings were, of course, capable of producing cytopathogenic effects and plaques in bovine kidney cell cultures. Certain of these strains were further characterized on the basis of their serologic relationships, the characteristics of plaques formed in bovine kidney cell cultures, and their ability to form plaques in kidney cell cultures from three heterologous species.

Neutralization tests using rabbit antiserums indicated that these strains belonged to two serological types. Antiserums against each type cross-reacted to a limited extent with heterologous strains.

Daily observations of inoculated bovine bottle cultures under agar overlay medium revealed that plaques were first visible macroscopically

TABLE 2

Incidence of enterovirus in rectal swabs from calves as a function of age

Calf no.	Age in weeks																								
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
530A	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
523G	−	−	−	−	−	−	−	−	+	−	+	−	−	−	−	−	..	..	..	..	..	..	..	..	..	..
522G	..	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	..	..	..	..	..	..	..	..
531A	..	..	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	..	..	..	..	..	..	..
510J	..	..	..	..	+	+	−	+	−	−	+	+	+	+	+	+	−	+	−	+	+	..	..	..	..	..
511J	..	..	−	−	−	−	−	−	+	+	+	+	+	+	+	+	−	+	..	..	..	..	..	..	..	..
689H	..	−	−	−	+	+	+	+	−	−	+	+	−	+	+	..	..	..	..	..	..	..	..	..	..	..
162S	−	−	−	−	−	−	−	−	−	−	−	−	−	−	..	..	..	..	..	..	..	..	..	..	..	..
532A	−	−	−	−	−	−	+	+	+	+	+	+	..	..	..	..	..	..	..	..	..	..	..	..	..	..
690H	−	−	−	−	−	−	−	+	+	+	+	+	..	..	..	..	..	..	..	..	..	..	..	..	..	..
533A	−	−	−	−	−	−	−	+	+	+	+	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
513J	..	−	−	−	+	−	+	+	+	−	+	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..

− Means no virus was isolated; +, a virus was isolated; ..,not tested.

on the third day after inoculation, at which time their diameter was about 1 mm. These plaques continued to enlarge, and others to appear, for several days. The rate of appearance of plaques of Type I was somewhat slower than that of Type II, so that a plaque-count equal to 90% of that ultimately recorded was observed about 24 hr later with Type I than Type II. Possibly as a result of the delayed appearance of the plaques, the mean diameter of the plaques of Type I was usually one or more millimeters smaller than that of Type II, other things being equal. An example of these differences is reported in Table 3.

Differences were also observed in the host ranges of these virus strains in heterologous kidney cell cultures. Prototype strains of the two virus types were diluted in tenfold increments and titrated in cell cultures of bovine, porcine, monkey, and human kidneys. No attempt was made to adapt the strains to the cultures in which they were tested. The results of this experiment are reported in Table 4.

Detection of mixed viral infections. The results of first tests applied to the rectal swabs have been expressed only in terms of whether viruses were detected. To determine whether both types of viruses might be recovered from a single rectal swab sample, an additional study was conducted.

Since the concentrations of viruses in the original rectal swab fluids were too low to be suitable for neutralization testing, sero-typing was performed with fluid harvested from primary virus isolations in cell culture. The harvested culture fluid was diluted in BSS to contain an estimated 50 plaque-forming units of virus per 0.1 ml. Aliquots of this virus suspension were incubated 60 min at room temperature with an equal volume of: (1) BSS, (2) BSS containing 5% antiserum I, (3) BSS containing 1% of antiserum II, or (4) BSS containing 5% of antiserum I and 1% of antiserum II. Duplicate bottle cultures of bovine kidney cells were used to assay each of these mixtures by the plaque technique (1). Whenever the results of the first test were not clear, virus was subcultured from selected plaques and retested in the same manner.

FIG. 1. Appearance of plaques on bovine kidney cell cultures five days after inoculation from a rectal swab taken from Calf 510J on November 18, 1959.

TABLE 3

Daily observations of the number and size of plaques of two virus types on bovine kidney cell culture

Days of incubation	Type I		Type II	
	Number of plaques	Mean diameter	Number of plaques	Mean diameter
		(mm)		*(mm)*
3	2	1	3	2
4	4	3	17	4
5	11	5	18	7
6	14	7	18	9
7	14	8	18	12

The first positive rectal swabs from six calves were successfully typed, and it was found that both Types I and II were present in samples from Calves 530A, 532A, and 690H, whereas only Type II could be indentified in the first positive samples from Calves 522G, 510J, and 511J. In the case of Calf 530A, an attempt was made to serotype all of the positive weekly rectal swabs obtained in the course of the study. Both virus Types I and II were identified in samples taken at 8, 10, 20, 22, 23, and 24 weeks of age. Only Type I was adentified in samples at 9, 13, 17, 18, 19, and 21 wk. The virus in the 15-, 16-, and 25-wk samples was lost. Only Type I could be demonstrated in the rectal swab taken from Cow 490G at the time of parturition.

DISCUSSION

The presence of enteroviruses has been demonstrated in a high proportion of rectal swabs obtained from calves of various ages in The Ohio State University dairy herd. Since the system of management of these animals presents no particularly unusual features, it is unlikely that this herd is unique with respect to the incidence of enteroviruses.

Only one of 12 calves was not found to be shedding virus during a prolonged period of weekly observations. However, no calf studied was found to be shedding virus at an age of less than 4 wk, although exposure was probably continuous from birth. Since antibody capable of neutralizing this virus has been demonstrated in a number of samples of colostrum (3), it is possible that these calves were protected by antibody derived passively through nursing.

Virus was detected in a rectal swab of only one of seven cows studied at the time of parturition. Since it is likely that all of these animals had been infected and shed virus at some time during their lives, the decreased incidence of detectable virus in adults may be taken as evidence of the development of active immunity.

Pure strains of viruses were established by isolation from plaques formed in bovine kidney cell cultures. It was shown by means of antiserums prepared in rabbits that these strains belonged to at least two separate serologic types. These types were also found to differ with respect to plaque morphology in bovine kidney cell cultures and efficiency of plaque formation in kidney cell cultures from three other species. The plaques produced by Type I in bovine kidney cultures were somewhat smaller on any given day of incubation. Type I apparently produced plaques relatively efficiently in monkey kidney cultures, less so in porcine kidney cultures, and not at all in human kidney cultures. Type II was relatively efficient in forming plaques in monkey kidney cultures, less so in human kidney cultures, and was not

TABLE 4

Per cent efficiencies of plaque formation by two serologic types of bovine enteroviruses in the cultured kidney cells of four species

Serologic type	Species of culture			
	Bovine	Porcine	Monkey	Human
I	100.00	0.12	42.00	0 [a]
II	100.00	0 [a]	52.00	0.05

[a] No plaques observed after inoculation with the most concentrated (10%) suspension tested.

found to produce plaques in porcine kidney cultures. Dr. S. A. Sattar of this laboratory has recently demonstrated that both of these types are ether-resistant.

Mixed infections with the two types have been found to be common in these calves under natural conditions. The incidence of virus of either type in rectal swabs could not be correlated with diarrhea or pneumonia, which occasionally occurred in mild form in some of the animals.

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NEUTRALIZATION OF BOVINE ENTEROVIRUSES BY COLOSTRUM, MILK, AND BLOOD SERUM [1]

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SUMMARY

Serum and colostrum samples from seven parturient cows, milk from these animals after seven or more days of lactation, and serum samples from their offspring before and after nursing, have been tested for their ability to neutralize two serologic types of bovine enteroviruses isolated from the same dairy herd. In most, the descending order of the neutralizing potencies of these samples was: colostrum, parturient cow serum, postnursing calf serum, prenursing calf serum, and normal milk. The neutralizing potency of normal milk was found to be negligible, and five prepartum milkings in one cow were found to decrease the antibody content of the mammary secretions to that of normal milk.

The efficiency with which ingested colostral antibody was absorbed into the blood stream was apparently quite high in newborn calves but somewhat decreased if nursing was deferred for 22 or even 12 hr after birth. There was no apparent correlation between the levels of neutralizing antibody in the serums of calves at the end of the first week of life and the age at which enteroviruses were first detected in rectal swabs from these animals.

The isolation of enteroviruses from dairy cattle has been reported recently by several laboratories (2, 11, 12, 16-20). Work in this laboratory has resulted in the isolation of two serologic types of bovine enteroviruses from a high percentage of calves in a herd of dairy cattle (4). Weekly rectal swabbings of these calves revealed that no enteroviruses could be recovered from animals less than 4 wk of age, despite the likelihood that exposure to the viruses was continuous from birth under normal conditions of rearing.

The purpose of the present study was to determine the levels of humoral neutralizing antibody against these enteroviruses in parturient cows and their calves by means of tne plaque technique. An attempt was made to evaluate the role of colostrum in the transference of passive antiviral antibody to the newborn calf under conditions of immediate and deferred postnatal nursing.

Early studies with antibacterial agglutinins indicated that cows which had high serum agglutinating titers produced calves which had no serum agglutinins. The colostrum of these cows was shown to be high in agglutinin titer, and the ingestion of this colostrum resulted in the passive acquisition of large quantities of serum agglutinins by the calf (15, 21). More recent electrophoretic studies have indicated that the serum of the newborn calf is virtually devoid of immune globulin but that this substance is acquired from colostrum under normal conditions of nursing (7, 8). The blood serum of the cow has been implicated as the source of colostral globulin (1, 13, 14).

MATERIALS AND METHODS

Cell cultures. Primary monolayer cultures were prepared by the trypsinization of kidneys from yearling or older cattle, with the subsequent growth of the dispersed cells in a culture medium consisting of: 0.5% lactalbumin enzymatic hydrolysate; 5.0% bovine serum; 94.5% Hanks' balanced salt solution (BSS); and 100 μg of dihydrostreptomycin, 200 units of penicillin, and 50 units of nystatin (Mycostatin [2]) per milliliter (6).

Neutralization tests. Neutralization tests were performed to measure by means of the plaque

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[2] Mycostatin is produced by E. R. Squibb and Sons, New York.

technique the ability of samples of colostrum, milk, and blood serum to neutralize enteroviruses of two serologic types isolated from animals of the same dairy herd (4). Samples of colostrum, milk, or blood serum to be tested were diluted in tenfold increments, with an initial dilution of 1:10 or 1:16. Equal volumes of the virus suspension (diluted so that in 0.1 ml a countable number of plaques would occur) and the diluted neutralizing substance were incubated together at room temperature for 1 hr. A control for the estimation of the actual concentration and volume of the virus was provided by incubating the same concentration and volume of the virus suspension with an equal volume of BSS under the same conditions. The BSS served as the diluent for the virus suspensions and the colostrum, milk, and blood serum samples; and 0.2-ml aliquots of each incubated mixture were inoculated into duplicate bovine kidney cell cultures in 4-oz prescription bottles from which the medium had been discarded (3).

These bottle cultures were incubated at room temperature for 1 hr after inoculation, with frequent agitation. Each culture was then overlaid with 12 ml of a medium consisting of: 1.0% Noble agar (Difco); 0.5% lactalbumin enzymatic hydrolysate; 0.0015% neutral red; approximately 98.5% BSS; and 100 μg of dihydrostreptomycin, 200 units of penicillin, and 50 units of nystatin per milliliter. These bottles were observed daily for evidence of plaques (23).

The extent to which each concentration of a colostrum, milk, or blood serum sample neutralized virus of each serologic type was expressed as a percentage of neutralization. This percentage was computed by dividing the total plaque-count for the duplicate bottles in question by the total plaque-count for the appropriate duplicate virus control bottles, subtracting the quotient from 1, and multiplying this difference by 100. As a rule, little importance was attached to neutralizations of less than 40%.

Animals investigated. The animals under investigation were members of The Ohio State University dairy herd. With the minor exceptions noted, the calves studied were being raised according to the normal system for this herd. That is, the newborn calves remained with their dams and nursed ad libitum during the first three days of life. They were then moved to the adjacent calf barn, where they received approximately 10% of their body weight per day of surplus herd milk or colostrum for several weeks and decreasing quantities thereafter to the age of five months, with free access to water, grain, and hay. Rectal swabs taken from these calves at birth and at weekly intervals thereafter were tested for the presence of enteroviruses in a manner described previously (4). The cows were tested in the same manner for enterovirus excretion at the time of parturition.

EXPERIMENTAL PROCEDURE AND RESULTS

The animals studied are grouped in pairs consisting of a cow and her newborn calf, each pair being designated a case. Numbers were assigned to the cases in order of chronological precedence.

Case 1. A heifer calf, 530A, was born to Cow 467A on July 5, 1959. Samples of colostrum and of blood from both animals were obtained at that time, after which the calf was allowed to nurse. Additional blood samples were taken from the calf at 48 hr, seven days, and 12 wk of age. A sample of normal milk was obtained from 467A on the 15th postpartum day. All of these samples were tested for the ability to neutralize both types of enteroviruses. Results of these neutralization tests are presented in Table 1.

It may be noted from Table 1 that the level of antibody in the colostrum, and seemingly in the 48-hr and seven-day calf serum samples exceeded that of the maternal blood serum at parturition. The cow was not found to be shedding enteroviruses at the time of parturition, but enteroviruses of both Types I and II were detected in rectal swabs from the calf beginning with the eighth week of life and continuously thereafter until weekly observations were terminated after 25 wk. It is, therefore, noteworthy that the levels of antibody against each type in the 12-wk serum sample did not differ significantly from those detected at birth.

Case 2. Cow 630H gave birth to a 130-lb bull calf after a complicated labor on August 15, 1959. Samples of colostrum and of blood from both animals were obtained at the time of parturition. Additional blood samples were collected from the calf at 48 hr and nine days of age. The calf died later, on the ninth day, with a diagnosis at necropsy of clostridial enterotoxemia. A sample of normal milk was taken from the cow on the 24th postpartum day. These colostrum, milk, and blood serum samples were tested for ability to neutralize virus of both types at dilutions of 1:16, 1:160, and 1:1,600. In no case did the extent of neutralization exceed 40%, so the concentrations of neutralizing antibody in all of the samples from these animals were regarded as negligible. Enteroviruses

TABLE 1

Percentages of neutralization of virus Types I and II by colostrum, milk, and serum samples from Cow 467A and Calf 530A

Virus type	Dilution of sample	Nature of sample: Cow serum	Colostrum	Normal milk	Pre-nursing calf serum	48-hr calf serum	7-day calf serum	12-wk calf serum
I[a]	1:16	100	100	58	51	100	100	67
	1:160	44	61	13	....	65	54	0
	1:1,600	0	25	7	....	0	7	0
II[b]	1:16	100	100	0	0	100	100	0
	1:160	14	78	0	....	40	17	0
	1:1,600	0	0	0	....	0	0	0

[a] Ninety-nine and one-half plaque-forming units per bottle.
[b] Sixty plaque-forming units per bottle.

were not detected in rectal swabs from the cow at parturition and from the calf at birth and at death.

Case 3. Cow 490G gave birth to a heifer calf, 523G, on September 9, 1959. A sample of blood had been taken from the cow six days previously. Samples of colostrum and of blood were obtained within an hour of parturition. In this case, the calf was separated from its dam at birth and received no sustenance of any kind until it was 22 hr old, after which it was allowed to nurse. Aside from a 10-ml sample, the cow had not been milked prior to nursing. Additional blood samples were taken from the calf at 48 hr, seven days, and 13 wk of age. Normal milk was collected from the cow on the 14th postpartum day. These colostrum, milk, and blood serum samples were tested for their ability to neutralize enteroviruses of both types. The results of these neutralization tests are reported in Table 2.

It will be noted that the neutralizing potency against both types of enteroviruses was somewhat lower in the sample of cow serum at parturition than that taken six days prepartum. It is also apparent that the levels of antibody against Type I in the serum and lacteal samples from 490G were not nearly as high as those against Type II. It should be mentioned in this connection that a rectal swab from 490G revealed that she was shedding enteroviruses of Type I at the time of parturition.

When contrasted to the results reported in Table 1, it appears that deferring nursing until 22 hr after birth resulted in a considerable decrease in the efficiency with which ingested antibody is transported to the blood stream of the calf. Although it was probably exposed to these enteroviruses from birth (3), weekly rectal swabs from 523G revealed no virus until the eighth week of life, at which time Type II was found being shed. Antibody titers against

TABLE 2

Percentages of neutralization of virus Types I and II by colostrum, milk, and serum samples from Cow 490G and Calf 523G

Virus type	Dilution of sample	Nature of sample: 6-day pre-partum cow serum	Parturient cow serum	Colostrum	Normal milk	Pre-nursing calf serum	48-hr calf serum	7-day calf serum	13-wk calf serum
I[a]	1:10	88	58	40	30	46	65	58	74
	1:100	54	54	0	0	....	54	56	61
	1:1,000	39	0	4	0	....	39	30	19
II[b]	1:10	100	100	100	41	17	66	73	83
	1:100	85	73	100	16	....	24	0	43
	1:1,000	60	33	71	0	....	22	12	2

[a] Twenty-eight and one-half plaque-forming units per bottle.
[b] Forty-one plaque-forming units per bottle.

TABLE 3

Percentages of neutralization of virus Types I and II by colostrum and serum samples from Cow 76S and Calf 162S

Virus type	Dilution of sample	Nature of sample: Cow serum	Colostrum	Pre-nursing calf serum	48-hr calf serum	7-day calf serum
I[a]	1:16	73	98	54	46	69
	1:160	77	63	....	2	19
	1:1,600	67	15	....	12	0
II[b]	1:16	8	92	0	3	5
	1:160	0	10	....	8	0
	1:1,600	0	0	....	0	0

[a] Twenty-four plaque-forming units per bottle.
[b] Thirty-eight and one-half plaque-forming units per bottle.

both virus types had apparently persisted at similar levels in the serum for 13 wk, which may be taken as an indication that an active immune response had taken place.

Case 4. Cow 76S gave birth to a bull calf, 162S, on October 3, 1959. Samples of colostrum and of blood from both animals were obtained 12 hr after birth, during which time the calf had been kept away from the cow. The calf was then allowed to nurse for the first time, and additional blood samples were taken from it at 48 hr and seven days of age. The results of neutralization tests performed with these colostrum and blood serum samples and both types of enteroviruses are reported in Table 3.

The results presented in Table 3 indicate that the levels of humoral antibody against Type I in Cow 76S were significantly higher than those against Type II. The neutralizing potency against both Types I and II of a sample of normal milk taken on the 13th postpartum day was found to be negligible, nor were the neutralizing antibody levels in any of the samples reported in Table 3 as high as those of their counterparts reported in Table 1. Nevertheless, weekly rectal swabs from the calf from the day of birth through the 13th week of life, when observations were terminated, and the rectal swab from the parturient cow were not found to contain enteroviruses.

Case 5. Cow 495A gave birth to a bull calf, 532A, on October 9, 1959. Samples of colostrum and blood from both animals were collected immediately after parturition, and the calf was allowed to nurse. Further blood samples were taken from the calf at 48 hr and seven days of age. The results of neutralization tests reacting these colostrum and serum samples with both types of enteroviruses are reported in Table 4.

The degree of neutralization brought about by the calf serum collected before nursing is the highest of any such sample tested. Never-

TABLE 4

Percentages of neutralization of virus Types I and II by colostrum and serum samples from Cow 495A and Calf 532A

Virus type	Dilution of sample	Nature of sample: Cow serum	Colostrum	Pre-nursing calf serum	48-hr calf serum	7-day calf serum
I[a]	1:16	90	100	96	94	100
	1:160	26	54	....	3	40
	1:1,600	19	25	....	14	0
II[b]	1:16	95	100	74	83	94
	1:160	9	13	....	22	4
	1:1,600	0	9	....	11	0

[a] Seventy-five plaque-forming units per bottle.
[b] Eighty plaque-forming units per bottle.

theless, some increase in the serum antibody level appeared to have taken place by the seventh day, presumably as a result of nursing. Despite these relatively high neutralizing antibody titers at seven days of age, the calf was found to be shedding both types of enteroviruses in its sixth week of life. Enteroviruses were not detected in a rectal swab from Cow 495A at the time of parturition. The neutralizing potency of a sample of normal milk taken from the cow on the seventh postpartum day was considerably lower than that of the colostrum or any of the serum samples reported in Table 4.

Case 6. Cow 646H gave birth to a heifer calf, 690H, on October 14, 1959. Samples of blood and the contents of the udder had been collected 12 days before parturition. Due to mastitis, the cow had been milked five times at 12-hr intervals before parturition. The sample obtained from the udder at the time of parturition did not have the appearance of colostrum. Blood samples were taken from both the cow and the calf immediately after parturition, and additional blood samples were obtained from the calf at 48 hr, seven days, and 8 wk of age. A sample of normal milk was taken from the cow on the 14th postpartum day.

Neutralization tests against the two types of enteroviruses indicated that the levels of antibody against these viruses in the sample taken from the udder at parturition were in the range regarded as negligible and were quite comparable to those measured in normal milk on the 14th postpartum day. Nevertheless, serum antibody levels against both types in the calf at 48 hr and seven days of age were higher that at birth.

A supplementary experiment was performed in which the serum and lacteal samples were heated at 56 C for 30 min and compared to unheated samples as to their ability to neutralize a strain of enterovirus Type II isolated from the calf at 7 wk of age. Such heating did not significantly decrease the neutralizing potencies of any of the samples. The concentration of antibody against Type II in the udder contents on the 12th prepartum day was much higher than those in the udder samples at parturition and 14 days thereafter. The neutralizing potency of the eighth week calf serum was somewhat lower than those of the 48-hr and seven-day serum samples.

Case 7. Cow 482A gave birth to a heifer calf, 533A, on October 19, 1959. Samples of colostrum and of blood from both animals were collected at parturition, and the calf was allowed to nurse. Additional blood samples were taken from the calf at 48 hr and nine days of age, and a sample of normal milk was collected from the cow on the ninth postpartum day. Although the antibody concentrations throughout were somewhat lower, the relative neutralizing potencies against both types of enteroviruses of the colostrum, milk, and serum samples from this pair of animals were quite similar to those reported in Table 1. The cow was not found to be shedding enteroviruses at parturition. Enteroviruses of undetermined type were first detected in a rectal swab from the calf during the fifth week of life.

DISCUSSION

Neutralizing antibodies against two types of bovine enteroviruses have been demonstrated in the serums of at least four of seven parturient dairy cows under natural conditions. It was shown in one case that the serum titer of these antibodies at parturition was somewhat lower than it had been six days previously. This finding seems to be in keeping with the results of electrophoretic studies on bovine serum immune globulin by Larson (13) and Larson and Kendall (14). Only one of these seven cows was shown to be shedding enteroviruses at the time of parturition, and in this cow the level of serum antibody against the enteroviruses type being shed was considerably lower than that against the other type of enterovirus prevalent in the herd.

The level of neutralizing antibody in the colostrum was generally higher than that in the blood serum of the cow from which it was obtained. The neutralizing potency of normal milk collected seven or more days postpartum was considered negligible, and five prepartum milkings in one case were found to decrease the previously high concentration of antibody in the udder contents to approximately that of normal milk. An experiment by Dr. K. V. Singh of this laboratory indicated that the colostrum sample obtained in Case 3 had little or no neutralizing effect upon a porcine enterovirus (ECPO-5).

Calf serums collected at birth had some ability to neutralize these enteroviruses, but the neutralizing potency was considerably higher at 48 hr and seven to nine days of age if the calf was allowed to ingest colostrum of high antibody content shortly after birth. Under these conditions, the antibody level of the calf serum at 48 hr might even exceed that of the dam at parturition. An apparent decrease in the efficiency with which ingested antibody was absorbed into the blood stream of the calf was

observed if nursing was deferred for 22 or even 12 hr after birth. A similar decrease in intestinal permeability to immune protein has been demonstrated by electrophoretic techniques by other workers (5, 24).

In those cases in which blood samples were taken from calves after they had been shown to be shedding enteroviruses, it appeared that the active immune response was somewhat inhibited if the early serum titers of passive antibody were high. A similar inhibition of active immunity by passive antibody was observed in swine by Hoerlein (9).

Examination of the serum neutralizing potencies in these calves after nursing revealed no apparent correlation with the ages at which enteroviruses were first detected in rectal swabs from these animals. An analogous system is found in human beings and the enteroviruses homologous to that species, including poliovirus. Koprowski et al. (10) have reported that passive, congenitally acquired antibody did not protect human infants from intestinal infection with ingested attenuated poliovirus, and Sabin (22) has found that serum titers of neutralizing antibody in older human beings were not correlated with protection of the alimentary tract against infection with attenuated poliovirus.

It is possible that the observed delay in the incidence of rectal enteroviruses in these calves, despite the probability of continuous exposure at least from the third day of life, may simply have been a result of the immaturity of the digestive tract. However, the lack of correlation between the calf's serum antibody level at the end of the first week of life and the age at which it was first found to be shedding enteroviruses might also have been due to the continued ingestion of milk and surplus colostrum. It is postulated that the neutralizing antibody in these ingesta may have persisted in the intestinal contents and acted locally upon the virus without altering the serum antibody level.

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TECHNICAL NOTES

XANTHINE OXIDASE ACTIVITY OF MILKS IN RELATION TO STAGE OF LACTATION, FEED, AND INCIDENCE OF SPONTANEOUS OXIDATION[1]

The development of spontaneous oxidation in milk has been attributed to its xanthine oxidase activity (1), copper content (3) and, more recently, to the copper and ascorbic acid content (8).

The data in this report were obtained in conjunction with an extensive study of the role of dihydroquercetin as an antioxidant (4). Xanthine oxidase activity is expressed as the reciprocal of the time, in minutes, required for complete reduction of methylene blue, using xanthine as the substrate (6). Spontaneously oxidized flavors were determined organoleptically and by the TBA test (2), at 48-hr intervals. The TBA values, expressed as μg malonaldehyde per 10 g sample, were derived from a standard curve for malonaldehyde.

Individual milk samples were received at regular intervals from the Field Department, Dairy Cooperative, Portland, Oregon, between February and May, 1960. They were brought to the laboratory within 3 hr after milking.

The scatter diagrams (Figure 1) depict the changes in xanthine oxidase activity as related to the stage of lactation and to the incidence of spontaneously oxidized flavors in milks produced on dry-lot feeding and, subsequently, by the same cows on pasture.

[1] Contribution from the Oregon Agricultural Experiment Station. Approved by the Director.

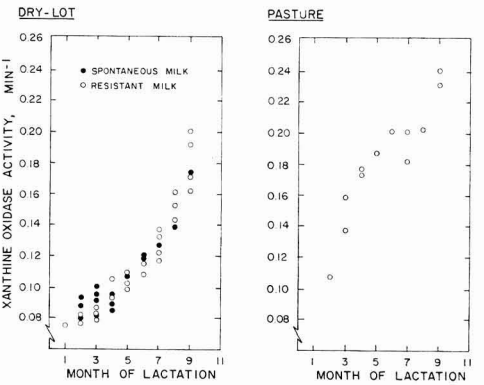

FIG. 1. Xanthine oxidase activity in relationship to stage of lactation and incidence of spontaneous oxidation under dry-lot and pasture regimes.
● = Spontaneous milk; ○ = resistant milk.

A direct relationship appears to exist between xanthine oxidase activity and the stage of lactation. The change from dry-lot feed, consisting of pelleted alfalfa, grain mix and alfalfa hay, to mixed-grass pasture, supplemented with grain mix, did not alter the relationship between xanthine oxidase activity and the stage of lactation.

TABLE 1
Inhibition of spontaneous oxidized flavor in milk during storage at 1 C for 96 hr by dihydroquercetin

Cow no.	Month of lactation	Treatment	0 hr TBA [a]	0 hr Flavor [b]	96 hr TBA	96 hr Flavor
2	2	Control	5.00	−	21.00	++
		+ DHQ [c]	5.00	−	11.5	−
3	2	Control	7.00	−	22.25	++
		+ DHQ	7.00	−	13.75	−
4	2	Control	5.25	−	17.00	+
		+ DHQ	5.25	−	9.00	−
5	4	Control	5.85	−	25.25	++
		+ DHQ	5.85	−	19.00	+
12	8	Control	5.00	−	12.75	−
		+ DHQ	5.00	−	11.25	−

[a] TBA results expressed as μg malonaldehyde per 10-ml sample of milk.
[b] Flavor evaluation: − oxidized flavor not detectable; ++ pronounced oxidized flavor.
[c] Dihydroquercetin dissolved in warm distilled water and added to milk at the 5 mg per cent level.

The addition of 5 mg per cent of the water-soluble antioxidant, dihydroquercetin, to milks produced during the dry-lot regime inhibited the development of spontaneous oxidation (Table 1). This antioxidant has been shown to be effective against copper-sensitive milks (5). The results indicate either that the lipid system in milks produced under dry-lot feed conditions is more susceptible to oxidation than those produced on pastures or that more effective natural inhibition against oxidation prevails in the latter milks.

It is evident that the activity of xanthine oxidase in milk is not a primary determinant of its susceptibility or resistance to spontaneous oxidation. This view supports that of Smith and Dunkley (7, 9).

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EFFECT OF ENZYMES AND BACITRACIN ON SILAGE QUALITY [1]

Recent experiments have indicated that zinc bacitracin produced alteration in the chemical composition of silage. Alexander et al. (1) reported higher percentages for dry matter, crude protein, and ash, but lower crude fiber in silage treated with bacitracin. Andrews and Martin (2) found that bacitracin tended to increase silage dry matter percentage and ether extract, but found little effect on pH, fiber, protein, or ash. Dexter (3) found the effect of bacitracin on pH to be inconsistent, but treated silage had excellent mild odor. Bacitracin added to white Dutch clover tended to reduce the pH but had no consistent effect on proximate constituents (8). In another experiment (7) this antibiotic increased the dry matter, NFE, and ash content of silage.

Leatherwood et al. (4) determined that the addition of a product containing cellulolytic and other enzymes to both alfalfa and barley at ensiling decreased the percentage of cellulose slightly and increased reducing substances and titratable acidity. Ralston et al. (6) reported that fungal protease consistently increased digestibility of all components of the proximate analysis, whereas pectinase, HT protease, and fungal amylase had no consistent effects. Treatment of alfalfa with *Aspergillus oryzae* and a nutrient culture of lactic acid bacteria increased silage consumption and gains of heifers (5).

Since the complex carbohydrates (crude fiber) are usually less completely digested by animals than the more soluble forms (NFE), an experiment was conducted to determine whether the addition of cellulase, hemicellulase, and pectinase to sorghum at the time of ensiling would result in degradation of polysaccharides or otherwise influence silage quality.

EXPERIMENTAL PROCEDURE

Atlas sorghum was harvested in the mature seed stage and chopped to about ½-inch lengths and preserved in 1-gal glass jars. Lids were sealed with plastic tape. A factorial type design 4 × 2 × 2 was used. Each enzyme treatment was replicated four times, each at two concentrations and each concentration with and without zinc bacitracin addition. The enzyme preparations used [2] contained a low percentage

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[2] The enzyme preparations were supplied by Miles Chemical Company, Clifton, New Jersey.

TABLE 1

Effect of enzymes on chemical composition of silages

	Control	Pectinase	Cellulase	Hemicellulase
Dry matter *(%)*	21.5	22.2	21.0	21.4
Crude protein *(%)*	7.98	7.83	8.16	8.08
Ether extract *(%)*[a]	9.09	9.22	11.34	8.43
Crude fiber *(%)*	22.08	21.48	23.05	21.85
N-free extract *(%)*[a]	52.70	53.77	49.12	53.53
Ash *(%)*	8.16	7.72	8.33	8.12
pH	4.35	4.20	4.15	4.15

[a] Significantly different among enzymes at $P < 1\%$.

of enzyme (exact concentration unknown) suspended in a diluent. The treatments were cellulase preparation, 2 g and 8 g/3,000 g; hemicellulase (CE-100) 2 g and 7 g/3,000 g; pectinase concentrate, 2 g and 4 g/3,000 g; and Silotracin,[3] 7.5 g/3,000 g.

The Silotracin was added in the dry form and the enzymes were applied in water suspension. The suspensions were prepared by shaking the enzyme preparation with distilled water, filtering and making up to a volume of 75 ml to permit spraying of the additive on to the silage (3,000 g) for each bottle. The control samples received an equal quantity of water. These silages were stored at room temperature for about three months before opening for examination. Each silage was evaluated organoleptically by three individuals, pH measurements were taken, and chemical analyses were made according to standard procedures.

RESULTS AND DISCUSSION

The influence of the carbohydrate enzyme additives on silage composition is shown in Table 1. No differences were found between the control and the enzyme treatments. However, significant differences were found among enzyme treatments in ether extract and nitrogen-free-extract content.

[3] The Silotracin had a potency of 5 lb zinc bacitracin per ton. This product was supplied by Commercial Solvents Corp., Terre Haute, Indiana.

TABLE 2

Effect of enzyme level on composition of silage

	High-level enzymes	Low-level enzymes
Dry matter *(%)*	21.27	21.82
Crude protein *(%)*	8.11	7.93
Ether extract *(%)*[a]	10.38	8.94
Crude fiber *(%)*	22.43	21.82
N-free extract *(%)*[b]	50.72	53.55
Ash *(%)*[a]	8.36	7.76
pH	4.12	4.22

[a] Significantly different at $P < 5\%$.
[b] Significantly different at $P < 1\%$.

There was no apparent degradation of the complex polysaccharides by any of these enzymes. In contrast, the cellulase-treated silage was about four percentage units lower in NFE than the other treatments. It appears that microbial activity might have been stimulated in some manner by the cellulase with the utilization of NFE and the synthesis of microbial fat.

The over-all influence of increasing the level of enzymes (Table 2) was to significantly increase ether extract and ash and decrease NFE. Since these effects of enzyme level were found to be independent of the type of enzyme, it appears that each of the three enzymes at optimum levels may have an effect on ether extract and NFE similar to that of cellulase. An increase in ash content with the level of enzyme suggests a loss of organic matter. This, too, may be the result of stimulated microbial activity.

The effect of bacitracin was mainly in increasing crude protein and decreasing NFE (Table 3). This contrasts with earlier reports

TABLE 3

Effect of addition of zinc bacitracin on composition of silage

	With bacitracin	Without bacitracin
Dry matter *(%)*	21.34	21.74
Crude protein *(%)*[a]	8.12	7.90
Ether extract *(%)*	9.83	9.21
Crude fiber *(%)*	22.56	21.66
N-free extract *(%)*[a]	51.32	53.24
Ash *(%)*	8.17	7.99
pH	4.28	4.15

[a] Significantly different at $P < 5\%$.

of higher dry matter content (1, 2, 7) with bacitracin supplementation, but it corroborates those showing increased crude protein (1) and decreased NFE (7).

The dry matter percentage of these silages was not appreciably altered by any of the treatments imposed. The pH values were also very similar for all treatments. Since pH was below five for all treatments, it is concluded

that adequate acid development was obtained in all silages.

The silage odors were found by judges to vary from mild and fruity to very strong and foul. Although agreement among judges was good, odor characteristics were not consistent within treatments. One silage sample was discarded when it was found to have extensive mold development, resulting from a punctured lid. (The missing value technique was used to obtain values for this entry.) Three strongly acid samples and one foul-odored specimen were detected. Each represented a different enzyme treatment and the control group, respectively.

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OUR INDUSTRY TODAY

DIRECT-STEAM INJECTION SYSTEM FOR PROCESSING FLUID MILK PRODUCTS [1]

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In recent years a trend has developed towards ultrahigh-temperature processing of fluid milk products. Several commercially manufactured units are available for heating fluids to various high temperatures. These can be classed as heat exchangers (plate or tubular) or direct-steam injectors.

The use of direct-steam injection heating in food processing has been discussed by Morgan (2) and the mechanics of control over the steam system have been established (1-3).

A number of potential advantages to be realized, particularly from high-temperature treatments, are better keeping quality in the finished product, removal of volatile flavor compounds when vacuum treatment is incorporated, and better process control in terms of quality characteristics of the finished product.

This paper will describe the system developed, with some adaptations for use in a research program involving physical and chemical changes in the constituents of products subjected to the process.

STEAM-INJECTION SYSTEM

Description of heater. A drawing of the steam-injection heater is given in Figure 1. Milk and purified steam enter the heater at the openings indicated and are mixed at high velocities in the heater. The constriction in the mixing chamber is provided to enhance the mixing process. Spacers may be added where indicated, to adjust the heater for proportion and velocities, to insure mixing, and to provide a wide range of processing volumes. Steam is supplied to the heater in sufficient quantity to raise the temperature of the mixture to the desired point. The equipment may also be operated with excess steam to provide better steam distillation of volatile compounds.

Description of process. A flow diagram of the entire heating system is given in Figure 2. Fluid product can be preheated in a vat, or continuously through a high-temperature short-time pasteurizer, as high as 190 F, depending on the factor(s) being studied. The preheated product is passed through a homogenizer to provide positive flow through the steam-injection heater. The product is mixed with steam instantaneously in the heater and is ejected into a holding tube of the desired length. Skimmilk has been heated from 160 to 300 F for times ranging from 2 sec (calculated) to 180 sec (measured). From the holding tube, the product passes into a chamber wherein sufficient vacuum is maintained to cool the product to approximately the preheat temperature by flash evaporation, which restores the original

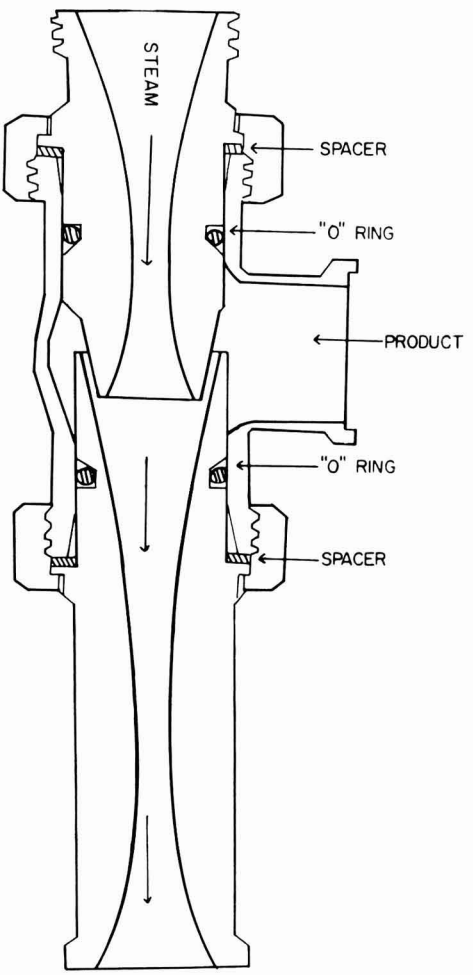

FIG. 1. Schematic diagram of the steam-injection heater.

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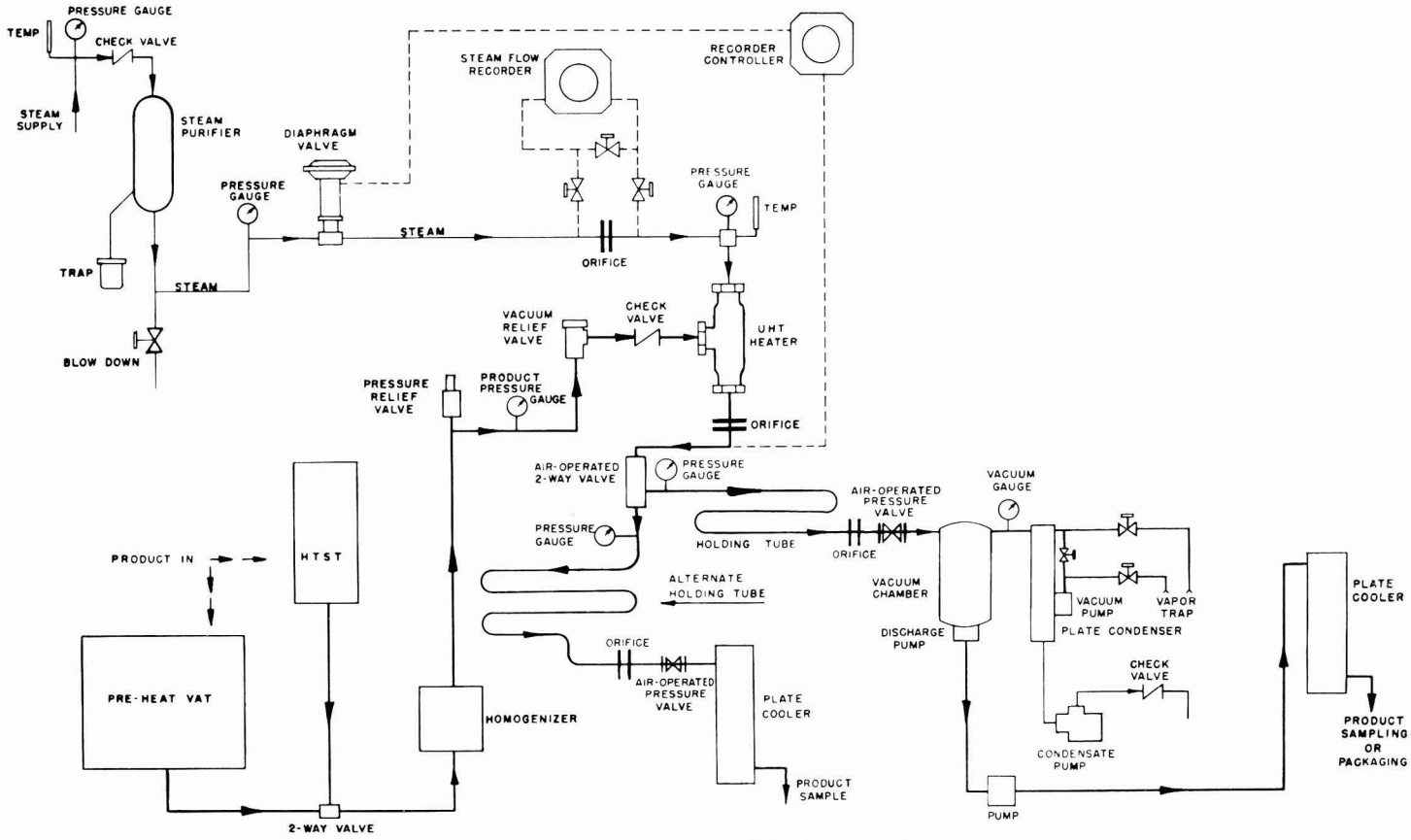

FIG. 2. Flow diagram of the steam-injection processing system.

solids concentration. The product is removed continuously from the bottom of the vacuum chamber and pumped through a plate cooler to lower the temperature to 40 F.

The desired heating temperature is automatically maintained by means of a Taylor recording controller (Series No. 122R) which operates a ¾-inch diaphragm valve in the steam supply line. Sufficient pressure is maintained on the holding tube by means of an air-operated pressure release valve to prevent flashing and to assure a smooth liquid flow during the entire length of the holding tube.

Flow rates of steam consumed may be recorded continuously by means of a Minneapolis-Honeywell recording flowmeter (Model No. 292D15) operating on the principle of a pressure differential across an orifice of known size. The recorder is connected to the steam supply line on each side of the orifice with ½-inch tubing as shown in the diagram.

The condensable vapors removed from the product by the flash evaporation treatment may be sampled from the condensate pump (Figure 2). The noncondensable vapors are sampled at the vapor trap as shown in the figure. In addition, if it is desired to cool the product without the use of vacuum and/or removal of volatile components, an alternate holding tube may be used which passes directly to a plate cooler or other cooling equipment. Analyses made on this latter product, however, must be corrected for dilution.

RESULTS AND DISCUSSION

Many devices have been developed for mixing live steam with fluid milk products for the purpose of rapid and efficient heat transfer. Since these devices have not been used for pasteurization, accurate temperature control has not been required. Therefore, very little emphasis has been placed on instrumentation and control. However, since direct steam-injection heating has become more widespread, it is desirable to have a system that is properly instrumented and controlled so that data can be obtained on the effects of a wide range of time and temperature treatments of milk and milk products.

Some of the problems encountered in bringing this system under control were:

1. Uniformity of flow rates with changes in temperature.
2. Correct volume and pressure relations between product and steam.
3. Maintenance of liquid conditions in the holding tube.
4. Location of the temperature sensing controller in relation to the incoming mixture of steam and product.

When a temperature of 300 F is desired, it is necessary to maintain a holding tube pressure of 60 to 70 lb (gauge). Obviously, pressures of this magnitude decrease the rate of flow of steam. Thus, it was necessary to use a ¾-inch steam-regulating valve, rather than a ½-inch valve, as previously calculated, to provide an adequate volume of steam. Positive pumps were found to allow some by-passing of product resulting in a reduction in rate of flow at the higher temperatures and pressures. This was corrected by using a homogenizer as the product pump. Check-valves were placed in the incoming product and steam lines to prevent backing-up in either of the lines.

The injection heater was designed with spacers so that the opening for product could be adjusted for changes in volume of product processed. Since the product flow was standardized at approximately 2,500 lb/hr, there was no need for changing the spacers once the optimum operating conditions were determined. This was found to vary some between fluid milk and ice cream mix.

A glass holding tube was used to determine the state of the mixture at the operating temperature. A hand-operated air-pressure control valve was located on the discharge end of the holding tube. Sufficient pressure was applied on the valve to maintain a liquid state in the holding tube. This pressure varied from about 10 lb at 160 F to 60 lb at 300 F. There was some vibration of the discharge valve as a result of the large pressure drop from 60 lb to a 25-inch vacuum. This vibration was eliminated by placing an orifice in the holding tube a short distance before the valve. Times in the holding tube were accurately measured with a Solu-Bridge automatic timer (Industrial Instruments Company) and were found to vary less than 0.05 sec under a given set of operating conditions.

It was necessary to place the temperature controller near the heater, to get an immediate response to temperature changes. This was especially true when long holding times were used. It was found that an orifice placed at the discharge of the heater reduced fluctuations that resulted from incomplete mixing and thus gave more uniform temperature control.

The degree of temperature control with the equipment as described is shown in Figure 3. It is noted that very accurate temperature control was maintained on the system over a range of temperature varying from 160 to 300 F. The major fluctuations in the chart, e.g., at 270 and 300 F, were due to adjustments in holding tube pressure. Also, the system was brought under control with large changes in temperature in a relatively short period of time.

Since the discharge valve on the holding tube is manually operated, some experience and skill are necessary to make the adjustments quickly. As more operating data on pressure relationships are obtained, it should be possible to preset the conditions for stable uniform operation.

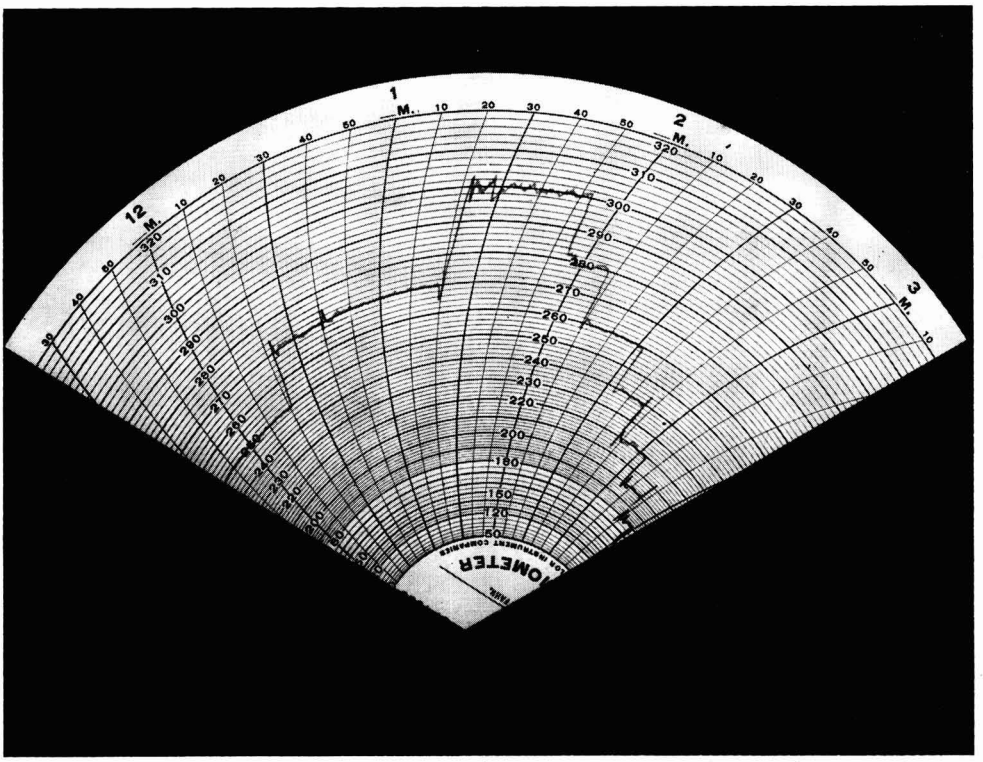

FIG. 3. Section of recorder chart showing temperature control at various operating temperatures.

SUMMARY

A steam-injection system for heating fluid milk products is described in which temperatures are automatically and accurately maintained as high as 300 F. Holding times are varied by adjustments in the length of the holding tube. Thus, accurate heat treatments of fluid products can be provided over a wide range of total treatments. The system is very adaptable for research and commercial use because of the wide range of treatments and volumes possible with the equipment.

The system has been used for the commercial processing of fluid milk and ice cream mixes in conjunction with a high-temperature short-time pasteurizer. It has also proved very useful in research on fluid milk, ice cream mix, and skimmilk for cultured milk products. The vacuum system is being used to provide quantities of volatile materials from milk for identification purposes. The entire system is cleaned easily in-place and should be very adaptable to an automated process.

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