# Journal of DAIRY SCIENCE

Vol. 47 January, 1964 No. 1


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# JOURNAL OF DAIRY SCIENCE

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# INSTRUCTIONS TO CONTRIBUTORS

The Style Manual for Biological Journals[1] has been adopted as the guide for authors. Publication of papers is limited to members of A.D.S.A., but only one author of a joint paper need be a member. Those of outstanding merit may be accepted from nonmembers. Papers when accepted become the copyright of the JOURNAL and can be reprinted only by the Garrard Press. Reproduction of graphs, tables, and illustrations for books and other periodicals may be authorized by the Editor-in-Chief.

To be eligible for publication in the JOURNAL, the data in papers must not have appeared previously in any other publication except proceedings of annual or biennial conferences or in symposia.

## 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. The summary aids in the dissemination of scientific information, reduces the work and cost of abstracting journals, and is convenient for readers.
   b. The summary must be on the first page, be brief, specific, and factual. It should not exceed 200 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. Except in most unusual cases, references to earlier work should be omitted.
3. Statement of the problem and pertinent investigations.
4. Objectives or reasons for research.
5. Experimental procedures.
6. Results.
7. Discussion. (6 and 7 may be combined.)
8. Conclusions are optional.
9. Acknowledgments.
10. References. The minimum of only pertinent references should be used. They must have title(s), 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.
11. Manuscripts must be typed double-spaced on 8½- by 11-inch bond paper. Multilithing on bond paper is acceptable. 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. *Pages should not be stapled together.*
12. Figures (graphs) should be made with black India ink on white drawing paper, tracing paper, or blue linen and the sheets should not exceed 8½ by 11 inches. Graph papers with yellow, green, and red lines should not be used, because the lines cannot be filtered out. Curves should be identified with the symbols ○, ⊙, ●, □, ■, △, ▲, ▼, +, or ×, and they should be about 0.8 mm thick, for the axes about 0.5 mm thick, and for grid lines about 0.4 mm thick. Grid lines are necessary only if readings are to be made from the curves. Letters on the abscissae, ordinate, and the figure should be in upper case and be about 3 by 5 mm and about 0.5 mm thick, to be readable when graphs are reduced to column width. Titles for figures (graphs) must be on separate sheets. Following is a well-made figure reduced to size of the printed page.

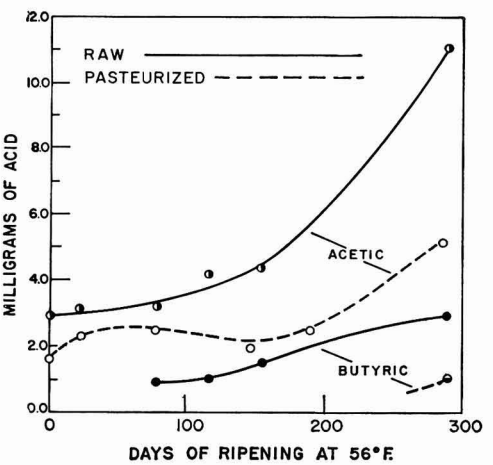



FIG. 1. Acetic and butyric acids in raw and pasteurized milk Cheddar cheese during ripening (milligrams in distillate obtained from 150 g of cheese oil).

[1] American Institute for Biological Sciences, 2000 P Street, N. W., Washington, D. C. Price $3.

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

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

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

    *Tables are costly. Use graphs whenever possible.*
14. Colored illustrations can be reproduced. Authors should submit detailed specifications to the Editor and costs will be supplied.
15. Abbreviations for titles of periodicals and for botanical, chemical, physical, mathematical, and statistical terms should conform to those in the Style Manual for Biological Journals.
16. Terms such as Cottage cheese, Cheddar cheese, Limburger cheese, etc., should be capitalized as indicated. Butteroil, skimmilk, buttermilk, etc., should be written as one word. Milk fat has replaced butterfat.
17. Critical reading of papers, before they are submitted, by persons other than the author(s) will help to clarify statements and eliminate errors.
18. All manuscripts must be submitted to the Editor-in-Chief in duplicate. The ribbon copy, only, of revised manuscripts should be resubmitted.
19. Receipts of manuscripts will be acknowledged. Authors will be notified within 30 to 60 days of the action taken by the Editorial Staff.

TECHNICAL NOTES

20. The technical note section of the JOURNAL is for the publication of research with narrow boundaries, announcement of new findings that may help other scientists, invention of research and teaching devices, establishment of priorities, and for useful information from projects that have been terminated before completion. *This section is not a repository for inferior research.*
    If the scope of the technical note permits, it may be organized the same as a research paper, except there is no summary at the beginning and the author(s) name(s) and affiliation(s) appear at the end. Otherwise organization is flexible.
    Technical notes may or may not be reviewed by the Editorial Board.

OUR INDUSTRY TODAY

21. This section of the JOURNAL is for papers of timely industry interest. If the papers contain applied research, they should be organized the same as research papers, although a summary at the beginning is not required. Regardless of the material in these papers, they should be organized in logical topics.
    Papers in this section may or may not be reviewed by the Editorial Board.

PUBLICATION OF EVENTS

22. Material for the announcement of meetings, short cources, and other special events of interest to the Association membership, must be received by the first day of the month and at least two full calendar months before the event is scheduled to take place. For example, the material for any event in February must be received by December 1. These statements do not apply to material for the Annual Meeting.

# PEOPLE AND EVENTS

## Robert E. Johnson Honored at Storrs

R. E. JOHNSON of Storrs was called into the College of Agriculture dean's office on November 1 at the University of Connecticut, where he found four distinguished educators waiting to greet him. They were DR. H. D. BABBIDGE, JR., the University President, A. E. WAUGH, Provost, W. B. YOUNG, Agriculture Dean, and DR. W. A. COWAN, Head of the Animal Industries Department. This group paid tribute to Professor Johnson for his 40 years of service as a member of the University of Connecticut faculty. The honored educator is Associate Professor of Dairy Husbandry. President Babbidge read a letter written by Gov. John Dempsey, praising Prof. Johnson for his remarkable record. The letter stated in part; "During the 44 years, including your undergraduate days, that you have spent at Storrs, you have witnessed a remarkable growth and development in our State University. You may well take pride in the considerable contribution you have made to that development. The cattle bred by your Animal Industries Department and the prizes they have won add a great deal to the Connecticut agricultural reputation."

R. E. Johnson

Professor Johnson's main duties over the years have been in teaching. He has developed and taught courses in dairy production—from fundamentals for undergraduates to advanced theory for graduate students. He also has been responsible for the development and management of the dairy herd, acknowledged as one of the top college herds in the country. He is an outstanding cattle judge and a close friend and advisor to the owners and managers of the outstanding dairy herds in the East.

His first love is teaching. He is never satisfied with the student who just learns the facts—he must be able to relate these facts to life that exists around him. "Only when the student is able to do this is he learning something and stimulated to learn more," Professor Johnson said on this fortieth anniversary.

Prof. Johnson, a native of Norwich, received his B.S. degree in dairy husbandry from the Connecticut Agricultural College (now the U of C) in 1922 and his M.S. degree from Iowa State College a year later. He is a member of the American Genetic Association, American Society of Animal Production, the American Dairy Science Association, Alpha Gamma Rho, social fraternity, Gamma Sigma Delta, honorary agricultural society, and Phi Kappa Phi.

## G. W. Shadwick Honored

DR. G. W. SHADWICK, director of technical services for Beatrice Foods Company in Chicago, has won an Indy award, industrial movie world's equivalent of the famed Hollywood Oscar. He produced a film entitled Milkman to Malaya, judged the best one-man film, and merited a special citation. The chemist and bacteriologist received his special Indy at the fifth annual industrial film awards dinner in Boston.


INDUSTRIAL PHOTOGRAPHY

ANNUAL *Industrial Film* AWARD

CERTIFICATE OF MERIT

Awarded to *Dr George W Shadwick*

for technical assistance and professional services rendered in the production of

*"Milkman To Malaya"*

by *Beatrice Foods Co.*

selected *Best One Man Film*

in the class of *Special Citations*

date *September 1963*

New York, N.Y.

James S. Watkins, Publisher

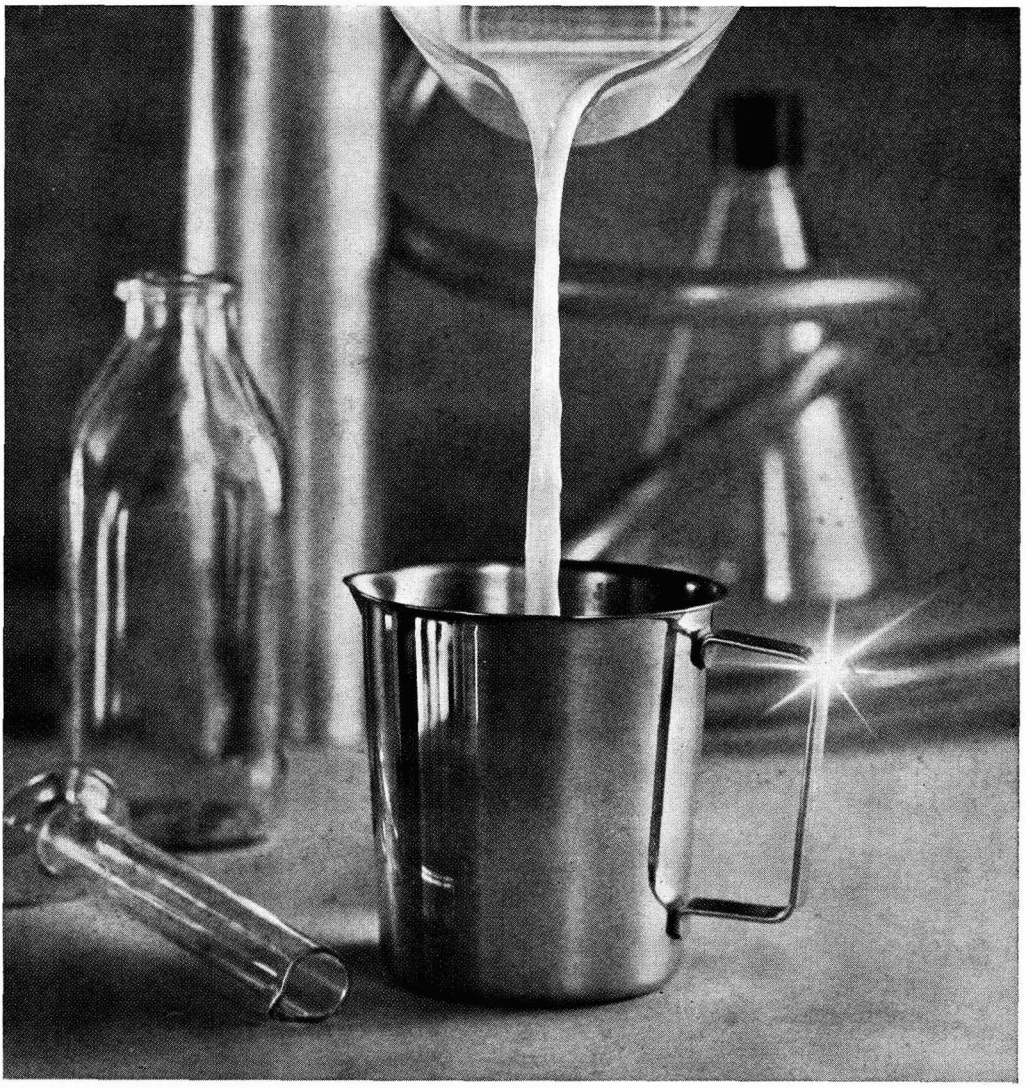
Mitchell M. Beeler, Editor

The film deals with the establishment of a Beatrice Foods plant in Kuala Lumpur, Malaysia. Dr. Shadwick made 3,000 feet of film, edited to about 1,500 feet, running for almost 45 minutes.

The production is not the dry, technical type of film usually associated with training and company productions. Dr. Shadwick produced his film as a travelogue type that appeals to the general public as well as to company personnel and stockholders.

The film portrays the first building of an overseas plant by Beatrice Foods Co. It is climaxed with the dedication of the plant that produces a canned, sweetened and condensed milk in a country that has no fresh milk.

The film, in sound and color, also includes the famed Kilauea Puna volcano eruption in Hawaii and scenes of aboriginal life in native Malayan villages. "Happiest people I ever saw," Dr. Shadwick said about the healthy, industrious aborigines he lived among for a week. The film also includes scenes in New Zealand, where the company buys milk that is processed into canned, condensed milk in Malaya.



# Why milk processors spell clean with a "K"

"K" is the eleventh letter in the alphabet . . . but for modern cleaning and sanitizing in milk processing plants it tells the whole story from A to Z. When you add the "K" from Klenzade you add a new concept in completely controlled sanitation programs. The Klenzade Complete Plant Quality Program is safer, simpler and quicker. From **Brite-Klenz HC-20** to **Heavy Duty Acid LC-30,** you can get all your detergents, sanitizers and application equipment from one reliable source. A Klenzade technical representative will be happy to help you improve your dairy products. Write today. We'll reply promptly.

KLENZADE

For Completeness, Quality, and Cost-Cutting, it's **KLENZADE PRODUCTS,** Dept. 56, Beloit, Wisconsin.

M. M. Badler (left), editor of Industrial Photography, presenting the Indy award to G. W. Shadwick, with G. W. Colburn holding the accompanying certificate of merit.

One part includes Indian Thaipusum, where Hindus perform incredible feats of penance like walking on fire and piercing their tongues with spikes or bodies with spears. The bloodless religious rites are performed while those seeking forgiveness are in mental trances.

Dr. Shadwick's film has been seen by groups all over the country and is being used by governmental agencies like the Department of Public Health and various agricultural services. Currently, Universities of Hawaii, Illinois, and Ohio State have copies of the film.

The film was originally produced, as were previous Shadwick productions, for annual stockholders' meetings. Thus, a nationally known chemist and bacteriologist who took up photography originally only to augment his teaching in college has realized the top prize in the world of industrial photography.

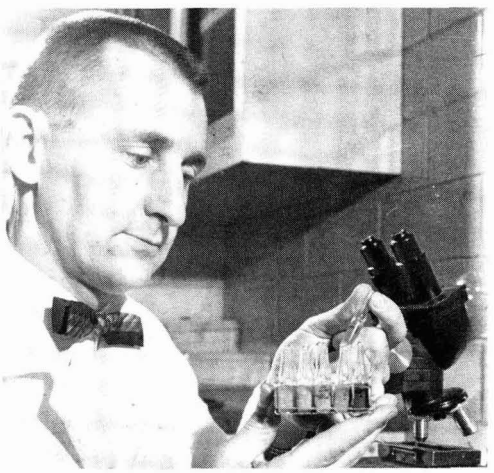
## N. L. VanDemark Appointed Head of Department of Dairy Science at Ohio State

N. L. VanDemark, professor of physiology in the University of Illinois Department of Dairy Science, has been named head of the Dairy Science Department at Ohio State University. He will begin his administrative duties on February 1.

Dr. VanDemark joined the U. of I. dairy science staff in 1948 as assistant professor of dairy cattle physiology. His major responsibilities have been teaching and research in dairy cattle reproduction. He has published more than 100 scientific papers on reproductive physiology and artifical insemination and is co-author with G. W. Salisbury of the text, Physiology of Reproduction and Artificial Insemination of Cattle.

He is one of the more popular instructors in the U. of I. College of Agriculture. In 1960, students in agriculture voted him the outstanding teacher.

Also active within the community, VanDemark is serving on the Champaign, Illinois, Unit 4 School Board, a position he has held since 1956. He has been chairman of the School Board's building committee for seven years.



VanDemark is a past president and program chairman of the Bondville, Illinois PTA. He served five years as Scout Master for Troop 20 of the Bondville Boy Scouts and is institutional representative for that troop.

In 1958, he received the $1,000 Borden Award in Dairy Production for contributions to the fundamental knowledge of reproduction in cattle. He also has been president of the National Committee for the Biennial Symposium on Animal Reproduction. In 1958, he was invited by the Austrian government to attend the Eighth Austrian Symposium on Animal Reproduction. He attended the CIBA Foundation Symposium on Mammalian Germ Cells in London in 1952.

VanDemark is a member of the American Dairy Science Association and served on that organization's editoral board from 1956 to 1961. He also is a member of the American Society of Animal Science; a Fellow of the American Association for the Advancement of Science; a member of the society for the Study of Fertility; Gamma Sigma Delta, and Sigma Xi.

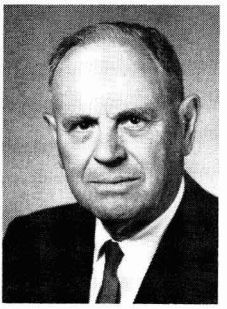
Before joining the U. of I. Department of Dairy Science in 1948, VanDemark was assistant in animal husbandry at Cornell University. During 1946-47, he served in Austria as livestock specialist for the Austrian Livestock Rehabilitation Program. He received his B.S. degree from Ohio State University in 1941, his M.S. degree from Ohio State in 1942, and his Ph.D. from Cornell University in 1948.

Dr. VanDemark served in World War II. He was promoted through the ranks from private to 2nd Lieutenant in the U.S. Infantry and counterintelligence.

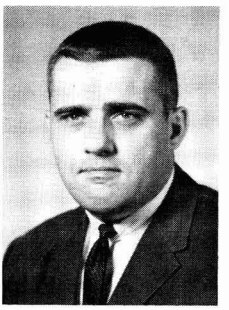
### New Appointments at North Carolina

DR. J. H. NAIR, a native of Chicago, Illinois, has recently joined the Department of Food Science at North Carolina State as visiting professor. Dr. Nair is a physical chemist and food technologist widely known in the United States and abroad. He was educated at Beloit College and Syracuse University. He has been visiting lecturer at Columbia University. Active for forty years in numerous scientific and technical societies, Dr. Nair has been president of the Association of Research Directors and of the American Institute of Chemists. For many years he has been listed in Who's Who in America and in American Men of Science. Beloit College in 1958 conferred on him an honorary degree of Doctor of Science.

DR. F. F. BUSTA, a native of Montgomery, Minnesota, has recently joined the staff at N.C. State as Assistant Professor in Food Science. Dr. Busta received his B.A. and M.S. degrees from the University of Minnesota and his Ph.D. from the University of Illinois. Dr. Busta's area of work will be concerned with the effect of ultrahigh temperatures on bacterial spores and other phases of ultrahigh-temperature sterilization.



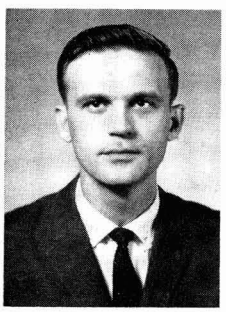
J. H. Nair



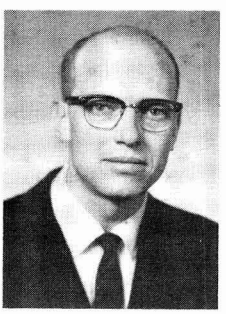
F. F. Busta

Mr. C. W. Moss, a native of Rural Hall, North Carolina, is now serving in the capacity of Research Associate in the Department of Food Science at N. C. State. Mr. Moss received his B.S. and M.S. degrees from N. C. State and worked in the Dairy Division of N. C. Department of Agriculture for about three years. His major area of work is concerned with food microbiology.

DR. R. J. BINGHAM, a native of Blackfoot, Idaho, has recently joined the staff at N. C. State as Assistant Professor in Food Science. Dr. Bingham received his B.S. degree at Utah State University and his M.S. and Ph.D. degrees at the University of Wisconsin. Dr. Bingham's major area of work will be in Food Chemistry concerning flavor research and ultrahigh-temperature processing.



C. W. Moss



R. J. Bingham

# STUDENT NEWS SECTION

W. W. SNYDER, Editor

A Section Devoted to News of Student Members

## SPOTLIGHT

The Oregon State University Dairy Club is featured this month. This far-Western club celebrated the 40th year since its founding by sending seven students to the 1963 American Dairy Science Association meetings at Purdue.

### The Oregon State University Dairy Club

I. R. Jones
Professor of Dairy Science

The first student Dairy Club at the then Oregon Agricultural College was organized in the school year of 1923-24 with Percy Murray, presently one of the owners of the Klamath Falls Creamery, Klamath Falls, Oregon, as its first president. At that time the late Professor P. M. Brandt was Head of the Department of Dairy Husbandry, with R. C. Jones for many later years extension dairyman of the USDA, and V. D. Chappel, dairy processing plant owner, Lodi, California, as other staff members. Senior Oregon State faculty members actively interested in the Dairy Club during ensuing years include I. R. Jones, 1925 to date; G. H. Wilster, 1929-60; H. P. Ewalt, 1932 to date; F. B. Wolberg, 1945 to date; G. A. Richardson, 1947 to date; J. O. Young, 1950-62; and R. W. Stein, 1952 to date.

The Club has been active continuously since 1923, with the exception of the World War II years. Many Oregon alumni of the Club have made and are making outstanding contributions as dairy farm and industry leaders as well as

in many related lines of business. Many have held or hold prominent positions in federal or state governmental agencies and in educational institutions.

The Club has been affiliated with the American Dairy Science Association as a student chapter since the development of this program by A.D.S.A. The 1963 student affiliate certificate identifies the chapter as the Oregon State University Dairy Club.

Current Club membership generally consists of both dairy and production and dairy processing students, although any student enrolled in general agriculture is eligible to belong. The membership roll usually consists of about 40 students. One faculty advisor from each of the two dairy groups is selected to assist the students in planning their program of activities. Present advisors are I. R. Jones and M. W. Montgomery.

The Club has traditionally been one of the most active of the nine student clubs in the School of Agriculture on campus, and cooperates with all-school student activities. The Club meets twice monthly during the school year. Talks by industry and educational leaders both on and off campus are featured at most meetings.

### Club Activities

The Club, since its origin, has engaged in fund-raising activities, with the proceeds helping finance student field trips, assisting with expenses of judging teams to the national contests, to the Pacific International Livestock Exposition at Portland, and the Grand National Livestock Exposition at San Francisco. Due to the distances involved, it has been possible for the Club to finance teams to the national intercollegiate contests only infrequently. Judging team members not only have given meritorious performances but also have enjoyed many interesting and lifelong-remembered acquaintanceships and experiences.

**Oregon's 1962 Pacific International dairy cattle judging team in front of trophy case, Withycombe Hall. From lert to right: Glen Ufford, Adolfo Velasco, Sharon Hobson, Marjorie Wheaton, and Coach Merle Peters.**

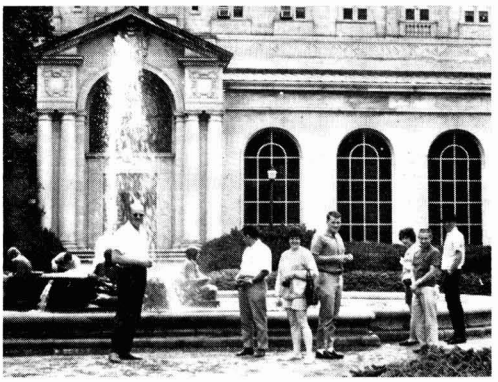
Oregon State Dairy Club members and advisors at the dairy bar with officers in front, including Don Claeys, 1963 president, extreme right, and 1962 president, Ed Hemenway, at his left.

The Club stimulates interest among students in preparing animals for show and arranging classes for judging by helping to organize the Little Pacific International, a student livestock show held on campus during the spring term each year. The Club provides trophies for the high contestants in dairy cattle judging and in fitting and showing. Each year the Club honors its outstanding freshman, senior, and graduate student members by giving plaques and cash awards of $25, $25, and $50, respectively. Club members assist in organizing judging classes during the annual Ag Weekend activities. Also, they act as leaders of groups during university visitation of prospective incoming high school students.

Oregon group experiences one of the unpleasant aspects of motor travel on its 1962 journey to the A.D.S.A. meetings.

Dairy students assist in the operation of the Dairy Bar in Withycombe Hall—location of the Department of Animal Science and the Dairy Processing Laboratory on the campus. The bar is a center for milk, ice cream, and other refreshments for students and staff members. Profits derived from the sale of certain food items benefit Club members by helping to finance field trips and other projects.

Club members make and sell Nutty Buddy ice cream bars not only at the Dairy Bar

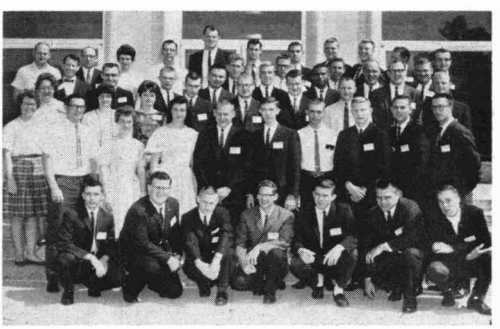


The Oregon State group visit Iowa State campus. From left to right: Professor Don Anderson, Carlos Lobo, Sharon Hobson, Eldon Boge, Marjorie Wheaton, Glen Ufford, and Stephen Hobson.

but also at student functions on campus. In other fund-raising projects, Club committees organize, prepare, and serve light refreshments or even complete lunch for 50 to 300 people attending meetings of state-wide organizations in Withycombe auditorium. Such meetings give the opportunity of meeting agricultural and industrial leaders from over the state.

A spring field trip is usually scheduled each year. This trip is made by cars or bus during the week of spring vacation. Generally, field trips are taken in different directions in successive years to allow the same students to observe the diversified areas in different sections of Western United States. Thus, in 1962 a 1,000-mile trip was made northward through the state of Washington to British Columbia, and in 1961 more than 2,500 miles southward through California. In addition to visiting dairy farms, breeding establishments, milk and dairy processing plants, the itinerary includes places of general educational value, historical interest, and scenic beauty.

The most extensive recent trip by members of the Club was taken by a delegation of seven Club members to the 1963 American Dairy Science meetings at Purdue. The Oregon State University delegates were accompanied by Extension Dairyman, D. E. Anderson, during the 17-day, 5,600-mile trip to Indiana. For several students it was their first trip eastward from Oregon. Legends on the photos will show a few of the highlights of the trip, including a group picture of the student delegates at Purdue from universities throughout the United States. Six of the seven Oregon State students were present for the picture.



**The 1963 A.D.S.A. student Affiliate members at Purdue, including six of seven Oregon State students.**

In addition to the interesting and educational meetings at Purdue, highlights of the trip were visits to university campuses at Utah, Wyoming, Colorado State, Nebraska, Iowa State, Wisconsin, and Minnesota, and places of interest in many cities, such as the Climatron in St. Louis and the Board of Trade in Chicago. Also included were visits to experimental farms, pure-

TEEN-AGE NUTRITION

# They're "starving" themselves!

*Nutritionally suboptimal diets among American teen-agers are usually traceable to faulty food consumption habits.*

Teen-agers are not immune to the immutable law of nutritional supply and tissue demand. They cannot forever escape the consequences of substandard nutrition on health, vigor, and performance. Nutrition surveys continue to disclose failures to meet the recommended dietary allowances of the Food and Nutrition Board, National Research Council for calcium, ascorbic acid, riboflavin, vitamin A, and protein in a disturbing proportion of the American teen-age population.

Teen-age food consumption patterns are studded with missed meals, erratic eating habits, food faddism, fear of fatness, and culinary conformity to the group. Teen-age food choices frequently reflect a misguided independence expressed as a defiance of good food habits inculcated in childhood. Teen-age girls in their zest for stylish slenderness and clear complexions have the least nutritious diets of any member of the family. As prospective mothers and homemakers they are most in need of nutritional guidance yet most resistant to advice and counsel unless properly motivated. Teen-age boys in their desire for athletic prowess and muscular magnificence are more interested in food quantity than in food quality. Ironically, teen-age nutritional omissions are rarely the result of privation. They stem instead from an ignorance of, or indifference to, the virtues of good nutrition.

Optimum nutrition in the teen-age period is best accomplished by eating a well-balanced diet containing at least a quart of milk—or its equivalent in other dairy products—with their wealth of calcium, B vitamins, and high quality proteins.

bred dairy herds, artifical breeding associations, processing plants, and research centers. Places of scenic beauty seen in the Rocky Mountains and Midwest included the Badlands of South Dakota and Yellowstone National Park.

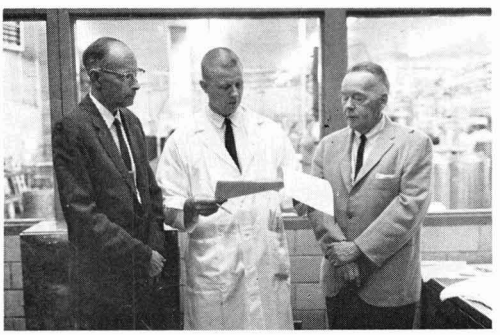
One of the highlights of the activities during the past year was the presentation of honorary Oregon State Dairy Club membership certificates to the American Dairy Princess, Sandra Tibeau, and Oregon Dairy Princess, Adrienne Ellison, at the Oregon Dairy Industry Convention banquet at the University attended by some 300 industry people mainly from Oregon. The princesses are portrayed in the picture with All-American quarterback, Terry Baker, of Oregon State University, winner of the Heismann Award and numerous other trophies and named as the outstanding 1962 football player in the United States.

1962-63 Oregon Dairy Princess Adrienne Ellison and American Dairy Princess Sandra Tibeau, both honorary Oregon Dairy Club members, toast Oregon's All-American quarterback and Heismann award winner, Terry Baker, and congratulate him upon his recognition as the winner of the National Youth Fitness Award at the Oregon Dairy Industries Convention.



### Sanitarians' Short Course Held at North Carolina

Twenty-six North Carolina sanitarians participated in the Food Sanitarians' Short Course conducted by the Department of Food Science November 4 through 26 on the North Carolina State campus. The cooperating agencies in this Short Course were: Food Science Department and North Carolina State Board of Health. Dr. F. B. Thomas, Food Science Department, served as chairman of the Short Course. Dr. M. E. Gregory, Food Science Department, coordinated the dairy phases of the Short Course.



John Andrews, M. E. Gregory, Carson Foard.

The Short Course has been revamped from the old Dairy Sanitarians' Short Course to now cover all the major food industries in North Carolina. Approximately one-half of the Short Course is related to the dairying phase and the remainder to poultry processing, fruits and vegetable processing, meats processing, and seafood processing.

### Fieldmen's Short Course at North Carolina

Over one hundred industry and regulatory personnel participated at the annual Dairy Fieldmen and Sanitarians Conference, November 25 and 26, on the North Carolina State campus.

G. S. Parsons, Extension Dairy Husbandry Specialist, coordinated the program. Some of

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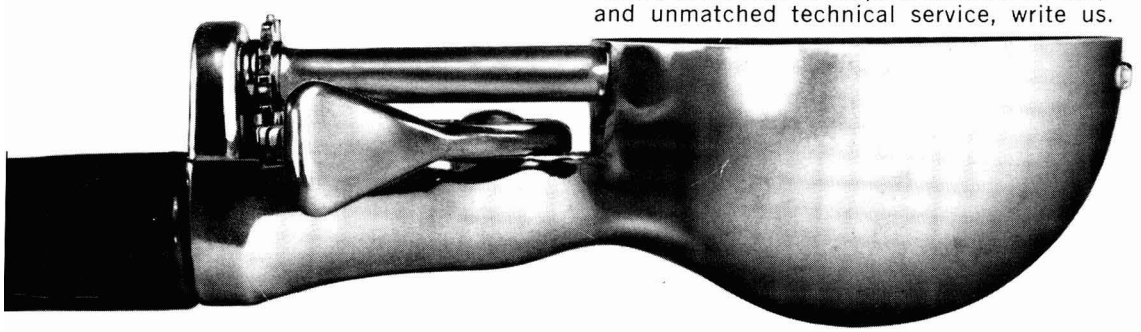



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the others participating on the program include: JOHN ANDREWS, E. T. CHANLETT, MAX DECKER, CHARLES DRAPER, W. S. FINCH, M. J.

**(L to R), E. T. Chanlett, G. S. Parsons, M. J. Foter.**

FOTER, R. W. HENNINGSON, G. W. HANSON, JR., R. O. HOWARD, T. S. HAGGAI, FRANCIS PATTERSON, PAMELA PRIDGEN, S. C. RICH, H. R. ROBERTS, G. L. WEIR, J. D. GEORGE, M. E. GREGORY, GEORGE HYATT, JR., FRED KNOTT, JAMES MIDDLETON, W. R. MURLEY, R. D. MOCHRIE, I. D. PORTERFIELD, DON PARDUE, W. M. ROBERTS, R. R. RICH, RAY RITCHIE, M. E. SENGER, M. L. SPECK, FRANK SARGENT, FRED WARREN.

### Dairy Conference at Kentucky

A conference featuring important problems in the dairy industry and flavor clinics was sponsored by the University of Kentucky and the State dairy industry in November.

PROFESSOR L. R. DOWD of the University of Connecticut emphasized flavor problems in ice cream and their thoughtful consideration. DR. J. J. JEZESKI of the University of Minnesota pointed out the importance of rancidity in milk and how it could be avoided. MR. N. E. THIEL of Sealtest Foods spoke about safety problems and stated that the dairy industry had one of the poorest safety records of any industry.

Other speakers were R. D. FINLEY of the Pet Milk Company, G. A. HOURAN of the DeLaval Separator Company, H. E. RANDOLPH of the University of Kentucky, and R. L. SMITH of Louisville, Kentucky.

### Southern Illinois University Sponsors Dairy Meeting

More than 100 dairymen from southern Illinois attended a meeting at Carbondale which featured economic, breeding, feeding, and managerial problems in dairy farming. The speakers were DR. W. H. ALEXANDER, of Louisiana State University, DR. D. A. WIECKERT of the University of Wisconsin, DR. H. H. OLSON and H. S. THURMON of Southern Illinois University, and W. A. WEEKS of Rockford, Illinois.

### Pet Milk Company Holds Managers' Meeting

On November 25, 1963, managers in the Pet Milk Company met at the Beach Club Motel in Naples, Florida. Awards were made to managers who had shown the most improvement in reducing costs and improving product quality. Four awards were given: three in milk and one in ice cream. These awards will be made annually, with the winners of the first awards retaining them if they are won a second time.

**B. K. Kemp, Charlotte, N. C., first place in milk; W. A. Pittman, Greensboro, N. C., third place in milk; R. B. Redfren, Production Manager; L. H. Gregory, Charlotte, N. C., first place in ice cream; and A. D. Redmond, Knoxville, Tenn., second place in milk.**

### Public Health Training Courses

The Public Health Service, through the Division of Environmental Engineering and Food Protection, will present the training course, Methods and Practices for State Milk Laboratory Survey Officers, March 9-13, 1964, to be conducted by the Training Program of the Robert A. Taft Sanitary Engineering Center in Cincinnati, Ohio.

Survey officers responsible for certification of laboratories examining milk supplies for interstate shipment are the eligible trainees. They should, preferably, have completed the course, Laboratory Examination of Milk or Microbiological Examination of Milk and Milk Products. Instruction in lectures, discussions, demonstrations, and laboratory covers the control methods employed in examining interstate milk supplies.

### Dairy Technology Societies

**Kansas**—January 13 meeting featured Professor Paul E. Johnson, Dairy Department, Oklahoma State University, with the topic Rancidity in Milk.

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**Massachusetts**—At the October meeting Professor Alec Bradfield of the University of Vermont gave an interesting talk and showed slides of his trip to Europe and visitation to Dairy Industry plants in England and on the Continent. Another speaker was Harold O. Clark, Flavor Specialist of the Vermont State Department of Agriculture.

At the November meeting Warren Church, engineer from H. P. Hood & Sons, Boston, and Chester Cheney of Eliot Creamery Inc., Milton, presented a very interesting and instructive discussion on the subject of Water.

**Metropolitan**—Speaker at the January 14 meeting was Ed Thom, Editor, Olsen Publishing Company, Milwaukee, Wisconsin. Olsen Publishing Company publishes Milk Dealer, Ice Cream Review, and Manufactured Milk Products Journal.

**Central Michigan**—Guest speaker at the January 15 meeting was Kenneth Van Patten, Assistant Chief, Dairy Division, Michigan Department of Agriculture. His topic: Updating Information on Michigan and USPHS Standards for Milk Processing.

**Western Michigan**—January 20 meeting featured Dr. Charles Stine of the Food Science Department, Michigan State University sity, as speaker. What's New in Dairy and Other Food Product Development was his topic.

**Oklahoma**—Dave Hatherley, APV Company, was speaker at the January 13 meeting, discussing Ultrahigh-Temperature Pasteurization.

**Philadelphia**—At the January 9 meeting of this group, Herbert Saal, Editor, American Milk Review, spoke on Trends in the Dairy Industry.

### Theses Completed

**Master's Degree:**

**August E. Branding.** Physiological effects of low levels of fiber in the diet of lactating cows on a high plane of nutrition. The Pennsylvania State University.

**Manfred Kroger.** Gas chromatographic analysis as an aid in organoleptic evaluation of cheese. The Pennsylvania State University.

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# RESEARCH PAPERS

## COMPARISON OF THE PROTEOLYSIS PRODUCED BY RENNET EXTRACT AND THE PEPSIN PREPARATION METROCLOT DURING RIPENING OF CHEDDAR CHEESE [1]

N. P. MELACHOURIS AND S. L. TUCKEY
Department of Food Science, University of Illinois, Urbana

SUMMARY

This investigation determined the differences in proteolysis produced by commercial rennet extract and a pepsin preparation during the ripening of Cheddar cheese. Preliminary experiments with the two enzymatic agents were made in fresh pasteurized milk. The bacterial factor was eliminated by the addition of toluene. The general course of protein degradation, as measured by nonprotein nitrogen production, was found to be higher when rennet extract was used. When the pH of milk was adjusted to four different levels from pH 6.5 to 5.3, both rennet extract and pepsin had maximum proteolysis at pH 6.3, with rennet extract liberating more nonprotein nitrogen than pepsin.

The proteolysis produced by rennet extract in Cheddar cheese during ripening was higher than that produced by pepsin as measured by the nonprotein nitrogen. However, there was almost no variation in the amino acid pattern of Cheddar cheese of the same age, regardless of clotting agent used. The concentrations of the amino acids were measurably different, but no qualitative differences were observed.

Organoleptic evaluation of the cheese revealed that both cheeses were of good quality. However, the texture and body of the cheese made with pepsin was slightly curdy for a longer period during ripening. Bitter flavor did not develop during the ripening period in any lots of cheese.

Pepsin and rennin have many similar properties. Because of this, pepsin may be used in the cheese industry to a greater extent in the future than it has in the past, either singly or in combination with rennet extract. It would appear from the decreasing number of dairy calves that the amount of rennet extract would tend to decrease in supply in the future. That pepsin has not been used to a greater extent is probably due to: a. The ample supply of rennet extract at a reasonable cost. b. The persistence in the minds of cheese-makers that pepsin produces a bitter flavor in cheese.

Numerous investigations have been made in which the properties of pepsin and rennet extract have been compared for making Cheddar cheese (1, 6, 11, 21). As early as 1903 Van Slyke et al. (19) compared the proteolysis produced by commercial pepsin and rennet extract in raw milk cheese and found they were comparable. Lucas (8) also found that pepsin had no undesirable effect on the flavor and body of the cheese, but the texture was somewhat inferior. Sherwood (18) reported that the use of pepsin resulted in less protein degradation during ripening, than when rennet was used. Maragoudakis et al. (10) observed that the total amount of amino nitrogen was higher during ripening in the cheese made with rennet extract than with a pepsin preparation. However, the work of Vivian (20) contributed to the idea that pepsin causes the development of bitter flavor. He noted that a bitter flavor developed in Cheddar cheese during the early stages of ripening when it was made with pepsin. Furthermore, Davis et al. (5) also reported that some lots of cheese developed a bitter flavor when pepsin was used as a coagulating agent.

Therefore, this investigation was undertaken for three reasons:

1. To compare the proteolysis produced by rennet extract and the commercial pepsin preparation Metroclot on milk protein as it exists in milk and in Cheddar cheese by measuring quantities of nonprotein nitrogen (NPN) liberated.
2. To determine whether pepsin and rennet extract hydrolyze casein in the same stages by determining quantity and sequence of

Received for publication September 9, 1963.

[1] The preparation Metroclot will be referred to as pepsin, although it is known that this material is a commercial pepsin preparation.

free amino acids liberated during Cheddar cheese ripening.

3. To determine if pepsin causes the development of bitter flavor in the cheese during ripening.

## EXPERIMENTAL PROCEDURE

*Proteolysis in pasteurized milk.* Fresh, whole milk was pasteurized at 62 C for 30 min and, after cooling, 1,200 ml was added to each of two, two-liter sterilized flasks and the milk adjusted to pH 6.3 with sterile lactic acid. Rennet extract was added to one flask and to the other pepsin at the rate of 3 oz per 1,000 lb of milk, and the milk was covered with 30 ml of toluene. The flasks were shaken for 21 to 30 days at 20 C. One-hundred-milliliter samples of milk were taken from the flasks at intervals for nitrogen analysis and filtered through No. 42 filter paper. From the filtered milk 10 ml was placed in a 50-ml volumetric flask and the protein precipitated by Rowland's procedure (16). For the nitrogen determination the method of Rao and Whitney was used (14). When the effect of pH on the rate of proteolysis in pasteurized milk was determined, five 500-ml flasks were used, and the milk was adjusted to pH 6.3, 6.0, 5.7, and 5.3 with sterile lactic acid. The remainder of the treatment and analysis of the samples were the same as previously described for milk adjusted to pH 6.3.

*Nitrogen distribution in cheese.* A slice ½-in. thick was taken from the longhorn-style cheese, grated, and mixed thoroughly. The fat from 5 g of ground cheese was extracted in a Soxhlet apparatus with 150 ml of ethyl ether, and the defatted sample was transferred to a beaker and dried, followed by the addition of 150 ml of deionized water. While the sample was agitated with a magnetic stirrer, 16 ml of 0.1 N NaOH was added dropwise. After 3 hr of stirring the cheese protein had been dispersed, and the solution was transferred to a 200-ml volumetric flask, and made up to volume with deionized water. Ten milliliters of the solution was pipetted into a 50-ml volumetric flask, one drop of 1N HCl added to adjust the pH to approximately 6.5, and NPN determined by the same procedure as described for milk.

*Determination of free amino acids in cheese.* A 1.5-g sample of the grated cheese was defatted and dried. Twenty milliliters of deionized water was added, the suspension was heated in a water bath at 40–45 C for 1 hr with periodic shaking (4), followed by filtration through No. 42 filter paper into a centrifuge bottle and addition of 120 ml of 95% ethanol. After keeping the mixture in the cold for 24 hr, the sample was centrifuged and filtered. The filtrate was evaporated to a volume of 10 ml and made up to 25 ml with 95% ethanol in a volumetric flask. The solution was used for resolution of the free amino acids on one-dimensional paper chromatograms (46 by 57 cm). The chromatographic solvents used were: 1. n-butanol–acetic acid–water (250:60:250) (3), for separations of: alanine, arginine, cystine, lysine, proline, tryptophane, and tyrosine. The length of development was 17–20 hr. 2. Phenol-water (4:1) (2), for separation of: aspartic acid, glutamic acid, glycine, serine, threonine, and valine. Length of development 17–20 hr. 3. Sec-butanol-3% $NH_3$ (3:1) (15), for separation of isoleucine and leucine. Time of development was 33 hr. 4. Sec-butanol–acetic acid–water (240:30:50) (15), for separation of histidine and phenylalanine. Time of development was 28 hr. 5. Benzyl alcohol saturated with water (7) for methionine. Length of development 36 hr. After development the chromatograms were dried at room temperature for 1 hr and then in an oven at 60 C for 15 min. Chromatograms developed with phenol were dried under the hood for 3 hr and in the oven for 15 min. Traces of the solvent were removed by washing the paper with ethyl ether. When benzyl alcohol was used as a solvent the length of drying time was 12 hr under the hood and 45 min in the oven. The amino acids were located by dipping the chromatograms in a 0.2% ninhydrin in acetone solution (15), dried until the first spots appeared, and the full color developed by heating in the oven at 60 C for 3 min. Quantitative estimations of individual amino acids were made by density measurements, using a Spinco Model R Analytrol.

*Cheese manufacturing procedure.* Cheddar cheese was made in accordance with good commercial practices, and the manufacturing conditions were kept as constant as possible for each replicate. Mixed milk of the University herd was used and standardized to 3.7 to 3.9% fat, and pasteurized at 143 F for 30 min. Approximately 0.8% mixed lactic starter culture was added and the milk ripened for 1 hr. However, the lots in which pepsin was used were ripened to pH 6.3. Rennet extract and pepsin were added at the rate of 4 and 3 oz per 1,000 lb milk, respectively. The curd was cooked at 102 F and milled when the acidity was approximately 0.51%. Amount of salt added was 3 lb per 1,000 lb of milk. All the cheeses were

ripened at 50± 2 F. The finished cheese contained approximately 35% fat, 32% moisture, and 2% salt (Table 1).

TABLE 1

Cheddar Cheese Manufacturing Record

| Manufacturing steps | Rennet 1[a] | Pepsin 1 | Rennet 2 | Pepsin 2 | Rennet 3 | Pepsin 3 |
|---|---|---|---|---|---|---|
| Amount of milk (*lb*) | 806.5 | 421.0 | 819.0 | 402.0 | 851.5 | 418.0 |
| Fat content of milk (%) | 3.95 | 3.95 | 3.85 | 3.85 | 3.70 | 3.70 |
| Acidity of milk (%) | 0.19 | 0.19 | 0.18 | 0.18 | 0.19 | 0.19 |
| Amount of starter (%) | 0.80 | 0.83 | 0.97 | 0.74 | 0.82 | 0.89 |
| Acidity of starter (%) | 0.85 | 0.85 | 0.89 | 0.89 | 0.85 | 0.85 |
| Setting temperature (*F*) | 86 | 86 | 86 | 86 | 86 | 86 |
| Marshall rennet test (*spaces*) | 4 | 2.75 | 4 | 2.5 | 4 | 1.75 |
| Amount of coagulating agent (*oz/1,000 lb*) | 4 | 3 | 4 | 3 | 4 | 4 |
| Time in coagulation (*min*) | 27 | 31 | 41 | 32 | 33 | 22 |
| Acidity of whey after cutting (%) | 0.14 | 0.14 | 0.13 | 0.14 | 0.12 | 0.14 |
| Temperature of cooking (*F*) | 102 | 102 | 102 | 102 | 101 | 104 |
| Time from addition of coagulating agent to draining of whey (*min*) | 135 | 135 | 141 | 125 | 138 | 113 |
| Acidity of whey at end of draining (%) | 0.17 | 0.18 | 0.18 | 0.19 | 0.20 | 0.22 |
| Time from addition of coagulating agent to milling (*min*) | 258 | 237 | 264 | 200 | 263 | 163 |
| Acidity of whey at milling (%) | 0.51 | 0.50 | 0.52 | 0.51 | 0.52 | 0.55 |
| pH of whey at milling | 5.30 | 5.30 | 5.17 | 5.18 | 5.15 | |
| Fat content of cheese (%) | 35.9 | 36.2 | 34.5 | 35.7 | 32.7 | 32.6 |
| Moisture content of cheese (%) | 32.26 | 31.58 | 34.35 | 32.46 | 35.01 | 36.47 |
| Salt content of cheese (%) | 2.160 | 1.956 | 2.102 | 2.029 | 1.721 | 1.905 |
| pH of cheese after two days | 5.22 | 5.19 | 5.18 | 5.21 | 5.20 | 5.14 |

[a] Numbers 1, 2, and 3 designate the cheese replicates.

## RESULTS AND DISCUSSION

*Milk.* Preliminary determination of proteolysis produced by rennet and pepsin in milk was done with a series of experiments designed to measure the rate and amount of protein hydrolysis by these enzyme preparations on native protein in the absence of the stimulating effects of acidity or enzymes of bacterial origin. Bacteria in the pasteurized milk were controlled by the addition of toluene. The data (Tables 2 and 2a) show that the proteolysis produced by rennet extract in pasteurized milk was statistically significantly greater than that produced by pepsin, which is in agreement with previous findings (18,19). When the NPN produced by the action of rennet extract and pepsin was compared in pasteurized milk adjusted to pH 6.3, 6.0, 5.7, and 5.3, both agents yielded the highest amount of NPN in milk adjusted to pH 6.3. Lower values were obtained at pH 6.0, 6.5, 5.7, and 5.3. In general, the NPN produced by the action of pepsin was lower than that produced by the action of rennet extract (Figure 1). The differences were statistically significant at all pH levels except at pH 5.7. It has been reported by Lundsteen (9) that the optimum pH for the coagulating action of rennin is 5.4. On the other hand, Berridge et al. (12) compared rennet extract and pepsin acting in milk at pH

TABLE 2

Proteolysis produced by rennet extract and pepsin in pasteurized milk adjusted to pH 6.3

| Days | Nonprotein nitrogen | |
|---|---|---|
| | Rennet | Pepsin |
| | (*mg/ml*) | |
| 0 | 0.3304 | 0.3304 |
| 1 | 0.3550 | 0.3444 |
| 2 | 0.3614 | 0.3547 |
| 5 | 0.4082 | 0.3750 |
| 8 | 0.4332 | 0.4243 |
| 11 | 0.4965 | 0.4786 |
| 14 | 0.5083 | 0.4813 |
| 19 | 0.5254 | 0.5120 |
| 22 | 0.5651 | 0.5197 |
| 26 | 0.6218 | 0.5803 |
| 30 | 0.7118 | 0.6491 |

TABLE 2a

Analysis of variance of the proteolysis produced by rennet extract and pepsin in pasteurized milk adjusted to pH 6.3

| Sources of variation | df | SS | MS | F |
|---|---|---|---|---|
| Total | 21 | 0.2538 | | |
| Days | 10 | 0.2486 | 0.0248 | S[a] |
| Rennet vs. pepsin | 1 | 0.0032 | 0.0032 | S[a] |
| Error | 10 | 0.0020 | 0.0002 | |

[a] Significant at 5% level.

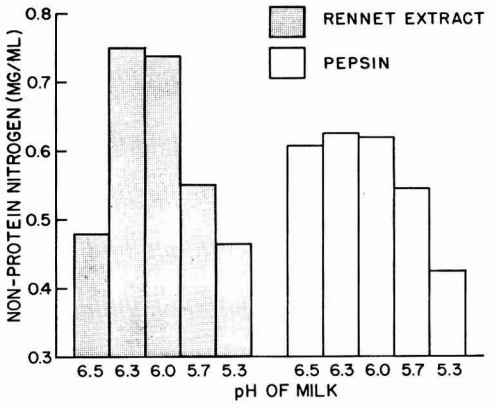


FIG. 1. Proteolysis produced by rennet extract and pepsin in pasteurized milk adjusted to four different pH levels at the end of 21 days at 20 C.

levels between 6.0 and 5.1 for a period of 2 wk, and found that both enzymes produced maximum proteolysis at pH 5.4. The data (Figure 1) are in partial agreement with the above finding, in that each proteolytic agent produced highest amount of NPN at the same pH level, but the acidity at which the highest amount of NPN occurred during this investigation was at pH 6.3–6.0. No adequate explanation can be advanced to account for the differences between the results of Berridge et al. and those obtained in these experiments. However, there is a limited amount of information about the effect toluene has on proteolysis (13, 17). Sherwood (17) reported that toluene, when used as an antiseptic, reduced total NPN liberated, but increased slightly a subpeptone fraction in milk when compared with samples without the toluene. It is not known whether toluene had any significant effect on NPN liberated in the samples of milk in the series of experiments reported here.

*Cheese.* By reference to Figure 2 it will be seen that the extent of protein breakdown occurring in the cheese made with rennet extract was higher than in the cheese made with pepsin (Replicates 1 + 2). This difference in proteolysis, statistically significant, was due to the effect of rennet extract, because the only important variable in the two lots was the coagulating enzyme preparations. The milk and starter culture, as well as the acidity at the time of milling, the pH during ripening, and the salt content of the two lots were the same. Greater proteolysis was also produced by rennet extract in pasteurized milk, as previously mentioned. Other investigators (6, 18, 19) also found that rennet extract produced greater proteolysis in Cheddar cheese than did pepsin.

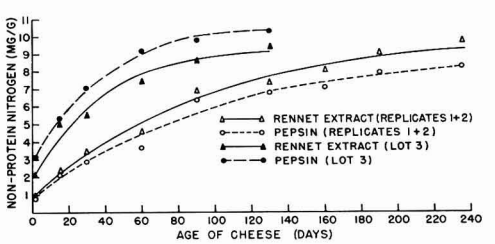


FIG. 2. Nonprotein nitrogen content of Cheddar cheese made with rennet extract and pepsin during ripening.

The amino acid pattern of Cheddar cheese made with rennet extract is well known. However, no attempt has been made to study the amino acids set free in Cheddar cheese made with pepsin during ripening. It was not known whether pepsin degraded cheese protein in a manner different than did rennet extract, or if the kind and amount of free amino acids liberated were the same. Free amino acids for both lots of cheese were determined at different intervals. A comparison of the data obtained at 2, 60, 130, and 235 days, as recorded in Table 3 and Figure 3, shows that there is almost no variation in the amino acid pattern of Cheddar cheese of approximately the same age made with rennet extract and pepsin. The concentrations of the amino acids were different in the two lots of cheese, but not any qualitative differences were observed. Isoleucine, methio-

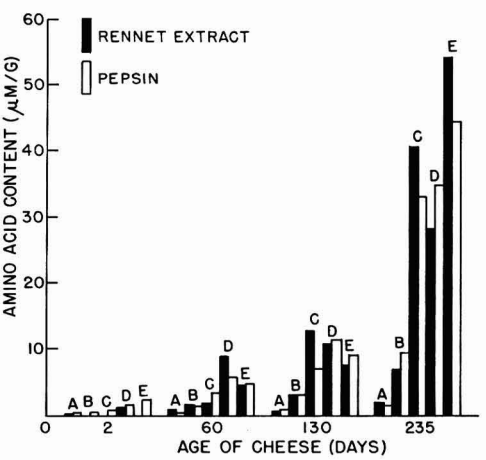


FIG. 3. Changes of the concentration of the amino acids tyrosine (A), methionine (B), valine (C), glutamic acid (D), and leucine (E) during the ripening of Cheddar cheese made with rennet extract and pepsin.

TABLE 3

Amino acids liberated in Cheddar cheese during ripening
(Replicate 1)

| | Treatment | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Rennet | | | | Pepsin | | | |
| | Age (*days*) | | | | Age (*days*) | | | |
| Amino acids | 2 | 60 | 130 | 235 | 2 | 60 | 130 | 235 |
| | ($\mu M/g$) | | | | | | | |
| Alanine | [-a] | 2.24 | 10.77 | 12.34 | — | 2.02 | 8.97 | 11.56 |
| Arginine | 0.28 | 2.46 | 3.55 | 5.79 | 0.57 | 1.95 | 4.87 | 4.76 |
| Aspartic Acid | — | 1.42 | 2.93 | 4.50 | — | 1.27 | 1.65 | 5.78 |
| Glutamic Acid | 1.22 | 8.84 | 10.61 | 28.09 | 1.70 | 5.64 | 11.42 | 34.76 |
| Glycine | — | 2.93 | 5.86 | 7.86 | — | 2.00 | 3.73 | 4.93 |
| Histidine | — | 1.28 | 1.57 | 4.58 | — | 1.62 | 2.00 | 5.91 |
| Isoleucine | — | — | 1.52 | 4.11 | — | 0.45 | 0.99 | 6.48 |
| Leucine | [+b] | 4.50 | 7.85 | 54.15 | 2.44 | 4.57 | 9.22 | 40.42 |
| Lysine | 0.61 | 2.12 | 5.33 | 8.61 | 2.87 | 2.94 | 6.36 | 9.57 |
| Methionine | — | 1.60 | 3.15 | 7.03 | 0.46 | 1.47 | 3.15 | 9.51 |
| Phenylalanine | — | 1.15 | 0.72 | 10.95 | — | 0.90 | 0.96 | 9.44 |
| Serine | — | 0.57 | 1.04 | 2.66 | — | 0.85 | 1.71 | 4.18 |
| Threonine | — | 2.68 | 3.86 | 3.94 | — | 3.77 | 3.02 | 4.61 |
| Tyrosine | 0.11 | 0.88 | 0.77 | 1.93 | 0.33 | 0.44 | 0.82 | 1.71 |
| Valine | — | 1.70 | 12.72 | 40.42 | 0.85 | 3.41 | 7.08 | 33.13 |
| | 2.22 | 34.37 | 72.25 | 196.96 | 9.22 | 33.30 | 65.95 | 186.75 |

[-a] Absent.
[+b] Present.

nine, and valine appeared later in the cheese made with rennet extract, but at 130 days the same amino acids were present in each lot of cheese. Glutamic acid, leucine, and valine were always present in the highest concentration for both lots of cheese, whereas tyrosine, aspartic acid, threonine, glycine, serine, histidine, isoleucine, and arginine showed lower concentrations.

The organoleptic evaluation of cheese did not reveal any marked differences in body, texture, and flavor between the two lots of cheese. The results (Table 4) indicate that the texture and body of the cheeses made with pepsin were criticized as curdy. These cheeses remained curdy for a longer time than did the cheeses made with rennet extract. The flavor of the cheeses made with pepsin was clean, but milder as compared to that of the cheeses made with rennet extract. Bitter flavor did not develop in any lot of cheese, regardless of the kind of coagulating agent used.

The third lot of cheese differed from the first two replicates in that the total amount of NPN produced was greater and the rate of production faster. Furthermore, the cheese made with pepsin showed greater proteolysis than did that made with rennet extract, as measured by the NPN production (Figure 2). Also, data obtained during this investigation showed that the concentration of free amino acids was higher in the cheeses of the third lot than in the first two replicates. Moreover, the cheese made with pepsin was also higher in amino acids than that made with rennet extract, but no qualitative difference in amino acids was observed. These apparently contradictory results to the first two replicates need explanation, because it is considered that manufacturing conditions were the main contributing factors to the differences. The conditions which contributed to a greater rate of proteolysis were: a) Initially slow starter, which later became very active in the milk used for the pepsin lot, contributed to an extended ripening time (172 min vs. 82 min), which resulted in greater acidity and bacterial population. This was in spite of the fact that the milk and the starter culture were the same for both lots of cheese. b) Increase in the amount of pepsin preparation used. Four ounces per 1,000 lb instead of 3 oz per 1,000 lb of milk were added. c) Reduction in the amount of salt used, so as to reduce the time of persistence of curdy body. d) Moisture content was approximately 2% greater in the third lot as compared with the first two replicates.

All these factors undoubtedly contributed to the greater proteolysis in the third lot. However, the quantity of pepsin used was perhaps

TABLE 4

Organoleptic evaluation of Cheddar cheese during ripening
(Replicates 1 + 2)

| Age *(days)* | Treatment | Texture-body | | Flavor | |
|---|---|---|---|---|---|
| | | Score | Criticism | Score | Criticism |
| 15 | Rennet | 29.0 | Slightly open, Slightly corky, curdy | 39.0 | Desirable, mild |
| 15 | Pepsin | 29.0 | Slightly open, Slightly corky, curdy | 39.0 | Desirable, mild |
| 30 | Rennet | 29.0 | Slightly open, curdy | 39.5 | Clean, mild |
| 30 | Pepsin | 29.0 | Slightly open, curdy | 39.0 | Clean, mild |
| 90 | Rennet | 29.5 | Good | 39.5 | Typical |
| 90 | Pepsin | 29.0 | Slightly curdy | 39.0 | Mild, clean |
| 130 | Rennet | 29.5 | Good | 39.5 | Typical |
| 130 | Pepsin | 29.0 | Slightly curdy | 39.0 | Mild, clean |
| 235 | Rennet | 29.5 | Good | 40.0 | Excellent |
| 235 | Pepsin | 29.0 | Slightly curdy | 39.0 | Mild, clean |

not as important as the other factors listed, because pepsin produced less proteolysis than rennet extract in milk samples, regardless of pH, when the two preparations were used in equal concentrations (Table 2).

The organoleptic evaluation data (Table 5) indicate that the texture and body of the cheeses made with pepsin preparation was slightly crumbly, whereas that of the rennet extract lot was slightly open. The crumbly defect is considered to be due to the short matting time (50 min) and not to any specific properties of the enzyme itself. The flavor was excellent and slightly acidy in the cheeses made with rennet extract and pepsin, respectively. In spite of the greater amount of pepsin used, and the higher acid content, no bitterness developed in the cheese made with pepsin. It would, therefore, appear that the pepsin preparation could satisfactorily be used as a coagulating agent for Cheddar cheese, recognizing that the rate of ripening or body breakdown would tend to be slower than when rennet extract was used, if all other manufacturing conditions were comparable.

TABLE 5

Organoleptic evaluation of Cheddar cheese during ripening
(Lot 3)

| Age *(days)* | Treatment | Texture-body | | Flavor | |
|---|---|---|---|---|---|
| | | Score | Criticism | Score | Criticism |
| 15 | Rennet | 28.5 | Slightly open, curdy | 39.0 | Desirable |
| 15 | Pepsin | 27.5 | Weak | 38.0 | Acidy |
| 30 | Rennet | 28.5 | Slightly open, curdy | 39.0 | Clean, mild |
| 30 | Pepsin | 27.5 | Slightly open | 38.5 | Acidy, not bitter |
| 60 | Rennet | 29.0 | Slightly open, curdy | 39.0 | Clean |
| 60 | Pepsin | 28.0 | Slightly open | 38.5 | Slightly acidy, not bitter |
| 90 | Rennet | 29.0 | Slightly open | 40.0 | Excellent |
| 90 | Pepsin | 28.0 | Crumbly, open | 39.0 | Slightly acidy, not bitter |
| 130 | Rennet | 29.0 | Slightly open | 40.0 | Excellent |
| 130 | Pepsin | 28.0 | Crumbly, open | 39.0 | Slightly acidy, not bitter |

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# USE OF NISIN IN PREPARING BEVERAGE-QUALITY STERILE CHOCOLATE-FLAVORED MILK

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SUMMARY

Nisin was used to aid heat sterilization of chocolate-flavored milk at levels ranging from 0 to 800 Reading Units per ml. Heat treatments given the chocolate-flavored milk ranged from an F value of 0.2 to 9.0. The relationship between the per cent thermophilic spoilage during incubation at 131 F for 3 wk and the level of nisin added before heat treatment of F = 3.0 was shown to exist. An F value required to prevent thermophilic spoilage for several concentrations of nisin was also demonstrated. Use of an F value of 3 and the addition of 80 Reading Units of nisin per ml as a heat sterilization aid resulted in a beverage-quality, commercially sterile chocolate-flavored milk.

Nisin is a polypeptide, produced by some strains of *Streptococcus lactis*, which has the unique property of inhibiting outgrowth from heat-damaged spores (2). In addition, nisin is readily inactivated by trypsin (5) and consequently cannot influence the intestinal flora. Thorough studies by Frazer et al. (3) and Hara et al. (5) showed nisin to be a safe substance for use in foods at the levels of treatment proposed. It occurs naturally in many dairy products (1). The activity of nisin is measured in Reading Units, and one Reading Unit is defined as that amount of nisin which will delay the growth of *Streptococcus agalactiae* in 1 ml of broth for about 16 hr. (Forty Reading Units are approximately equivalent to 1 ppm of nisin.)

Because of its safety and its unique property of inhibiting outgrowth from heat-damaged spores, nisin has been studied as an aid to the heat sterilization of canned foods. Gillespy (4), using a process of 80 min at 240 F, found that all control cans of beans in tomato sauce spoiled after 14 days at 130 F; whereas, none of the cans containing about 100 Reading Units of nisin per gram of can contents, and given the same heat treatment, spoiled. He also reported that 22% of control cans of peas, receiving a process of 25 min at 240 F (F = 3.0), spoiled; whereas, none of the cans containing 100 Reading Units of nisin per gram spoiled. (The F value referred to here is the equivalent of heat at the can center in terms of minutes at 250 F, assuming instantaneous heating and cooling.)

Campbell, Sniff, and O'Brien (2), using tomato juice packed in thermal death time cans, found that spoilage could be prevented with nisin at a level of 14 parts per million (560 Reading Units per gram) and a process of only 6 min at 206 F. Seventy-seven per cent of the control cans without nisin spoiled in one month at room temperature; none of the cans containing nisin spoiled during 18 months.

Wheaton (8) inoculated cream-style corn and chow mein with spores of thermophilic flat-sour organisms (about 2,700 per gram) and spores of PA3679 (about 40,000 per gram) and processed them in thermal death time cans at F = 4 and F = 3, respectively. All cans containing no nisin and inoculated with the thermophilic flat-sour organism spoiled when held 60 days at 131 F. No spoilage occurred in the products containing 1 ppm of nisin. Cans inoculated with PA 3679 were held one year at 98 F, during which all cans without nisin spoiled, whereas only 6 to 9% of those containing 2.5 ppm of nisin spoiled.

In view of the limited amount of work on the use of nisin as a heat processing aid, and because of its unique properties, it appeared desirable to study one product over a range of heat treatments and a range of levels of nisin. Chocolate milk was chosen, since it has a pH of about 6.6 and a high carbohydrate level; and, since cocoa generally contains relatively high numbers of thermophilic spore-forming organisms. In addition, the effect of heat treatment on flavor could be judged better than might be the case with some other foods.

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EXPERIMENTAL PROCEDURE

Chocolate-flavored milk was prepared containing 3.5% fat, 7.5% solids-not-fat, 7.3% sucrose, 1.2% cocoa, and 0.1% stabilizers. The dry ingredients were mixed in skimmilk, the necessary amount of cream added, and the product heated to 145 F. It was then homogenized at 2,500 lb/in.$^2$, and cooled to 60 F or colder if held more than 2 hr before filling. Immediately before processing, various portions were removed and nisin added, as Nisaplin.[2] The various lots of product were then filled into 300 × 402 cans (13 fluid ounces each), sealed, and processed to the desired F values in a continuous Food Machinery Corporation cooker. After processing, some cans from each lot were incubated at 131 F for 3 wk, some at 110 for 3 wk, and some at 85 for six months. Spoilage was evidenced by a drop in pH, by a change in appearance of the product, or by swelling of the cans.

Spore counts were made on the unprocessed chocolate-flavored milk according to the method described in the Laboratory Manual for the Canning Industry (6) for thermophilic flat-sour organisms. Since the same lot of cocoa was used for all experiments, only a few counts were made. These averaged 1.4 flat-sour spores per ml.

[2] Supplied as megaunit Nisaplin by Aplin and Barrett, Ltd., Yeovil, Somerset, England.

RESULTS

Data presented in Table 1 show that without nisin spoilage occurred at F values below 9.0. When F values as low as 0.2 were used, thermophilic spoilage did not occur when 400 Reading Units of nisin were added per ml before processing. At an F value of 3.0, all of the cans without nisin exhibit thermophilic spoilage after 3 wk of incubation at 131 F. However, when 80 Reading Units of nisin are added per ml prior to a heat treatment of F = 3.0, none of the cans spoil during 3 wk of storage at 131 F. This difference is illustrated in Figure 4. The cans on the left containing no nisin are curdled and some discolored; those on the right, with 80 Reading Units of nisin per ml, are normal.

The fiducial limits of the observed per cent spoilage, calculated by the method of Spiegel (7), are given in the last column of Table 1. It will be noted that sample sizes of 200 cans or more are reliable at the 95% levels of probability to less than 2% thermophilic spoilage. A Gram stain was made of each of the spoiled cans having a pH lower than normal. Micro-

TABLE 1

Effect of nisin on thermophilic spoilage of chocolate-flavored milk given various heat treatments

| "F" value used | R. U. of nisin [a] added | No. of cans tested | No. of cans spoiled | Per cent thermophilic spoilage | Range [b] |
|---|---|---|---|---|---|
| 0.2 | 0 | 10 | 10 | 100.0 | 39.1–100.0 |
| | 40 | 24 | 24 | 100.0 | 58.0–100.0 |
| | 400 | 28 | 0 | 0.0 | 0.0– 11.2 |
| | 800 | 28 | 0 | 0.0 | 0.0– 11.2 |
| | 1,200 | 28 | 0 | 0.0 | 0.0– 11.2 |
| 0.5 | 40 | 24 | 24 | 100.0 | 58.0–100.0 |
| 1.0 | 0 | 13 | 13 | 100.0 | 44.0–100.0 |
| | 40 | 155 | 6 | 3.9 | 1.8– 8.2 |
| | 80 | 24 | 1 | 4.2 | 0.2– 15.7 |
| | 120 | 24 | 1 | 4.2 | 0.2– 15.7 |
| | 200 | 24 | 0 | 0.0 | 0.0– 13.8 |
| 3.0 | 0 | 57 | 57 | 100.0 | 72.3–100.0 |
| | 10 | 24 | 7 | 29.1 | 14.9– 49.2 |
| | 20 | 24 | 2 | 8.3 | 2.2– 25.9 |
| | 40 | 172 | 1 | 0.6 | 0.1– 3.2 |
| | 80 | 200 | 0 | 0.0 | 0.0– 1.9 |
| 5.0 | 0 | 156 | 4 | 2.6 | 1.1– 6.4 |
| | 5 | 100 | 0 | 0.0 | 0.0– 3.7 |
| | 20 | 100 | 0 | 0.0 | 0.0– 3.7 |
| | 40 | 233 | 0 | 0.0 | 0.0– 1.6 |
| 9.0 | 0 | 48 | 0 | 0.0 | 0.0– 6.8 |

[a] Forty Reading Units are equivalent to 1 ppm nisin.
[b] Calculated for 95% level fiducial probability from observed spoilage.

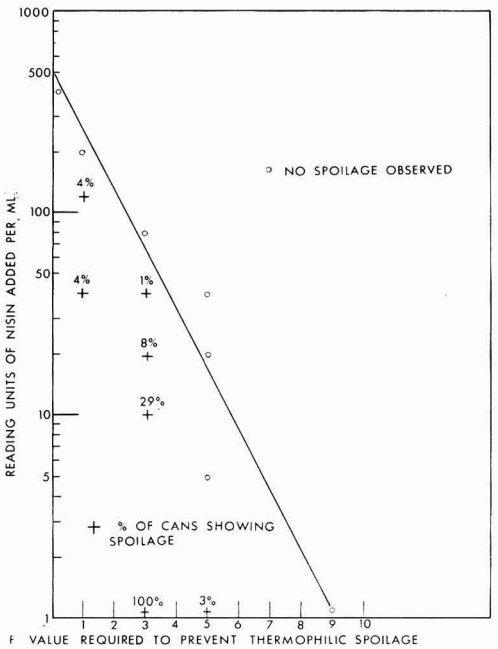


FIG. 1. Relation between level of nisin and heat treatment required to prevent thermophilic spoilage in chocolate-flavored milk.

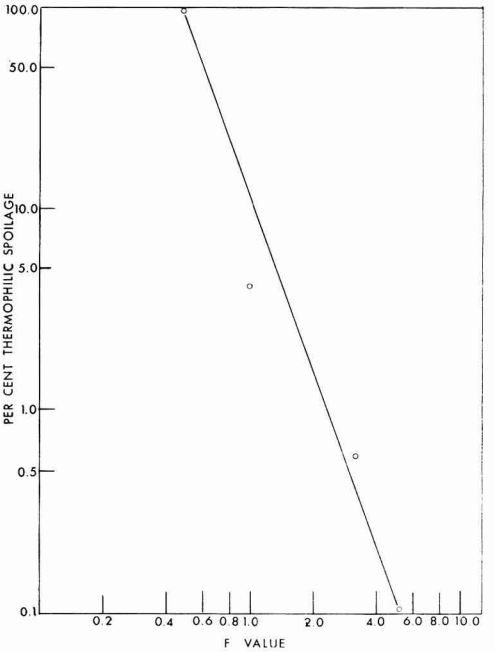


FIG. 2. Relation between per cent thermophilic spoilage and heat treatment of chocolate-flavored milk containing 40 R. U. nisin.

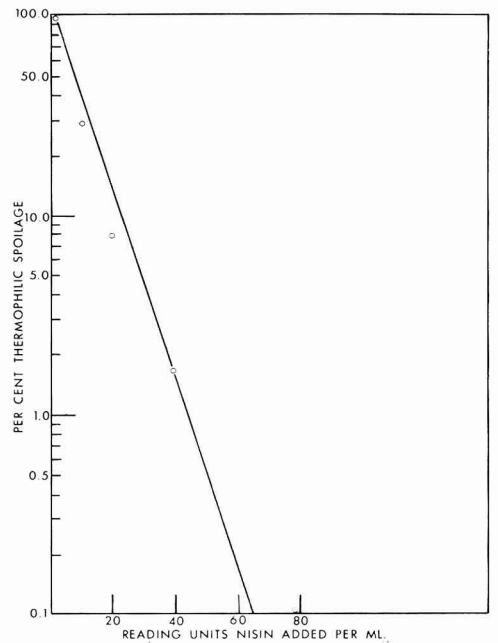


FIG. 3. Relation between per cent thermophilic spoilage of chocolate-flavored milk and level of nisin added before heat treatment of F = 3.0.

scopic observations of such slides showed gram-variable and gram-negative rods and spores typical of the flat-sour type of spoilage, with one exception. This can was examined, found to be defective, and was excluded from the data. The flora of the can contents was mixed, indicating post-sterilization contamination (which is not controlled by nisin). Samples processed at F = 0.2 containing 400 Reading Units per ml were also examined microscopically after incubation for 3 wk at 131 F, even though flavor and appearance were satisfactory and no organisms were observed.

These data are presented graphically in Figure 1, which illustrates the relationship between the heat treatment and the level of nisin required to prevent thermophilic spoilage in chocolate-flavored milk processed in 300 × 402 cans. Figure 1 indicates that the logarithm of the Reading Units of nisin added is inversely proportional to the F value required to prevent thermophilic spoilage in the range studied for chocolate-flavored milk.

There is, however, a level of heat treatment beyond which nisin is ineffective. Samples of chocolate-flavored milk were prepared containing 800 R. U. of nisin per ml. After sealing, the cans were processed in boiling water at

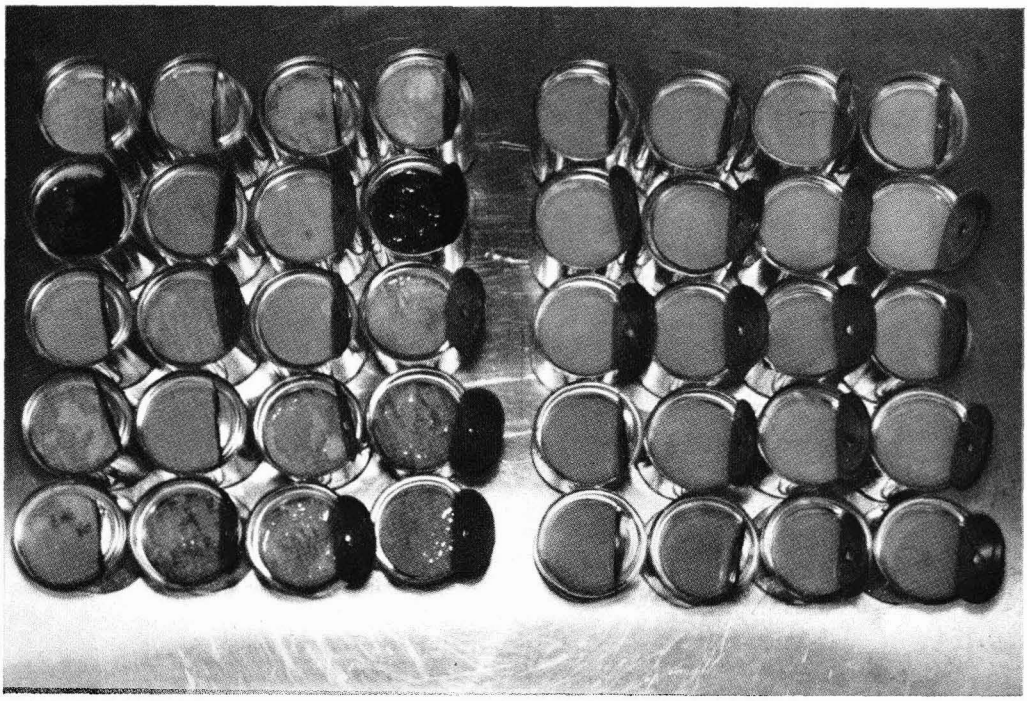

FIG. 4. Chocolate-flavored milk after storage at 131 F for 3 wk. Both lots were processed at F = 3.0. Cans at left contained no nisin; cans at right contained 80 R. U. per ml.

209 F for 30 min. None of the cans containing nisin spoiled during storage at 131 F for 3 wk; however, 44% spoiled during storage at 98 F for this same period of time. All of the control cans without nisin spoiled at both storage temperatures. The nisin thus controlled thermophilic spoilage under these conditions, but did not control mesophilic spoilage.

When the level of nisin was held constant at 40 R. U. per ml and the F value varied, it was observed that the logarithm of the per cent spoilage appeared to be inversely related to the logarithm of the F value between 0.5 and 5.0, as shown in Figure 2.

Figure 3 shows the relation between the per cent thermophilic spoilage and the level of nisin required to prevent spoilage with a constant heat treatment of F = 3.0. The logarithm of the per cent spoilage appears to be inversely related to the units of nisin added in the range of 0 to 80 R. U. per ml.

To determine the effect of spore concentration on the efficacy of nisin, spores of *B. stearothermophilus* were added to chocolate-flavored milk at levels of 840, 8,400, 84,000, and 840,000 spores per can. No spoilage occurred in the 26 cans at the lowest level of inoculation or in the 25 cans containing 8,400 added spores per can. However, three spoiled out of 26 at the 84,000 level and two spoiled out of 25 at the 480,000 level. Since the untreated product contained 530 spores per can, it appears that spore concentration must be increased by between 10- and 100-fold before spoilage results are influenced.

A flavor comparison was made between the chocolate-flavored milk processed to F = 9.0 and that processed to F = 3.0. Of the 12 panel members, ten chose the product given the lower process. Another panel was then conducted to compare unprocessed chocolate-flavored milk with that processed to F = 3.0 and containing 80 R. U. of added nisin. In this case, the panel was evenly divided, six preferring the unprocessed product and six the processed product. Using freshly processed product, this panel was repeated on three separate occasions with identical results. From a flavor standpoint, therefore, it would appear that a chocolate-flavored milk of beverage quality may be produced by using nisin as an aid to heat processing at F = 3.0.

When samples containing 80 R. U. of nisin were stored at 110 F for 3 wk, there was no

spoilage, whereas those without nisin spoiled during this period. No spoilage was observed after six months of storage at 85 F in samples containing 80 R. U. of nisin processed at F = 3.0, whereas 10% of the cans without nisin spoiled during this storage period. The flavor of the nisin-containing product was quite acceptable after this storage period.

While it was shown that nisin at high levels prevented thermophilic spoilage at F values below 2.5, such processes are not to be recommended, since these are below the generally accepted botulinum cook for low-acid foods. It should also be pointed out that nisin has not yet been approved as a heat-sterilization aid by the U.S. FDA.

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# COMPOSITION OF THE CASEINS OF BUFFALO AND COW MILK

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SUMMARY

The composition of buffalo milk casein and cow milk casein was almost identical in the numbers and in quantities of amino acids as estimated by paper chromatography.

Pepsin hydrolysis was slower for buffalo milk casein than it was for cow milk casein, regardless of period of incubation, substrate concentration, and enzyme concentration. This was also true for trypsin, papain, and pancreatic digestion of the two types of caseins.

A peptide isolated from peptic digestion of both caseins contained aspartic acid, glutamic acid, lysine, serine, glycine, arginine, threonine, tyrosine, valine, leucines, and phenylalanine.

The chemical and biochemical nature of casein has attracted much attention. The comprehensive review of Pyne (24) indicates that most of these studies were confined to casein from cow milk.

Substantial literature has also accumulated on the nature of action of proteolytic enzymes on casein. Mellander (21) observed a difference in the peptic digestion behavior between caseins from human and cow milk. The pattern of proteolysis resulting from different enzymes on casein was also discussed by Christensen (8). Bahadur and his coworkers (3, 4), in a series of reports, demonstrated the effect of pH, temperature, buffer, etc., on the action of papain on casein. Enzymatic hydrolysis of casein isolated from the milk of different species of animals was reported by Bennich, Johansson, and Mellander (7). Digestion experiments on milk proteins by proteolytic enzymes were also described by Kinoshita (18). Recently, Graae and Rasmussen (15) have provided further information on the action of trypsin on casein. These studies were all with cow milk casein.

Buffalo milk is an important source of milk proteins in India, Egypt, and other countries. This has resulted in initiating research on the chemical and nutritional differences which may exist between buffalo milk and cow milk. Despite this, not much information is available (9) and this field remains available for critical studies.

To study the nutritional properties of casein of buffalo milk, a comparative study on buffalo milk and cow milk caseins was undertaken. The purpose of this investigation was twofold: First, to examine the amino acid make-up, and secondly, to study the hydrolytic pattern of the caseins with different enzymes.

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## MATERIALS AND METHODS

*Preparation of casein.* Casein was prepared from skimmilk (cow or buffalo) after diluting with equal volume of water by the isoelectric precipitation at pH 4.5, using 10% HCl. The precipitate was allowed to settle and the supernatant fluid decanted off. Sufficient 95% ethanol (approximately twice the volume of casein) was then added to it and after mixing thoroughly in a Waring Blendor, the suspension was allowed to stand for 2 hr; the supernatant was then decanted. The casein was washed thrice more with alcohol and finally washed on a Büchner funnel with petroleum ether (40-60°) and air-dried by spreading over a filter paper. The dried casein was powdered and then defatted by Soxhlet extraction with petroleum ether for a period of 8 hr. The defatted casein was allowed to dry again, after which the final particles were sieved through a 100-mesh sieve to get a uniform casein sample. Samples prepared in this way were found to keep well for several months at room temperature.

*Other chemicals.* All amino acids used as standard and ninhydrin for identification and quantitative evaluation of casein amino acids were purchased from the British Drug House, England. Pepsin and papain were products from Constantino & Company, Italy. Trypsin used was supplied by E. Merck, Germany. All other reagents used were obtained from the commercial sources and were of analytical grade.

*Acid hydrolysis of caseins.* Hydrolysis of casein was done by refluxing 200 mg of casein with 20 ml of 6 N HCl for a period of 24 hr (12). After hydrolysis, the casein hydrolysate was made free of excess of HCl by repeated evaporation of the hydrolysate under vacuum over NaOH pellets. Finally, the acid-free dried material was dissolved in 5 ml of 10% isopropyl alcohol, adding a few drops of toluene

in each tube to prevent bacterial contamination. These hydrolysates were then used for analyzing the respective amino acids (except tryptophan). Completion of the hydrolysis of samples was ascertained by occasionally estimating the amino nitrogen by the Van Slyke method (17). The hydrolysates were kept in a refrigerator at 4° when not in use.

*Quantitative estimation of amino acids by paper chromatography.* Separation and identification of individual amino acids in casein hydrolysate were carried out by two-dimensional paper chromatography, using phenol-water and *n*-butanol:acetic acid:water (4:1:1) as solvents (12). Quantitative determinations of individual amino acids from the two-dimensional paper chromatogram were made by the method described by Giri, Radhakrishnan, and Baidynathan (13) and by Bagchi, Ganguli, and Roy (2). The circular paper chromatographic technique of Ganguli (11) was found most suitable for comparative study between individual amino acids present in the hydrolysates of the two types of caseins studied. For this, hydrolysate of each of the caseins was spotted on the two halves of the circumference of a circle drawn at the center of the circular filter paper. Tryptophan, which gets destroyed by acid hydrolysis, was estimated from separate samples of casein and calculated from the ultraviolet absorption spectra readings (5).

*Preparation of casein solution for enzymatic studies.* The substrate solution for the action of different proteolytic enzymes was prepared by dissolving casein in the respective buffers used for the individual enzymes, by heating it on a boiling water bath for 2 min with occasional shaking and finally making up the concentration of casein to 2% with the buffer. Usually, casein dissolved in about 2 min of heating. No significant change in the pH of the casein solution was observed after dissolving the casein in the buffer. Cow milk casein (CMC) was found to be dissolved somewhat faster than buffalo milk casein (BMC). Prolonged heating for dissolving the caseins in buffers was always minimized to prevent dephosphorylation due to heat (6). This was checked by estimating the inorganic phosphorus liberated during the preparation of the casein solutions.

*Enzymatic hydrolysis of casein.* The rate of proteolysis of caseins with pepsin, trypsin, and papain was determined by the method of Kunitz (19). Both spectrophotometric assay by reading the optical density at 280 mμ (20) and colorimetrically at 660 mμ (16), using Folin's phenol reagent (10), were used for the measurement of the activity of pepsin; whereas, in other enzymes, a Beckman spectrophotometer, Model DU, and cell having 1-cm light path, was used for the spectrophotometric method. The following assay systems were used:

(a) *Assay system for peptic digestion.* The procedure followed for peptic hydrolysis of casein was according to Anson (1), as described by Herriot (16). The complete system contained 5 ml of a 2% solution of casein in 0.06 N HCl and 1 ml of pepsin containing 2.93 mg. The mixture was incubated at 25° for a definite period, after which the reaction was stopped by adding 10 ml of 5% trichloroacetic acid, and the precipitated protein removed by filtration. A blank was run by stopping a similar mixture at zero-minute period. Readings were corrected for such blank values.

(b) *Assay system for trypsin digestion.* An almost similar procedure was adopted in which the reaction mixture contained casein (2%) dissolved in phosphate buffer adjusted to pH 7.3 and trypsin (2.93 mg). Incubation temperature was between 39-40°, after which reaction was terminated as in (a) above. All values were corrected for blank.

(c) *Assay system for papain digestion.* The incubation mixture contained 5 ml of casein (2%) in phosphate buffer, pH 7.4, and papain (2.93 mg). Incubation was done at 25°. The rest of the procedure was as in (a) above.

(d) *Pancreatic digestion of casein.* Rat pancreas was used as the source of proteolytic enzymes, to study the rate of casein hydrolysis. The animal was killed by a blow on the head and the pancreas immediately removed in a beaker dipped in ice. A homogenate of the tissue (1 g) was then prepared in 12 ml of phosphate buffer at pH 7.6, using an ice-cold Potter-Elvehjem all-glass homogenizer. The homogenate preparation was always made fresh for each set of experiments. The assay system consisted of 2.0 ml of casein (2% in phosphate buffer, pH 7.6), buffer, 50 μmoles; pancreatic homogenate, 1 ml; incubation at room temperature (35°) for definite periods. Reaction was terminated at regular intervals by adding 10 ml of 5% trichloroacetic acid to the incubation mixture; 0.5 ml of filtrate was then diluted to 3 ml and the optical density of this diluted solution was read at 280 mμ for determining the rate of proteolysis (19).

*Isolation and characterization of peptide from peptic digestion of casein.* For the isolation of the peptide which appears during the action of pepsin on casein, incubation was carried out as in (a) above and reaction allowed to continue for 9 hr at 25°, after which

the undigested protein was precipitated with 10 ml of 95% ethanol and the supernatant dried in a vacuum desiccator over $H_2SO_4$. The dried material was dissolved in a definite volume of water and then subjected to unidimensional paper chromatographic analysis, using phenol-water as solvent. A guide strip was cut out and sprayed with ninhydrin solution, to detect the peptide. For analyzing the peptide for its amino acid composition, the peptide area was eluted with water from the corresponding unsprayed position having a similar $R_f$ value on the paper chromatogram. The eluted solution was hydrolyzed by a micro technique (23) in a sealed tube with 6 N HCl in an oven at 100° overnight. The hydrolysate was evaporated and subjected to two-dimensional paper chromatography in a way similar to that described for casein hydrolysate, for the identification of the amino acids in the peptide. A control experiment was also run with the enzyme alone.

## RESULTS AND DISCUSSION

Results of the experiments on (a) evaluation of amino acid make-up of casein after acid hydrolysis and (b) enzymatic hydrolytic pattern of casein are presented below:

(a) *Acid hydrolysis of cow milk casein and buffalo milk casein.* Results of the quantitative determination of amino acids in BMC and CMC, using the method of Ghosh Majumder, Dutta, and Ganguli (12), are shown in Table 1. It may be seen from the table that the number of amino acids present in BMC is almost identical to that of CMC. Even quantitatively, the percentage proportion of individual amino acids is very close to each other, with the exception of arginine. Available data on composition of amino acids of CMC (14) indicate they are comparable with those of ours and, therefore, on this basis it may be concluded that both types of caseins do not differ much in their amino acid composition, either qualitatively or quantitatively. However, a lower value of arginine in BMC was observed compared to that of CMC.

(b) *Enzymatic hydrolysis of caseins.* As pepsin is the enzyme responsible for digestion in the stomach, it was thought of interest to carry out some kinetic studies on this enzyme reaction.

(1) *Effect of period of incubation.* The first study made was on the rate of hydrolysis of casein samples with a similar concentration of pepsin for different periods. Reactions were stopped at desired intervals and results of such study were expressed either as increase in optical density at 280 mμ or colorimetrically at 660 mμ, as indicated above. Table 2 represents values for such experiments, with samples incubated for a period of 30 min, whereas Figure 1 shows the rates of hydrolysis with increase in period of incubation with the two types of casein samples. Results in the table indicate that at a period of 30 min of incubation with pepsin, BMC is not hydrolyzed to the same extent as that of CMC. This lower value for BMC was consistently observed in all trials, whether the rate was measured spectrophotometrically or colorimetrically. Referring to Figure 1, it may be observed that the difference in rates become more prominent at the later stage of incubation period than at the initial stage of the reaction.

(2) *Effect of substrate concentration.* Rates of hydrolysis of caseins were next studied with increasing concentrations of substrate, keeping incubation period to 30 min and with the same enzyme concentration in every incubation mixture. Figure 2 shows that at higher substrate concentrations (20 mg and upwards) pepsin acts on CMC at a faster rate compared to BMC, whereas at lower concentration (10 mg) the rates in the two cases are almost similar.

(3) *Enzyme concentration.* Typical rate curves presented in Figure 3 show that with any of the enzyme concentrations used, the

TABLE 1

Amino acid composition of buffalo and cow milk caseins as determined by paper chromatography

| Amino acid | Casein source: Buffalo milk | Cow milk (a) | Cow milk (b) |
|---|---|---|---|
| | *(g/100 g)* | | |
| Aspartic acid | 27.28 | 28.63 | 7.1 |
| Glutamic acid | | | 22.4 |
| Alanine | 2.37 | 2.98 | 3.0 |
| Arginine | 2.78 | 4.71 | 4.1 |
| Cysteine | ..... | ..... | 0.3 |
| Glycine | 8.85 | 8.90 | 2.7 |
| Serine | | | 6.3 |
| Histidine | 1.62 | 1.38 | 3.1 |
| Lysine | 7.56 | 8.47 | 8.2 |
| Methionine | 2.01 | 2.48 | 2.8 |
| Proline [a] | +++ | +++ | 11.3 |
| Threonine | 3.74 | 4.31 | 4.9 |
| Tyrosine | 4.21 | 4.80 | 6.3 |
| Leucines | 13.26 | 13.55 | 15.3 |
| Phenylalanine | 4.46 | 4.99 | 5.0 |
| Tryptophan [b] | 1.45 | 1.30 | 1.7 |
| Valine | 5.58 | 6.58 | 7.2 |

[a] Compared visually; [b] Estimated by ultraviolet spectroscopy (5); (a) authors' values, (b) reported values (14).

TABLE 2

Rates of hydrolysis of buffalo and cow milk caseins by pepsin

| Nature of casein | Optical density at 280 mμ | | | Tyrosine formed *(mg)* | | |
|---|---|---|---|---|---|---|
| | 0 min | 30 min | Difference | 0 min | 30 min | Difference |
| Buffalo | 0.14 | 0.63 | 0.49 | 0.160 | 0.576 | 0.416 |
| Cow | 0.16 | 0.75 | 0.59 | 0.080 | 0.656 | 0.576 |

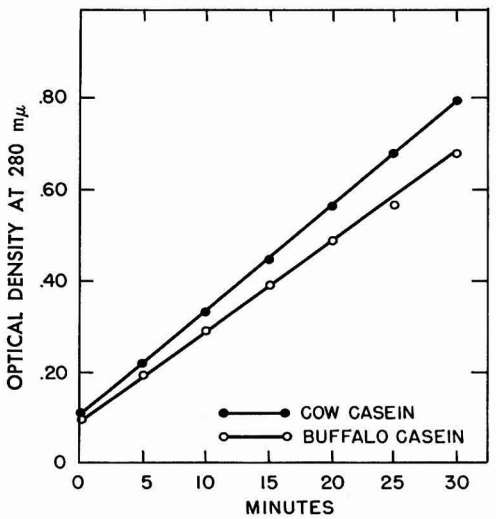


FIG. 1. Hydrolytic rates of caseins from buffalo and cow milk by pepsin with period of incubation.

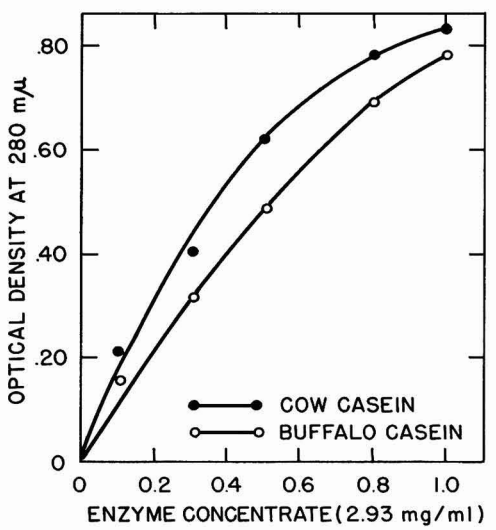


FIG. 2. Effect of substrate concentration on the rates of hydrolysis of buffalo and cow caseins by pepsin. Blanks for all individual substrate concentrations were prepared for corrections in final readings.

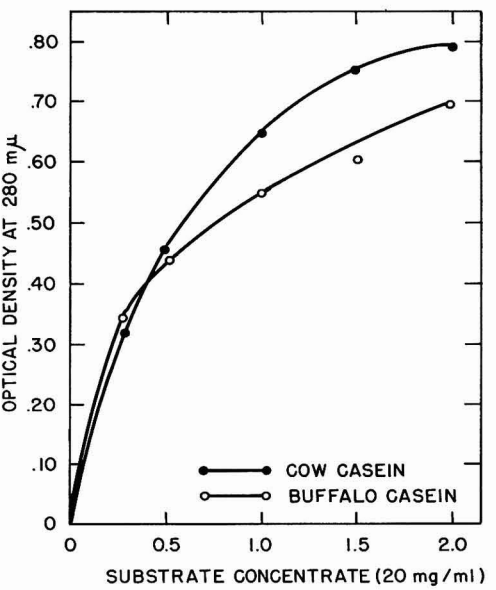


FIG. 3. Effect of pepsin concentration on the rates of hydrolysis of caseins from buffalo and cow milks. Separate blanks were used for different enzyme concentrations.

BMC was always hydrolyzed at a slower rate than CMC.

All of the casein samples were found to give always some reading originally present in the solution when read at 280 mμ and this was also observed by other workers (8) and which again was found to be higher in the case of BMC than CMC. An explanation suggested by Christensen (8) was that some protease, apparently being associated with casein, starts acting on it as soon as casein is in solution. Our results, which corroborate this observation, indicates that possibly a similar situation is responsible for such initial contribution of optical density at 280 mμ.

(4) *Nature of peptide appearance during pepsin action on casein.* With the above results at hand, it was thought reasonable to investigate the nature of the peptides being released during the action of pepsin on the two caseins. After a period of 9 hr of action of pepsin, a

peptide was isolated from the incubation mixture by the paper chromatographic technique. This peptide had an $R_f$ value of 0.85 in phenol-water and appeared in both BMC and CMC. There was no peptide detected in control experiments with the casein or enzyme alone, indicating it was formed only by the action of pepsin on caseins. Acid hydrolysis of the peptide revealed that it consisted of 12 amino acids and again this was identical with both types of caseins. The following are the amino acids present in the peptide: aspartic acid, glutamic acid, lysine, serine, glycine, tyrosine, arginine, alanine, threonine, valine, leucines, and phenylalanine. It shows, therefore, that although there is an apparent difference in rates of hydrolysis by pepsin, the release of peptide during the action of this enzyme is, however, identical.

The amino acid composition of the peptide isolated from peptic digest of casein is found to have a similar composition to that of a phosphopeptide isolated by controlled HCl hydrolysis on rabbit milk casein, reported by Sundararajan, Sampath Kumar, and Sarma (25), and also to a phosphopeptone obtained by enzymatic degradation of casein by Mellander and De Verdier (22). As these peptides have similarity in their amino acid make-up, the peptide reported here looks to be, therefore, a phosphopeptide. Based on this analogy and the results, it is tempting to infer that the release of phosphopeptide by pepsin on BMC and CMC are also identical. However, this point needs further confirmation by determination of phosphorus content of the peptide in addition to amino acids of the peptide.

(c) *Trypsin and papain hydrolysis of caseins.* Rates of proteolysis of the two types of caseins with trypsin are shown in Table 3. Results show that trypsin also behaved similarly to the reaction with pepsin.

The action of papain as shown in Table 4 also indicates an identical lower value for BMC proteolysis. It seems, therefore, from the results with all three proteolytic enzymes studied that they affect a slower rate of hydrolysis with BMC as compared to CMC, under the experimental conditions described.

TABLE 3

Rates of hydrolysis of buffalo and cow milk caseins by trypsin

| Nature of casein | Optical density at 280 mμ | | |
|---|---|---|---|
| | 0 min | 30 min | Difference |
| Buffalo | 0.025 | 0.130 | 0.105 |
| Cow | 0.005 | 0.153 | 0.148 |

TABLE 4

Hydrolysis of buffalo and cow milk caseins by papain

| Nature of casein | Optical density at 280 mμ | | |
|---|---|---|---|
| | 0 min | 30 min | Difference |
| Buffalo | 0.073 | 0.278 | 0.205 |
| Cow | 0.100 | 0.350 | 0.250 |

(d) *Pancreatic digestion of caseins.* To assess the fate of these caseins under the multiple action of different proteolytic enzymes simultaneously, experiments were designed to carry out the casein digestion with pancreatic homogenate, which is supposed to have a combination of proteolytic enzymes and was considered to stimulate the physiological environment. Figure 4, showing these results, indicates that rat pancreatic homogenate hydrolyzed BMC at a slower rate than CMC. These results lead to the general indication that the proteolytic enzymes either individually or in combination show distinctly different behavior towards these two types of caseins.

The probable superiority of CMC over BMC seems, therefore, to exist in the rate of its enzymatic digestion. It may be pointed out that a similar observation as to human milk casein in relation to pepsin or gastric juice action was pointed out by Mellander (21). In that study, the author attributed the less com-

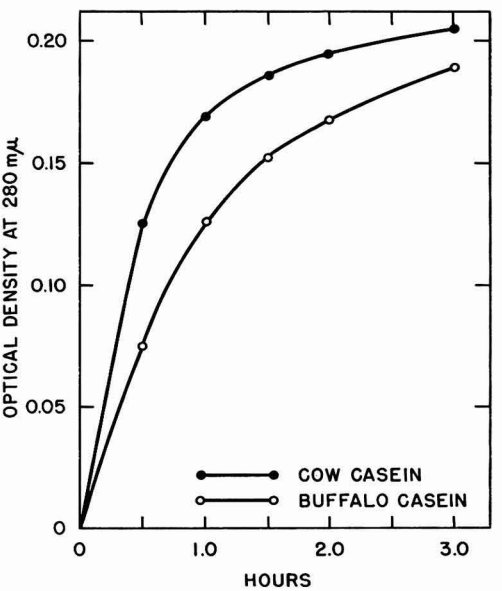


FIG. 4. Rates of hydrolysis of caseins by pancreatic homogenate.

plete peptic and tryptic digestion of human milk casein to the generation of enzyme-resistant phosphorylated peptides. In another study, Bennich et al. (7) have shown that the rates of enzymatic hydrolysis of caseins from different species do differ. Human milk casein proved to be more resistant to in vitro digestion with bovine trypsin or chymotrypsin or human pancreatic juice than caseins from the milks of cow, sheep, mare, reindeer, or whale, indicating that caseins differ in their hydrolysis by the proteolytic enzymes. Our results with individual enzymes and with pancreatic homogenate indicate clearly that BMC is yet another example which is not hydrolyzed just similarly to CMC.

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# CALCEIN AS AN INDICATOR FOR THE DETERMINATION OF TOTAL CALCIUM AND MAGNESIUM AND CALCIUM ALONE IN THE SAME ALIQUOT OF MILK

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SUMMARY

A method has been developed for calcium and magnesium determination in milk on the basis of the different behavior of these two cations towards the indicator calcein. The determination of both cations can be performed by back titration with calcium chloride solution in the same aliquot of a diluted milk containing an excess of Ethylenediamine tetraacetate (EDTA). Total calcium and magnesium can be obtained in milk at pH range 12.0 to 12.5, whereas calcium alone can be obtained from pH 13.0 to 13.5. Comparison of the values obtained for total calcium and magnesium by the calcein method with those obtained by the Eriochromeblack T method indicates no significant difference. The recoveries of calcium added to milk are complete. Concentrations of phosphates and proteins four times those present in the average milk and of heavy metals as present in average milk do not influence the results. Variations in the added amount of indicator between 0.05 and 0.15 ml and in the dilution of the sample with water in the ratio from 1:10 to 1:30 do not influence significantly the results. The method is rapid and reproducible, with a standard deviation of ±0.14 meq/liter of milk for total calcium and magnesium, or ±0.19% of the measured value, and ±0.12 meq/liter of milk for calcium alone, or ±0.18% of the calcium content.

Since the complete and effective study of the complex phenomena which occur in milk, especially under the variety of the conditions during processing, presupposes the knowledge of the amount of each of the divalent cations, and in some cases even the form in which they exist at a given moment, an analytical procedure that is rapid and accurate is needed. On the other hand, in many research problems the available material for study is very small, and one cannot afford to waste an appreciable amount for the separate analysis of both calcium and magnesium.

While flame photometry has been employed for some time for calcium determination in milk (9, 12), complexometric procedures based on Schwarzenbach and coworkers' method (18) have been developed, after the removal of the interfering materials, for the separate determination of both calcium and magnesium in milk (1, 4, 7) or of calcium only (13). A combination of flame photometry and complexometry for rapid determination of calcium and magnesium has been developed by Murthy and Whitney (14) and Have and Mulder (5).

The discovery of new, more specific indicators, especially calcein (3, 10), stimulated new procedures for calcium and magnesium determination in biological systems. The problems of the interfering constituents, especially the phosphates and the proteins, received more attention. No interference of phosphates or proteins was observed during the determination of calcium and magnesium in blood plasma or milk (8) or in the determination of calcium alone in casein solution (16). In previous work (15) it has been shown that the presence of phosphates and proteins does not interfere during the direct titration of the total calcium and magnesium with disodium dihydrogen ethylenediamine tetraacetate (EDTA) solution with Eriochromeblack T as indicator.

The interference of trace heavy metal ions has been eliminated by the addition of cyanide or triethanolamine (3, 10) or by using a technique developed for the removal of them (2).

This paper describes a method, based upon the difference in the complexing behavior of the calcium and magnesium, for the separate determination of the ions in the same aliquot of a diluted milk sample by the back titration of excess EDTA with standard $CaCl_2$ solution at two different pH values, pH 12.0 and 13.0, with calcein as indicator.

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## METHOD

### *Reagents*

Standard EDTA solution. Dissolve 10 g of disodium dihydrogen ethylenediamine tetraacetate dihydrate (TitraVer, Hach Chem-

ical Company, Ames, Iowa) and 2 g of sodium hydroxide pellets in water, and dilute to one liter with water. This solution has a titer of approximately 0.05 meq/ml and is standardized against the standard calcium solution.

Standard calcium chloride solution. Dry $CaCO_3$ at 100 C to constant weight and dissolve about 2.5 g (exactly weighed) in a minimum of hydrochloric acid. Transfer to a liter volumetric flask and dilute to volume with water. This solution contains approximately 0.05 meq Ca/ml.

$$\left(\frac{\text{g } CaCO_3}{100.09} \times 2 = \text{meq Ca/ml}\right)$$

Potassium hydroxide 8 N.

Calcein indicator solution. Dissolve 0.2 g of bis-N,N-di(carboxymethyl) amino methylfluorescein (G. Frederick Smith Chemical Company, No. 222) in 25 ml of 0.1 N sodium hydroxide and dilute to 100 ml with water. The solution should be stored in the dark (2).

Deionized water was used in the preparation of all reagents and throughout the study.

*Procedure*

Transfer 25 ml of milk into a 500-ml volumetric flask and dilute to volume with water. Add 5 ml EDTA to 50-ml aliquots of the diluted milk in 125-ml Erlenmeyer flasks.

Calcium and magnesium: Add 8 N potassium hydroxide to increase the pH to 12 (0.15 ml 8 N KOH is usually sufficient), and three drops of indicator. The color of the sample becomes pink. Back titrate with standard calcium chloride solution (from a microburet, 5-ml capacity, calibrated in 0.01 divisions) to a definite and permanent green color (first end point).

Calcium: To the same aliquot add an additional amount of 8 N potassium hydroxide to increase the pH to 13 (1.5 ml is usually sufficient). The color of the sample changes again to pink. Continue the back titration with calcium chloride solution until the color again becomes a definite and permanent green (second end point).

To determine the end point accurately the Erlenmeyer flask containing the sample should always rest on a black background. The titration is continued until, with further addition of calcium solution, no change in color takes place. During the titration for calcium only, the green color appears momentarily and fades again. It is helpful to develop completely the color of the end point in one of the aliquots by over-titration. Then, with this color as a guide, the titration of the other aliquots is continued until the colors match.

*Calculations:*

$$\text{meq (Ca + Mg)/liter} = \frac{\text{ml EDTA sol. employed}^a \text{ (first end point)} \times \text{normality of EDTA sol.} \times 1{,}000}{\text{ml of milk in the sample titrated}}$$

$$\text{meq Ca/liter} = \frac{\text{ml EDTA sol. employed}^a \text{ (second end point)} \times \text{normality of EDTA sol.} \times 1{,}000}{\text{ml of milk in the sample titrated}}$$

$$\text{meq Mg/liter} = \text{meq (Ca + Mg)/liter} - \text{meq Ca/liter}$$

## RESULTS

*Back titration of the calcium and magnesium at pH 12 and 13.* During the course of establishing the influence of the pH on the use of the calcein in the determination of the calcium in milk, two distinct titration values were obtained. Consideration of these values suggested that the one obtained at the pH 12.5 and below represented both calcium and magnesium and the other obtained at pH 13.0 and above represented only calcium. This observation, along with the observation of Korbl and Vydra (10) that at alkali concentrations below 0.1 M the presence of magnesium caused the fluorescence of the indicator to reappear, suggested the possibility of quantitative determination of both total calcium and magnesium and calcium alone in a single aliquot by employing different pH values.

To test this possibility, a calcium chloride solution (0.0518 meq/ml) and a magnesium chloride solution (0.03991 meq/ml) were prepared, and equal volumes of these two solutions were mixed to yield a solution containing both cations. Aliquots of 3 ml of each of the three solutions were transferred to 125-ml Erlenmeyer flasks, 5 ml EDTA solution (1 ml $\simeq$ 1.041 ml calcium solution) was added to each, and the mixtures were diluted to 50 ml with water. The contents of half of the flasks were adjusted with KOH to pH 12 and of the other half to pH 13. Three drops of calcein indicator were added to each and the mixtures were back-titrated with calcium chloride solution. The results (Table 1) indicate that at pH 12 both calcium and magnesium are measured completely, whereas at pH 13 only calcium is determined.

[a] ml EDTA sol. employed = ml EDTA sol. added $- \frac{\text{ml Ca sol. employed}}{\text{ml Ca sol.} \simeq 1 \text{ ml EDTA sol.}}$

TABLE 1

Back-titration of calcium and magnesium solutions at pH 12 and 13 with calcein as indicator (0.2698 meq EDTA added to each)

| pH | Solutions: Ca content | Solutions: Mg content | Ca required for back titration [a] | Ca or Mg observed | |
|---|---|---|---|---|---|
| | *(meq)* | | | | *(%)* |
| 12 | 0.1555 | 0.0000 | 0.1142 | 0.1556 | 100.07 |
| | 0.0000 | 0.1197 | 0.1504 | 0.1194 | 99.72 |
| | 0.0778 | 0.0599 | 0.1325 | 0.1374 | 99.82 |
| 13 | 0.1555 | 0.0000 | 0.1144 | 0.1554 | 99.93 |
| | 0.0000 | 0.1197 | 0.2698 | 0.0000 | 0.00 |
| | 0.0778 | 0.0599 | 0.1917 | 0.0781 | 56.74 |

Analysis of variance of Ca or Mg observed *(%)*

| Source of variance | Degrees of freedom | Mean square | Significance [b] |
|---|---|---|---|
| Solutions [c] | (5) | 6,742.913 | V.S. |
| $Ca_{12}$ vs. $Ca_{13}$ | 1 | 0.036 | N.S. |
| ($Ca_{12}$, $Ca_{13}$, and $Mg_{12}$) vs. $(Ca + Mg)_{12}$ | 1 | 0.215 | N.S. |
| $Mg_{12}$ vs. $(Ca + Mg)_{12}$ | 1 | 0.016 | N.S. |
| $Mg_{13}$ vs. $(Ca + Mg)_{13}$ | 1 | 6,439.479 | V.S. |
| [$Ca_{12}$, $Ca_{13}$, $Mg_{12}$, and $(Ca + Mg)_{12}$] vs. [$Mg_{13}$ and $(Ca + Mg)_{13}$] | 1 | 27,274.820 | V.S. |
| Error | 18 | 0.092 | |

[a] Average of four replicates.
[b] V.S. = very significant; N.S. = not significant.
[c] Subscripts in the comparisons refer to pH.

*Comparison between the Calcein and the Eriochromeblack T methods.* Since the direct complexometric determination of the total calcium and magnesium in milk (Eriochromeblack T method) has been proved to be satisfactory (15), a comparison of the values obtained by this method with those of the calcein method should establish whether the proposed method measures all the calcium and the magnesium in milk. Therefore, several samples of milk were analyzed by both procedures. The results (Table 2) indicate no significant difference between the two methods.

*Recovery of added calcium in milk.* The recovery of known amounts of calcium added to milk is an additional way of testing whether the proposed method measures all of the existing calcium in milk. Therefore, varying amounts

TABLE 2

Comparison of the Calcein method with the Eriochromeblack T method for the determination of total calcium and magnesium in milk

| Milk sample no. | Calcium and magnesium content (average of 4 replicates): 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| | *(meq/liter)* | | | |
| Calcein method | 75.61 | 75.05 | 75.40 | 72.97 |
| Eriochromeblack T method | 75.64 | 75.11 | 75.37 | 73.12 |

Analysis of variance

| Source of variance | Degrees of freedom | Mean square | Significance [a] |
|---|---|---|---|
| Method (M) | 1 | 0.025 | N.S. |
| Milk (m) | 3 | 11.106 | V.S. |
| M × m | 3 | 0.010 | N.S. |
| Error | 24 | 0.031 | |

[a] N.S. = not significant; V.S. = very significant.

of calcium were added to aliquots of diluted milk. Titration at pH 13 according to the proposed procedure (Table 3) yielded recoveries of the added calcium from 99.42 to 100.67%. Application of the Chi-test yielded a value of 2.002 for $\chi^2$, and therefore, these values do not differ significantly from 100%, indicating complete recovery.

*Comparison between direct and back titration.* While back titration provides satisfactory results, this procedure is not as simple nor as potentially accurate as the direct one. Therefore, the two procedures were compared in the milk at three different pH values, 12, 13, and 13.5. The back titration was performed as described above; whereas, in the direct titration, 8 N KOH was added to 50-ml aliquots of the diluted milk to obtain the proper pH followed by three drops of calcein indicator, and the sample was titrated with EDTA solution to the disappearance of the green color. While by the back-titration procedure, a satisfactory end point was obtained for all pH values employed, an acceptable although somewhat uncertain end point was obtained only at pH 13.0 by the direct titration. Results (Table 4) indicate significant difference between the two procedures, with the direct titration yielding much higher values from the back titration. At pH 12 the end point is indistinct, whereas at pH 13.5 the end point is very uncertain.

To help explain the behavior of these titrations in the milk, pure solutions containing known amounts of calcium and magnesium were analyzed by direct and back titration at the same pH values. Results (Table 5) indicate that calcium alone can be determined in either of the two ways at all pH values employed. When magnesium is present, however, only the back titration and the direct titration at pH 13.5 provide the correct values. Therefore, the back titration was employed throughout this work.

*Effect of variables.* Since variations in the pH, the concentration of the indicator, and the dilution of the sample may affect the determination of calcium and magnesium with the new method, it is important to know the extent and the range of their effects. Therefore, the influences of these variables were investigated independently.

1. To establish the effect of the pH on the calcium and calcium plus magnesium de-

TABLE 3

Recovery of added calcium in milk by the calcein method

(Calcium content of milk sample 0.1541 meq [a])

| Calcium added | Calcium observed (average of 2 replicates) | Calcium recovered | |
|---|---|---|---|
| *(meq)* | | | *(%)* |
| 0.0500 | 0.2039 | 0.0497 | 99.42 |
| 0.1001 | 0.2546 | 0.1005 | 100.44 |
| 0.1501 | 0.3050 | 0.1508 | 100.50 |
| 0.2001 | 0.3556 | 0.2015 | 100.67 |
| 0.2502 | 0.4045 | 0.2504 | 100.09 |

[a] Average of four replicates.

TABLE 4

Comparison of the direct and back-titration method for determination of calcium and magnesium in milk with calcein as indicator

| | Calcium and magnesium content [a] | | Calcium content [a] | | | | | |
|---|---|---|---|---|---|---|---|---|
| | pH 12 | | pH 13 | | | | pH 13.5 | |
| Milk sample no. | 1 | 2 | 3 | 4 | 5 | 6 | 1 | 2 |
| | | | *(meq/liter)* | | | | | |
| Direct titration | Indistinct end point | | 68.74 | 66.23 | 66.78 | 64.49 | Uncertain end point | |
| Back-titration | 66.02 | 66.15 | 66.47 | 63.86 | 64.74 | 62.62 | 56.62 | 56.56 |

Analysis of variance (pH 13)

| Source of variance | Degrees of freedom | Mean square | Significance [b] |
|---|---|---|---|
| Method (M) | 1 | 36.607 | V.S. |
| Milk (m) | 3 | 22.648 | V.S. |
| M × m | 3 | 0.103 | N.S. |
| Error | 24 | 0.050 | |

[a] Average of four replicates.
[b] V.S. = very significant; N.S. = not significant.

TABLE 5

Comparison of the direct and back-titration method for the determination of calcium and magnesium in pure solutions with Calcein as indicator

| Method | Solutions | | pH 12 | | | pH 13 | | | pH 13.5 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Ca-content | Mg-content | Ca and Mg observed [a] | $\chi^2$ | Significance [b] | Ca observed [a] | $\chi^2$ | Significance [b] | Ca observed [a] | $\chi^2$ | Significance [b] |
| | *(meq)* | *(meq)* | *(meq)* | | | *(meq)* | | | *(meq)* | | |
| Direct titration | 0.1497 | 0.0000 | 0.1499 | 4.20 | N.S. | 0.1501 | 5.04 | N.S. | 0.1500 | 5.85 | N.S. |
| | 0.0000 | 0.0293 | Indistinct end point | | | 0.0017 | 92.33 | V.S. | 0.0000 | ..... | ..... |
| | 0.1497 | 0.0293 | Indistinct end point | | | 0.1518 | 26.03 | V.S. | 0.1498 | 4.20 | N.S. |
| Back-titration | 0.1497 | 0.0000 | 0.1498 | 3.40 | N.S. | 0.1498 | 3.11 | N.S. | 0.1499 | 3.78 | N.S. |
| | 0.0000 | 0.0293 | 0.0292 | 3.25 | N.S. | 0.00001 | 3.03 | N.S. | −0.00005 | 3.46 | N.S. |
| | 0.1497 | 0.0293 | 0.1787 | 6.41 | N.S. | 0.1498 | 3.07 | N.S. | 0.1499 | 3.55 | N.S. |

[a] Average of four replicates.
[b] N.S. = not significant; V.S. = very significant.

terminations, aliquots of a diluted milk were adjusted by a pH meter to pH 12.0, 12.5, 13.0, and 13.5 with KOH and back-titrated with calcium chloride solution. In addition, a series of aliquots was adjusted to pH 12.5, back-titrated with calcium solution, and after the appearance of the green color, the pH increased to 13. Then the titration was continued to the final end point to determine if the values for calcium are the same when the titration has been performed on a separate aliquot or on the one used for calcium and magnesium determination.

2. The influence of the concentration of the indicator was investigated by the addition of three different amounts of indicator solution: 0.05 ml, 0.10 ml (which represents about three drops), and 0.15 ml.
3. The influence of the dilution of the milk sample with water was investigated at three levels: 1:10, 1:20, and 1:30. The amounts of indicator and the pH were adjusted accordingly.

Results (Table 6) indicate that there is no significant variation in the calcium and magnesium determination between pH 12.0 and 12.5 nor in the calcium determination between pH 13.0 to 13.5. The values for calcium are the same whether the titration has been performed on the same aliquot used for calcium and magnesium or on a separate aliquot. The concentration of indicator and dilution of the sample do not significantly affect the titration throughout the ranges investigated.

*Influence of phosphates, proteins, and trace heavy metals.* The effect of phosphate concentration upon the test was established by determining the total calcium and magnesium and the calcium content of the diluted milk containing varying amounts of a solution of $Na_2HPO_4$ (0.950 g P/liter) as indicated in Table 7. The analysis of variance of these results indicates that concentrations of phosphate four times the total phosphates present in the average milk do not have any significant effect upon the results.

To investigate the influence of the concentration of the protein, calcium- and magnesium-free skimmilk was prepared by shaking 200 ml of skimmilk for 1 hr with 250 g cation exchange resin (Amberlite IRC-50 equilibrated with sodium malate buffer $\Gamma/2 = 0.14$, pH 6.7). Varying amounts of this milk were added to the diluted milk sample as indicated in Table 7, and the pH adjusted accordingly. The analysis of variance of the results obtained indicate that a fourfold increase in the protein content of the sample did not significantly affect the titration, although the performance of the test is easier at lower concentrations of proteins.

The amount of the trace heavy metals in the milk normally is not enough to cause any difficulty in the test. The agreement of the values obtained by the proposed method with the values obtained by the Eriochromeblack T method, where the addition of the sodium sulfide prevents the interference of these metals, suggests that they do not significantly interfere with the test. Nevertheless, a direct comparison with the present method between aliquots containing a screening agent and those without is necessary. Therefore, to half of a series of aliquots of diluted milk an excess of potassium cyanide was added (10 ml of 1 M KCN per aliquot), and all the aliquots were titrated according to the new procedure. The results (Table 7) indicate no significant difference between the aliquots with and without screening agent.

*Reproducibility.* To determine the reproducibility of the proposed method, 14 different milk

TABLE 6

Effect of variables on the calcium and calcium plus magnesium determination in milk

| pH | | | Conc. of indicator | | | Dilution of sample | | |
|---|---|---|---|---|---|---|---|---|
| | | | Volume of indicator solution | | | | | |
| pH | Ca and Mg content [a] | Ca content [a] | | Ca and Mg content [a] | Ca content [a] | Ratio milk to water | Ca and Mg content [a] | Ca content [a] |
| | *(meq/liter)* | | *(ml)* | *(meq/liter)* | | | *(meq/liter)* | |
| 12.0 | 75.61 | | 0.05 | 75.75 | 67.57 | 1:10 | 75.70 | 67.65 |
| 12.5 | 75.56 | | 0.10 | 75.69 | 67.71 | 1:20 | 75.68 | 67.71 |
| 13.0 | | 64.55 | 0.15 | 75.75 | 67.74 | 1:30 | 75.65 | 67.53 |
| 13.5 | | 64.46 | | | | | | |
| 12.5 [b] | 75.59 | | | | | | | |
| 13.0 [b] | | 64.58 | | | | | | |

Analysis of variance

| Source of variance | Degrees of freedom | Mean square | Significance [c] |
|---|---|---|---|
| 1. Calcium plus magnesium | | | |
| pH | 2 | 0.002 | N.S. |
| Error | 9 | 0.034 | |
| Indicator | 2 | 0.005 | N.S. |
| Error | 9 | 0.020 | |
| Dilution | 2 | 0.002 | N.S. |
| Error | 9 | 0.021 | |
| 2. Calcium | | | |
| pH | 2 | 0.016 | N.S. |
| Error | 9 | 0.009 | |
| Indicator | 2 | 0.036 | N.S. |
| Error | 9 | 0.026 | |
| Dilution | 2 | 0.035 | N.S. |
| Error | 9 | 0.021 | |

[a] Average of four replicates.
[b] Both Ca and Ca + Mg were determined in the same aliquot.
[c] N.S. = not significant.

samples were analyzed in quadruplicate for calcium and seven for calcium and magnesium. The standard deviation of a single determination from its mean was observed to be ±0.14 meq/liter of milk for calcium and magnesium, or ±0.19% of the measured value, and ±0.12 meq/liter of milk for calcium alone, or ±0.18% of the calcium content.

## DISCUSSION AND CONCLUSIONS

Dilute solutions of calcein are known to fluoresce to yield a yellowish-green color (3). This fluorescence fades with increasing alkalinity and disappears above pH 12.0 (3, 10). However, even above pH 12.0, the calcein complexes of the alkaline earth metals possess a strong fluorescence. It has been noted by Korbl, Vydra, and Pribil (11) that even the alkali metals cause a weak fluorescence with calcein, with potassium being the least effective. Therefore, to keep the end point as distinct as possible, KOH was employed in this test for the adjustment of the pH. While Diehl and Ellingboe (3) considered that magnesium did not form a complex with the indicator, Korbl and Vydra (10) observed that it also causes calcein to fluoresce at alkali concentrations below 0.1 M.

When pure solutions containing calcium, magnesium, and the mixture of the two were analyzed in this study (Tables 1 and 5) at pH 12, calcium was easily titrated and completely recovered with both back and direct titrations. When magnesium is present, even though it would be in soluble form at pH 12.0, complete recovery of both calcium and magnesium was obtained only by the back titration. With the direct titration the end point was not distinct, since green coloration remained at the end of the titration. This is probably due to the relative magnitudes of the formation constants of MgEDTA and MgCalcein complex, since that of MgEDTA is lower than that of the CaEDTA (log $K_{Mg\text{-}EDTA}$ = 8.69, log $K_{Ca\text{-}EDTA}$ = 10.70) (18), whereas that of the MgCalcein complex is probably higher than the corresponding calcium complex. For the complexes of calcon, which are structurally similar to calcein, the formation constant of the Mg-

TABLE 7

Influence of phosphates, proteins, and trace heavy metals on the calcium and total calcium and magnesium in milk

| Phosphates | | | Proteins | | | Trace elements | | |
|---|---|---|---|---|---|---|---|---|
| Added phosphate solution [a] | Ca and Mg content [b] | Ca content [b] | Added cation exchanged milk [a, c] | Ca and Mg content [b] | Ca content [b] | Added KCN solution [a] | Ca and Mg content [b] | Ca content [b] |
| *(ml)* | *——(meq/liter)——* | | *(ml)* | *——(meq/liter)——* | | *(ml)* | *——(meq/liter)——* | |
| 0.0 | 75.68 | 66.89 | 0.0 | 75.68 | 66.89 | 0.0 | 75.33 | 64.47 |
| 2.5 | 75.74 | 66.86 | 2.5 | 75.72 | 66.88 | 10.0 | 75.29 | 64.44 |
| 5.0 | 75.66 | 66.87 | 5.0 | 75.73 | 66.87 | | | |
| 7.5 | 75.58 | 66.76 | 7.5 | 75.81 | 67.06 | | | |

Analysis of variance

| Source of variance | Degrees of freedom | Mean square | Significance [d] |
|---|---|---|---|
| 1. Calcium plus magnesium | | | |
| Conc. of phosphates | 3 | 0.012 | N.S. |
| Error | 12 | 0.018 | |
| Conc. of proteins | 3 | 0.013 | N.S. |
| Error | 12 | 0.024 | |
| Trace heavy metals | 1 | 0.005 | N.S. |
| Error | 6 | 0.008 | |
| 2. Calcium | | | |
| Conc. of phosphates | 3 | 0.013 | N.S. |
| Error | 12 | 0.009 | |
| Conc. of proteins | 3 | 0.033 | N.S. |
| Error | 12 | 0.010 | |
| Trace heavy metals | 1 | 0.002 | N.S. |
| Error | 6 | 0.009 | |

[a] Vol added to diluted milk sample containing 2.5 ml of the original milk.
[b] Average of four replicates.
[c] Ca and Mg content of the cation-exchanged milk is zero.
[d] N.S. = not significant.

complex is higher than that of calcium (log $K_{Mg} = 7.64$, log $K_{Ca} = 5.58$) (6). Therefore, enough free magnesium probably exists in equilibrium with the MgEDTA complex at this pH during the direct titration to interact with calcein and to confuse the end point. At pH 13, when calcium alone was present, it was completely recovered with both back and direct titrations. However, when calcium was titrated in the presence of magnesium, the calcium was completely recovered only by the back titration; whereas, with the direct titration the values observed were between those for calcium alone and for total calcium and magnesium. The recovery of the magnesium from the pure magnesium solutions was zero with the back titration, but some small amounts were observed with the direct titration. Although magnesium is precipitated as $Mg(OH)_2$ above pH 12.5 (6), nevertheless, even at pH 13 the concentration of the hydroxyl ions is not sufficiently high to reduce the concentration of magnesium ions below the traces necessary to interact with calcein. The green coloration observed, which cannot be due to the fluorescence of the indicator, since calcein is brown above pH 12 (3), requires some excess of EDTA to be completely depressed. Thus, the direct procedure yields erroneously high values for calcium when magnesium is present. At pH 13.5 the concentration of the hydroxyl ions is enough to cause complete precipitation of magnesium and the values obtained for calcium, when it is titrated alone or in the presence of magnesium, by both direct and back procedures are correct.

When milk is employed instead of pure solutions, at pH 12, the back titration (Table 2) provides values for total calcium and magnesium not significantly different from those obtained by the Eriochromeblack T method (15). The presence of the proteins and phosphates does not interfere with the determination, even when their concentration is four times the normal concentration in milk (Table 7). The end point by the direct titration (Table 4) is

not distinct, as could be predicted from the results with the pure solutions, due to the behavior of the magnesium present. At pH 13 the back titration provides an accurate measurement of calcium content of the milk. Addition of various amounts of calcium to the milk were completely recovered (Table 3), and the presence of the phosphates and proteins had no influence on the determination (Table 7). In the direct titration of the milk, the presence of the magnesium results in high values for calcium, as in pure solutions. At pH 13.5, contrary to the behavior of the pure solutions, the end point is uncertain in the direct titration of the milk sample. The pink color appears very early and fades again upon standing. The establishment of the permanent pink color requires successive additions of EDTA and about 20 to 30 min of time. The reproducibility is poor. This behavior appears to be due to the formation of some floccules, which may contain some calcium complexed with the phosphates or the proteins. As a result, titration with EDTA causes an early end point. Upon standing, the calcium gradually is released and titrated with EDTA. On the contrary, back-titration provides the correct values for calcium at this pH.

The back-titration in all cases, in pure solutions and in milk, provides the correct values, because an excess of EDTA is present in the sample before the addition of the alkali. The EDTA binds all of the calcium and magnesium, including that bound to proteins and phosphates. Then, when all of the free EDTA has been titrated by the calcium solution at pH 12, the first trace of magnesium displaced from the MgEDTA complex by the added calcium produces a green color with the calcein; thus, this titration measures the total calcium and magnesium. If the titration is performed at pH 13 to 13.5, the added calcium complexes first the free EDTA. Further addition of calcium displaces the magnesium from the MgEDTA complex, and the green color appears momentarily and fades again. This is because the freed magnesium reacts with the calcein, but because the pH is very high it is precipitated as $Mg(OH)_2$. As soon as the magnesium is completely replaced, then the first traces of the calcium yield a definite and permanent green color and this end point measures the calcium content only.

The test can be performed over a broad range of dilution of the sample and concentration of indicator, as is indicated in Table 6. The calcein is superior to murexide in this respect, since Schouwenburg (17) indicates that the amount of the murexide has some influence on the calcium determination. The pH control necessary for the test is not too exacting, since accurate values for total calcium and magnesium can be obtained throughout the pH range of 12.0-12.5, whereas calcium alone can be determined accurately from pH 13.0 to 13.5 (Table 6). The presence of the heavy metals, at least in the concentration of the average milk, has no influence on the test (Table 7). The method is very reproducible, the standard deviation being $\pm 0.14$ meq/liter of milk for total calcium and magnesium, or $\pm 0.19\%$ of the measured value, and $\pm 0.12$ meq/liter of milk for calcium alone, or $\pm 0.18\%$ of the calcium content.

Since the titration for both cations is performed in the same aliquot of the sample, the method provides a fast, easy, and accurate measurement of both cations.

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# SOME IMMUNOLOGICAL RELATIONSHIPS OF α-LACTALBUMIN AND β-LACTOGLOBULIN IN MILKS OF VARIOUS SPECIES [1]

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SUMMARY

Separate rabbit antisera prepared against cow α-lactalbumin and β-lactoglobulin were found in Oudin diffusion tests and on Ouchterlony plates to react with the milk of some, but not all, of a variety of species. A high degree of cross reaction was found with the milks of other ruminants (goat, sheep, and water buffalo), but not with the milks of the nonruminants tested (camel, horse, rat, mouse, guinea pig, pig, dog, and rabbit). The antiserum to cow β-lactoglobulin reacted with sheep milk about three times as strongly as with cow, goat, and buffalo milk, and the antiserum to cow α-lactalbumin reacted about twice as strongly with the goat and buffalo milk as with cow or sheep milk. Comparative analyses using crystalline β-lactoglobulins isolated from cow, sheep, and goat milks indicated that the antiserum to cow β-lactoglobulin reacted more strongly with sheep and goat β-lactoglobulin than with cow β-lactoglobulin itself. When the analyses are corrected on this basis, it is possible to analyze sheep and goat (and probably buffalo) milk for their β-lactoglobulin contents by using the antiserum to cow β-lactoglobulin. These results appear to agree with the contents of β-lactoglobulin indicated in these milks by other procedures.

Immunological methods based on the Oudin diffusion procedure have been used in this laboratory for the quantitative evaluation of the proteins β-lactoglobulin and α-lactalbumin in cow milk and other complex systems containing these milk proteins (6, 7). In the course of using these methods in investigations on the synthesis of protein in the mammary secretory cell of the cow, it was noticed that both of the rabbit antisera to these two proteins reacted with the milk of some other but not all species tested. As a result, a more thorough investigation was undertaken for information on the immunological relationship of α- lactalbumin and β-lactoglobulin in the milk of these various species.

## MATERIALS AND METHODS

The preparation of separate rabbit antisera to cow α-lactalbumin and β-lactoglobulin isolated from mixed herd milk, and their use in conducting the quantitative Oudin diffusion tests in capillary tubes, have been discussed previously (6, 7). Milk samples collected from the various species were diluted 1:20 and 1:100 with 0.15 M NaCl for use in the Oudin tests and duplicate samples at each dilution plated over the agar layer containing the specific antisera in the capillary tubes. The tubes were sealed, placed at 37 C, the diffusion zone measured at 24-hr intervals for 72 hr, and the results averaged.

Ouchterlony tests utilizing agar plates (2% Difco Bacto agar in 0.15 M NaCl plus 0.05% phenol) were conducted by cutting holes in the agar, placing the antiserum in the center well, and placing various dilutions of the milk of the different species in the equidistant-placed wells surrounding the center well. Control plates were set up also, using the antiserum in the center well with 0.15% (in 0.15% M NaCl) solutions of α-lactalbumin and β-lactoglobulin in the side wells. The rabbit antisera to α-lactalbumin and β-lactoglobulin used in the Ouchterlony tests were ten times more potent than the level used in the Oudin tests (6, 7).

## RESULTS

Data shown in Table 1 indicate the response of the antisera to cow α-lactalbumin and β-lactoglobulin to the milks from the various species. These may be conveniently divided into two main groups, ruminants and nonruminants. All the ruminant milk samples tested reacted in the Oudin and Ouchterlony tests with the two antisera as strongly or more strongly than did the cow milk itself. Slight possible reactions were

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TABLE 1

Immunological reaction of the milk of various species with antisera to cow α-lactalbumin and β-lactoglobulin

| Species milk | No. samples analyzed [a] | Antiserum to cow α-lactalbumin | Antiserum to cow β-lactoglobulin |
|---|---|---|---|
| Ruminants: | | | |
| Cow | >20 | + | + |
| Sheep | 4 | + | +++ |
| Goat | 4 | ++ | + |
| Water buffalo | 1 | ++ | + |
| Nonruminants: | | | |
| Camel | 1 | 0 [b] | 0 [b] |
| Horse | 2 | 0 [b] | 0 [b] |
| Rat | 2 | 0 | 0 |
| Mouse | 2 | 0 | 0 |
| Guinea pig | 1 | [c] | 0 |
| Pig | 2 | 0 | 0 |
| Dog | 2 | 0 | 0 |
| Rabbit | 2 | 0 | 0 |

[a] Each sample from an individual animal was run at least in duplicate in both the Oudin and the Ouchterlony procedures.

[b] A slight possible reaction (appearing quite nonspecific) was noted with camel and to a lesser degree with horse milk in the Oudin procedure (see text).

[c] Analysis not run.

found in the Oudin tests for both of the antisera with horse and camel milk. These reactions were quite unlike those found with the milk of the ruminants, in that clearly defined diffusion zones on the Oudin tubes were not present. Instead, a small amount of smeary-looking precipitate formed along one side of the tube for a short distance into the agar layer containing the antiserum. No reactions were found between the two antisera and either horse or camel milk in the Ouchterlony tests, as was also true for all of the other milks from the nonruminants in either test.

These results prompted a closer look at the extent of the reaction shown by milks of the other ruminants. Oudin tests were conducted with these milks against the two antisera, the diffusion distance noted for given lengths of time, and an apparent α-lactalbumin and β-lactoglobulin content calculated, based on the standard curves for the reaction between cow α-lactalbumin and β-lactoglobulin and their respective antiserum. Results shown in Table 2 indicate that the antiserum to cow β-lactoglobulin reacted similarly with goat and buffalo milk, but three times as strongly with the sheep milk as compared to the cow milk. The antiserum to cow α-lactalbumin reacted about the same with sheep milk as with the cow milk, but reacted

TABLE 2

Quantitative cross reaction in the Oudin diffusion procedure of the milk of several species with antisera to cow α-lactalbumin and β-lactoglobulin

| Ruminant species | Number samples [a] | Apparent α-lactalbumin content in milk | Apparent β-lactoglobulin content in milk [b] |
|---|---|---|---|
| | | *(mg/100 ml)* | |
| Cow | >20 | 120 | 300 (300) |
| Sheep | 2 | 147 | 930 (610) |
| Goat | 2 | 223 | 300 (240) |
| Water buffalo | 1 | 265 | 327 |

[a] Each sample run at least in duplicate analyses.

[b] Note later (Table 3) that crystalline sheep and goat β-lactoglobulin react quantitatively with the antiserum to cow β-lactoglobulin more strongly than does cow β-lactoglobulin. The values in parentheses represent the actual content of β-lactoglobulin in cow, sheep, and goat milk corrected to the actual quantitative relationship between it and the antiserum to the cow β-lactoglobulin.

about twice as strongly as this with the goat and water buffalo milk.

The availability of crystalline β-lactoglobulin from goat and sheep milk [3] then made it possible to examine more closely the quantitative aspects of the antiserum to cow β-lactoglobulin with the sheep and goat β-lactoglobulin samples. These results, shown in Table 3, indicate that in a given time at the same con-

[3] See Acknowledgments.

TABLE 3

Comparative quantitative reaction of antiserum to cow β-lactoglobulin with crystalline cow, sheep, and goat β-lactoglobulin

| Species | Species β-lactoglobulin [a] | Apparent β-lactoglobulin by analysis [b] |
|---|---|---|
| | *(μg/ml)* | |
| Cow | 20.0 | 20.0 |
| | 35.0 | 35.0 |
| | 50.0 | 50.0 |
| Sheep | 20.0 | 25.5 |
| | 35.0 | 49.0 |
| | 50.0 | 76.0 |
| Goat | 20.0 | 26.5 |
| | 35.0 | 47.0 |
| | 50.0 | 68.5 |

[a] These represent single β-lactoglobulin samples analyzed in duplicate. The 20 μg/ml level was analyzed on two other occasions with similar results.

[b] Values shown for the sheep and goat β-lactoglobulin samples were calculated from the standard curve for the cow β-lactoglobulin.

centration level the samples of goat and sheep β-lactoglobulin showed somewhat greater diffusion zones than did the sample of cow β-lactoglobulin. Thus, these samples of goat and sheep β-lactoglobulin apparently express a slightly stronger immunological relationship to the antiserum to cow β-lactoglobulin than does cow β-lactoglobulin itself. For both the sheep and the goat β-lactoglobulin samples, plots of the diffusion distance divided by the square root of time versus the β-lactoglobulin concentration on a logarithmic scale (see 6, 7) resulted in a straight-line relationship. It is apparent from this quantitative relationship that with different standard curves the antiserum to cow β-lactoglobulin may be used equally well to determine the β-lactoglobulin content of sheep and goat milk. One other difference was also noted, in that with time (i.e., after 48 or 72 hr) the clarity of the diffusion zone boundary became less marked in the tubes containing the sheep and goat β-lactoglobulin samples than in the bovine. This was not enough to obscure the analysis; however, it does point up a difference from cow β-lactoglobulin.

## DISCUSSION

Iit is readily apparent that the immunological similarity of α-lactalbumin and β-lactoglobulin in the milks of these species follows closely their zoological relationship. All of the milks from the ruminants tested showed an extremely close relationship, the milks of the two species close to the ruminants (camel and horse) showed a slight reaction, and the other tested species showed no reaction.

Unfortunately, the unavailability of β-lactoglobulin isolated from water buffalo milk and α-lactalbumin isolated from sheep, goat, and water buffalo milk makes it impossible, at present, to evaluate the quantitative relationship between them and the antisera to these two cow proteins. Since the water buffalo is also a species of bovine (subfamily Bovinae) more closely related to the cow than the sheep or goat (subfamily Caprinae), it is probable that a close quantitative relationship exists between water buffalo β-lactoglobulin and the antiserum to the cow β-lactoglobulin. The same close relationships may also be speculated to hold for α-lactalbumin in these species.

Results shown in Tables 2 and 3 indicate that sheep milk contains about two times as much β-lactoglobulin and goat milk about the same amount as cow milk. This conclusion, in general, agrees with results that would be predicted from electrophoretic studies run on the whey proteins in these milks (2).

It is well known that cow, goat, sheep, and water buffalo milk contain many common antigens (3, 8, 10). These ruminants are used extensively in various parts of the world for milk production. In some of these areas, problems exist of adulteration of the milk of one species or family with that of another (3, 8, 10). Results of the present investigation indicate that it would not be possible to use the antiserum to cow β-lactoglobulin or α-lactalbumin to distinguish between these milks.

It should not be overlooked, however, that it would be possible to determine the presence of one of these ruminant milks mixed with the milk of one of the species that does not react to the antiserum to cow β-lactoglobulin or α-lactalbumin. Thus, for example, the adulteration of camel, horse, pig, or some of the other species milk with goat, buffalo, sheep, or cow milk could be readily detected.

It is of interest to speculate on the possible structure of α-lactalbumin and β-lactoglobulin in sheep, goat, and buffalo milk as compared to cow milk. It is now known that cow β-lactoglobulin exists in at least two forms, A and B, which differ in composition by a few amino acid residues (1, 4, 9). It has been shown that immunologically these two forms are indistinguishable, in that antiserum to mixed cow β-lactoglobulin A and B reacts equally well with either the A or B forms (7), and specific antiserum to either A or B reacts with the other form equally as well (5). Thus, even though the β-lactoglobulin in sheep and goat milk reacts strongly with the antiserum to this cow protein, it does not preclude, and is probable that these proteins have, some differences in amino acid composition.

## ACKNOWLEDGMENTS

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# DIFFERENTIAL THERMAL ANALYSIS OF THE MELTING AND SOLIDIFICATION OF MILK FAT [1]

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SUMMARY

Differential thermal analysis (DTA) heating curves were recorded for milk fat cooled slowly, rapidly, and tempered; for the same fat from which 7% of HMG fraction was removed, and the same fat after randomization of the fatty acids on the glycerides. Thermal treatments led to differences in DTA curves that could be explained by the formation of mixed crystals of different glyceride groups. DTA cooling curves of milk fat, milk fat from which 7% of HMG was removed, and randomized milk fat are presented. DTA curves of conventionally churned and continuously made butters indicate that the latter type contains more mixed crystals, which may be an explanation for its different physical properties. Tributyrin was found to be a more satisfactory reference compound for DTA than was dioctylphthalate.

The melting and solidification of milk fat are of importance in a number of processes and it is well known that the consistency of butter can be influenced by temperature treatments of the cream or of the finished butter, or both (6, 7). Various investigators have ascribed the resulting consistency changes to either polymorphism or mixed crystal formation. As was pointed out in a recent review (4), there is no experimental evidence of any kind to relate such consistency changes to polymorphism. A considerable body of evidence now available indicates that the phenomenon of mixed crystal formation is the probable cause of the consistency differences in milk fats which have received different thermal treatments (3, 5, 7, 11). The present report deals with the application of differential thermal analysis (DTA) to the study of melting and solidification of milk fat. The technique of DTA has been applied to fats by Haighton and Hannewijk (8), Kaufmann and Schnurbusch (10) Jacobson et al. (9), and Perron et al. (13). It has been applied to the study of milk fat by a number of Soviet workers and their results have been reviewed (4).

## EXPERIMENTAL PROCEDURE

In DTA, a sample of fat contained in a tube is placed in a well of a brass block and another tube is filled with a reference compound which does not melt or crystallize over the temperature range used. When the block is heated and the fat sample melts, a negative (endothermic) heat effect occurs and the sample temperature falls below the temperature of the reference compound. This temperature differential is measured with two thermocouples arranged to make possible direct recording of $\Delta T$ as a function of the actual temperature during heating or cooling (12). If the copper block has provision for heating, differential thermal heating curves can be recorded. In this work two blocks were used, one contained a centrally located 200-w heating element, the other a central cooling well through which cold methanol from a Townson and Mercer minus 70 bath could be circulated. Heating rate regulation was obtained with a rheostat; cooling rate regulation, by adjustment of the methanol flow. DTA heating curves were recorded with a heating rate of 2.5 C/min and DTA cooling curves with a cooling rate of 4 C/min. The dimensions of the blocks used were: diameter 55 mm, height 60 mm, and of the sample tubes: inside diameter 10 mm, height 55 mm. The tubes were filled with 3.5 ml of sample or reference compound. $\Delta T$ with this experimental setup was of such a magnitude that the DTA curves could be recorded directly with a Sargent 1 mv recorder.

The milk fats were obtained from butter by melting, washing with water to remove nonlipid material, drying under vacuum, and filtration. Interesterification was carried out with sodium methoxide catalyst, as described earlier (1).

## RESULTS AND DISCUSSION

In the work published to date on the DTA of fats, the reference compound used has been dioctylphthalate (DOP) or a closely related compound. It is claimed that this compound does not solidify at a temperature of $-70$ C (8). In our experience DOP shows crystallization between $-20$ and $-70$ C and, therefore, is not a satisfactory reference compound.

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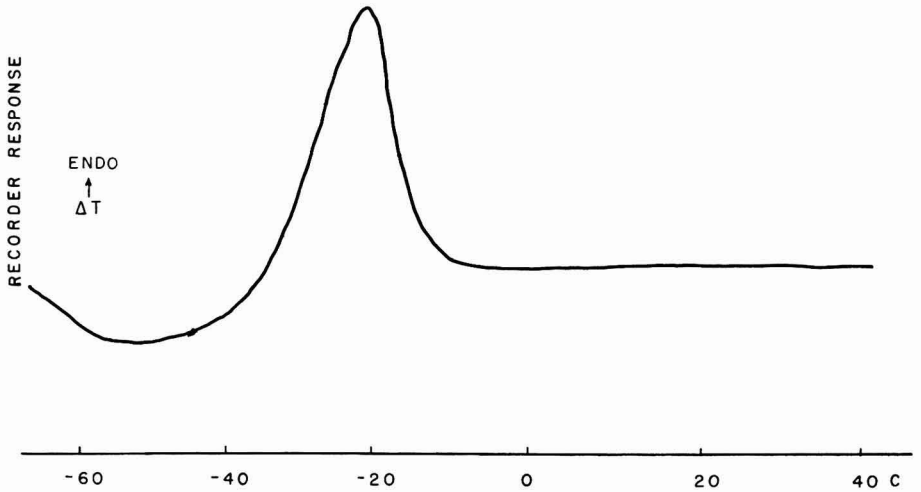


FIG. 1. DTA heating curve of dioctylphthalate, reference compound tributyrin.

Very few materials satisfy the conditions required of a reference for DTA, but it was found that tributyrin (TB) does not crystallize at −70 C or above. When one of the wells of the block was filled with DOP and the other with TB, a DTA curve was obtained which is shown in Figure 1. This curve indicates that DOP shows an endothermic heat effect as a result of melting between −40 and −10 C. TB is now used as a DTA reference compound in this laboratory.

DTA heating curves of the same milk fat with different temperature histories are presented in Figure 2. The fat represented by Curve A was cooled rapidly by immersion of the block containing the liquid fat sample into a methanol bath at −20 C. The curve indicates one main melting area with a peak at about 20 C and a shoulder of a high melting fraction at about 35 C. The melting point of the fat as indicated by this curve is about 37 C, corresponding well with the figure obtained with conventional melting point methods. When this fat was slowly cooled by keeping the block with the liquid fat sample in a Dewar flask in the refrigerator for 4 hr and subsequently in the sharp freezer for 12 hr, Curve B of Figure 2 was obtained. The curve shows three distinct melting areas, one between 5 and 10 C, one between 20 and 25 C, and an area for the high-melting glyceride fraction. This last part of the curve is displaced toward higher temperatures as compared to the corresponding area in curve A, Figure 1. When this fat was cooled rapidly and subsequently tempered by immersion of the block in a water bath at 25 C for 1 hr with subsequent cooling to −20 C, Curve C of Figure 1 was obtained. The tempering treatment has resulted in a separation of the

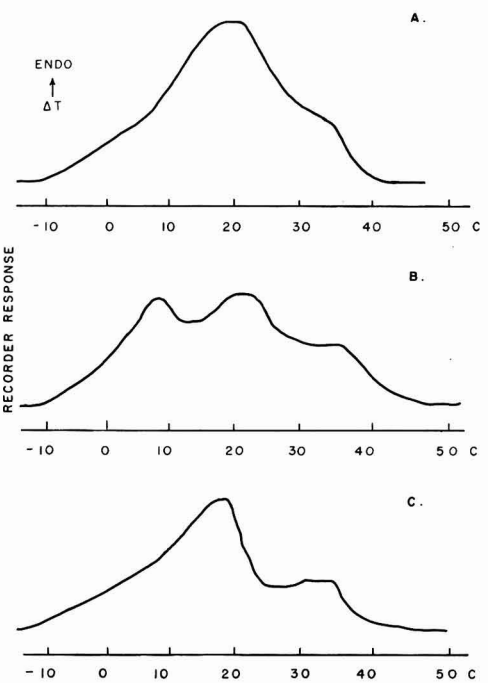


FIG. 2. DTA heating curves of milk fat with different temperature histories.
A—rapidly cooled
B—slowly cooled
C—tempered for 1 hr at 25 C
(For details of treatments, see text)

high-melting fraction and the main-melting fraction when compared with Curve A of Figure 2. The main-melting peak in Curve C is displaced towards lower temperatures as compared with Curve A. The three curves of Figure 2 indicate that it is possible to separate the lower and higher melting fractions of milk fat by appropriate thermal treatment. Dilatometric studies (5) have provided strong evidence for mixed crystal formation in rapidly cooled milk fat. The evidence obtained by DTA in this study presents further support for the theory of mixed crystal formation as the cause of differences in properties of rapidly and slowly cooled milk fat.

It has been shown (2) that the higher-melting glyceride fraction (HMG) of milk fat greatly influences the crystallization behavior of the fat. About 7% of the HMG was removed from the milk fat used for the aforementioned experiments by solvent crystallization from acetone, as described previously (1). DTA heating curves of this modified fat were run after both rapid and slow cooling, and the curves obtained are presented in Figure 3. The main difference between Curve A of Figure 3 and Curve A of Figure 2 is that the removal of HMG has resulted in the disappearance of most of the area between 30 and 40 C. This again is confirmation of melting points determined by conventional methods (1). Slow cooling of the modified fat resulted in a DTA heating curve (Figure 3B) with two well-separated peaks, the first one at lower temperature, the second one at slightly higher temperature than the corresponding areas of the curve representing the unmodified fat (Curve B, Figure 2).

Further indication of the effect of glyceride composition on the melting behavior of the fat was obtained by randomization of the fat. Interesterification with sodium methoxide leads to a random rearrangement of fatty acids between glycerides. The rearranged milk fat was rapidly cooled and the DTA curve obtained is presented in Figure 4. This curve is strikingly different from the one obtained with the unmodified milk fat (Curve A, Figure 2). The rearranged fat seems to consist of a lower-melting fraction with a peak at about 10 C and a higher-melting fraction with a peak at 37-40 C. The melting point of the rearranged fat was increased to about 45 C. Slow cooling of the rearranged milk fat led to a separation of the first peak into two, with maxima be-

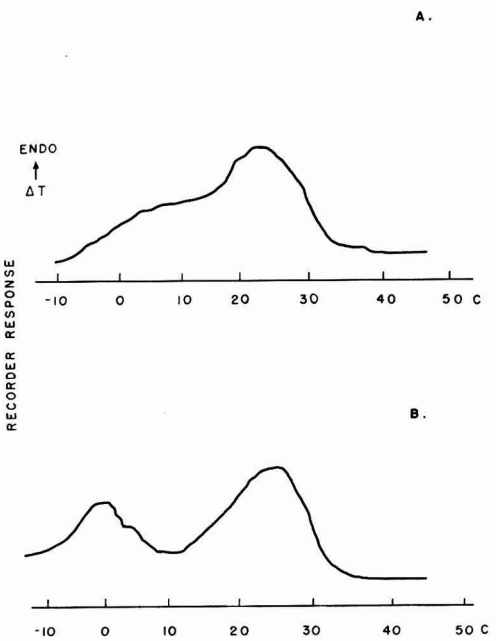


FIG. 3. DTA heating curves of milk fat from which about 7% HMG was removed by solvent crystallization.
A—rapidly cooled
B—slowly cooled

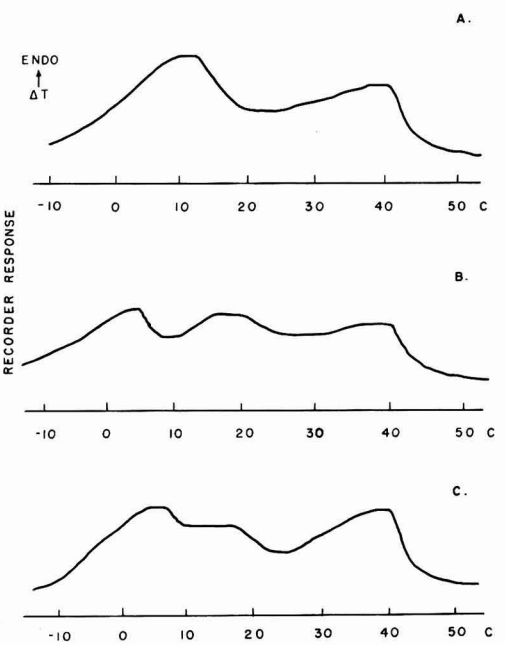


FIG. 4. DTA heating curves of randomized milk fat.
A—rapidly cooled
B—slowly cooled
C—tempered for 1 hr at 25 C

tween 0 and 5 C and between 15 and 20 C (Curve B, Figure 4). Tempering of this fat for 1 hr at 25 C resulted in Curve C, Figure 4, which is very similar to Curve B. All curves of Figure 4 show similar patterns for the HMG fraction. In the rearranged milk fat this HMG fraction seems to be a well-defined glyceride group separated by its melting point from the other glyceride groups; thus, different thermal treatments do not affect the melting properties of the HMG fraction, contrary to the pattern of the unmodified fat.

DTA cooling curves were recorded with milk fat, the same fat from which about 7% of the HMG was removed, and rearranged milk fat (Curves A, B, and C, Figure 5). The unmodified fat shows three ranges of crystallization; the major crystallization occurred when the temperature reached 5-0 C. Removal of the HMG fraction led to a decrease in starting temperature of crystallization and a flattening off of the second crystallization area. Rearrangement of the fat resulted in a distinct increase in the area of the curve, indicating a greater amount of solid fat, which is in accord with the dilatometric results of solid fat measurements (5). The relationship of the area under these DTA peaks to the amounts of melting or solidifying fat has not been established as yet, and further work on this aspect of DTA is in progress.

In commercial butter-making, conditions of relatively slow and rapid cooling of the fat are encountered in the conventional churning process and the continuous butter-making method (6). DTA curves of samples of churned and continuously made butter are presented in Figure 6. As could be expected, the churned butter curve consists of two peaks, whereas the curve of the continuously made butter has only one broad peak. This is further evidence for the theory of mixed crystal formation explaining

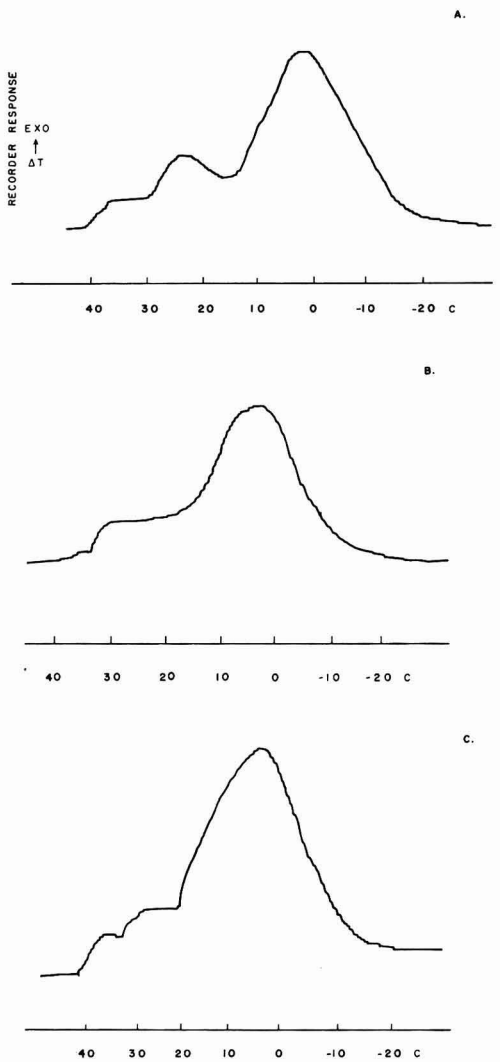


FIG. 5. DTA cooling curves.
A—milk fat
B—milk fat from which about 7% HMG was removed by solvent crystallization
C—randomized milk fat

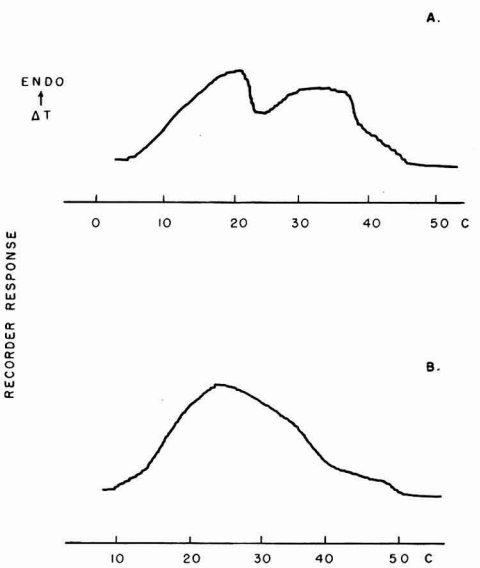


FIG. 6. DTA heating curves of butter.
A—conventional churned butter
B—continuously made butter

differences in physical properties of these types of butter, obtained on the butter itself rather than on fat obtained from it.

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# EFFECT OF FEEDING SAFFLOWER OIL ON THE FATTY ACID COMPOSITION OF MILK [1, 2]

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SUMMARY

Eight Guernsey and Jersey cows received, according to a three-period switchback design: one of two rations, 15% added safflower oil or a control diet, with both rations equalized for energy and protein content. Yield of milk, milk fat, and 4% FCM, as well as fat per cent, were all significantly depressed ($P < 0.01$) by the oil. Similarly, decreases were noted in all saturated fatty acids determined in the milk fat. In contrast, the content of unsaturated fatty acids was approximately doubled, with oleic acid contributing the major increase (19.2%). The palmitoleic acid concentration in the milk fat increased significantly, although this acid was absent in the safflower oil. Linoleic acid, which contributed 81% of the fatty acids in the oil fed to the cows, gave less increase. While the linolenic acid content was also elevated, this was not statistically significant.

Dietary triglycerides are known to undergo a number of changes in the rumen of cows. Among these is the hydrogenation of unsaturated fatty acids accompanied by the production of geometric *(trans)* and positional isomers (4, 9, 11, 13, 14). These alterations account for the many 18 carbon unsaturated fatty acids in ruminant milk fat and also partially explain why the milk fat of ruminants may not resemble dietary fat. Hydrogenation is accomplished by the rumen microflora, particularly the protozoa (5). Brown et al. (2) noted an increase in the 18-carbon monoenoic (18:1) and dienoic (18:2) content of milk fat when cottonseed oil was fed at the rate of 6% of the grain intake. A decrease in milk production was also observed. Tove and Mochrie (15) observed that, when ground soybeans were fed as part of the concentrate ration, the content of 18:1 and 18:2 in the milk fat increased. However, the increase of 18:2 was slight. When the rumen was by-passed by infusing a cottonseed oil emulsion into the jugular vein of cows, it was possible to increase enormously the 18:2 content of milk fat. The small increase in 18:0 content of milk fat, when soybean oil was fed, was attributed to hydrogenation of unsaturated fatty acids in the rumen.

The current interest in unsaturated dietary fats for human consumption led us to attempt to increase the 18:2 content of milk fat by feeding relatively large quantities of linoleic acid in the form of safflower oil. The resulting milk fat was analyzed by gas-liquid chromatography (GLC) and the results are reported herein.

## EXPERIMENTAL PROCEDURE

Eight Guernsey and Jersey cows from the University of Connecticut herd were divided into two equal groups with respect to breed and, insofar as possible, production level, but not with respect to stage of lactation or age. The cows were fed a basal ration consisting of 2 lb of medium-quality chopped alfalfa-timothy hay per 100 lb of body weight and 1 lb of a commercial grain mixture per 2 lb of 4% FCM. The hay allowance was held constant, whereas the grain allowance was based on the previous seven-day 4% FCM yield. After 3 wk, safflower oil [4] replaced 15% of the grain allowance in one group of cows, with the amount of grain decreased to a level providing the same energy intake as before, and soybean oil meal was added in amounts sufficient to provide the same protein content as before. After 5 wk, the ra-

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[4] Prepressed Safflower Oil, Pacific Vegetable Oil Corp., Richmond, California.

tion for each group was reversed, and this was repeated again in another 5 wk, thus completing a three-period switch-back design (1). The first week of each period was allowed for adjustment of the cows to their respective ration, and these data were not utilized. The fatty acid composition of the safflower oil, determined as described below, was (wt % as methyl ester): 16:0, 7.9; 18:0, 1.3; 18:1, 8.8; 18:2, 81.0; 18:3, 1.0.

Aliquots from pooled morning and evening milkings from each cow were obtained every seventh day. The fat content was determined by the Babcock procedure. The remaining portion of the sample was frozen and held at −20 C. Fatty acid analysis was subsequently accomplished by extraction of 25 ml of the sample by the silica-gel procedure (6), conversion of the fat to methyl and butyl esters by acid-catalyzed transesterification, and the component acids determined by GLC of the esters (3).

## RESULTS

The distributions of 13 of the fatty acids present in the milk fat are presented in Table 1. These figures represent the weight per cent of the methyl esters according to dietary treatment. The fatty acids referred to in Table 1 collectively as 15's include three acids: myristoleic (14:1), pentadecanoic (15:0), and a branched fifteen carbon acid (15:br) (3, 8), which have similar retention times and could not be separated by the technique used. Pentadecanoic acid and 15:br comprise the major portion of this peak (8). The feeding of safflower oil caused a highly significant ($P < 0.01$) depression in the levels of all the saturated fatty acid concentrations, except for 16:0, which was significant only at $P < 0.05$. All of the monounsaturated acids were consistently increased ($P < 0.01$). The dienoic acid 18:2, which comprised 81.0% of the fatty acids in the safflower as fed to the cows, was increased relatively less than in the monoenoics. While the change in the trienoic 18:3 was relatively larger than for the dienoics, the response among cows was less consistent, resulting in a nonsignificant effect.

The changes that occurred with time in the combined unsaturated fatty acids in the milk throughout the feeding trial are depicted in Figure 1. The effect of the oil ration upon the milk fat was evident soon after safflower oil was included in the diet.

The milk and fat production statistics are reported in Table 2. Feeding oil resulted in

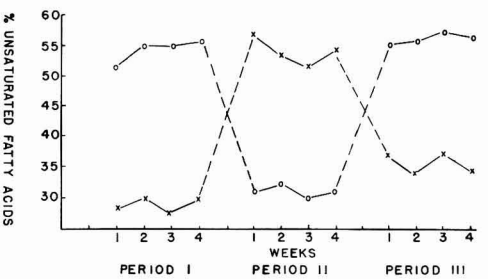


FIG. 1. Changes in total unsaturated fatty acid content brought about by switch-back feeding safflower oil to two groups of four cows.
Group x:—control-oil-control
Group o:—oil-control-oil

TABLE 1

Effect of feeding safflower oil on the fatty acid distribution in milk fat

| Acid | Treatment means | | Treatment difference | Confidence limits of the difference | |
|---|---|---|---|---|---|
| | Control | Oil | (oil-control)[a] | 95% | 99% |
| | *(wt % as methyl esters)* | | | | |
| 4:0 | 4.107 | 1.713 | −2.394 | ±0.695 | ±1.053 |
| 6:0 | 2.506 | 0.844 | −1.662 | ±0.213 | ±0.322 |
| 8:0 | 1.546 | 0.446 | −1.100 | ±0.416 | ±0.630 |
| 10:0 | 2.935 | 0.991 | −1.944 | ±0.448 | ±0.678 |
| 12:0 | 3.151 | 1.357 | −1.795 | ±0.504 | ±0.764 |
| 14:0 | 10.801 | 5.757 | −5.044 | ±0.025 | ±0.038 |
| 15's | 2.735 | 1.741 | −0.994 | ±0.455 | ±0.689 |
| 16:0 | 25.989 | 20.327 | −5.662[b] | ±0.514 | ±7.780 |
| 16:1 | 1.662 | 2.830 | +1.168 | ±0.651 | ±0.986 |
| 18:0 | 13.661 | 11.805 | −1.856 | ±1.179 | ±1.787 |
| 18:1 | 27.440 | 46.640 | +19.200 | ±3.230 | ±4.890 |
| 18:2 | 1.942 | 2.867 | +0.925[b] | ±0.719 | ±1.090 |
| 18:3 | 1.126 | 2.858 | +1.732[c] | ±2.692 | ±4.078 |

[a] All differences, except where indicated, are significant at the $P < 0.01$ level.
[b] Significant at $P < 0.05$ level.
[c] Not significant.

TABLE 2
Effect of safflower oil on milk and fat production

| | Treatment means [a] | | Treatment difference | Confidence limits of difference | |
|---|---|---|---|---|---|
| | Control | Oil | (oil-control) | 95% | 99% |
| Milk *(lb per day)* | 20.081 | 17.169 | −2.912 | ±1.517 | ±2.300 |
| Fat *(%)* | 5.241 | 3.559 | −1.682 | ±0.900 | ±1.364 |
| Fat *(lb per day)* | 0.970 | 0.508 | −0.462 | ±0.128 | ±0.193 |
| 4% FCM *(lb per day)* | 23.28 | 15.50 | −7.78 | ±2.090 | ±3.16 |

[a] All values are significant at the $P < 0.01$ level.

highly significant ($P < 0.01$) depression in daily yield of both milk and fat.

## DISCUSSION

Addition of 15% safflower oil to the ration of a cow produced significant qualitative and quantitative alterations of the milk fat. The 19% increase in 18:1 concentration of the milk fat can be attributed to the partial hydrogenation of 18:2 in the rumen. No evidence of complete hydrogenation was found in the present experiment, in contrast to the findings of Tove and Moehrie (15), who noted a large increase in 18:0 when soybean oil was fed. This probably reflects differences in the quantities of fat fed, since considerably more fat was fed in the present study than in the investigation of Tove and Moehrie. Thus, in the present experiment the concentration of unsaturated oil in the diet probably exceeded the reducing potential of the rumen microflora (5). Whether the rumen flora would eventually become adapted to this change in environment is not known.

The amounts of 16:0 and 18:0 were significantly depressed during the 5-wk feeding period. If complete saturation of 18:2 had occurred, an increase in the amount of 18:0 would have probably been noted. Although 16:0 was significantly decreased, the presence of approximately 8% of this acid in the oil fed to the cows may have been responsible for this smaller change which occurred in the milk fat.

The significant increase of the 16:1 concentration ($P < 0.01$) as a result of the oil feeding may be attributed to the partial hydrogenation and partial oxidation of the dietary 18:2. There was apparently no 16:1 present in the safflower oil. Evidence of the oxidation of 18 carbon acids to palmitic acid has been demonstrated previously (7, 17); however, increases of palmitoleic acid have not been reported. Tove and Moehrie (15) noted little change in the level of 16:1.

Maynard et al. (10) found a rapid rise in unsaturated fatty acids of milk fat (as measured by iodine number) within 24 hr after feeding a highly unsaturated oil to cows. Similar findings were apparent in the present experiment. However, after the initial rise in unsaturation, a gradual increase in 18:1, with a concomitant decrease in 18:2, occurred throughout the 5-wk oil feeding period. Hilditch (7) has stated that once the maximum effects on the milk fat of feeding a given oil have been reached, this level will be maintained. Since this was based on determination of iodine numbers alone, variations occurring among the unsaturates were not determined. Brown et al. (2) have reported significant increases in the 18:3 content of milk fat when 5% cottonseed oil was fed to cows, although this acid was not present in the cottonseed oil. A similar but not statistically significant effect was noted in the present experiment.

Cod liver oil has also been observed to decrease the fat content of milk (12). The decrease has been attributed to the degree of unsaturation of the oil (mostly unsaturated $C_{20}$ and $C_{22}$ fatty acids), since the fat-depressant effect can be destroyed by hydrogenation of the oil prior to feeding (4). Moore et al. (12) did not observe a decrease in the fat content of milk when cod liver oil was administered per os in small amounts at frequent intervals. Evidently, the concentration of the unsaturated fatty acids in the rumen at any one time was insufficient to produce the necessary changes in rumen metabolism.

Since feeding 15% safflower oil caused such large decreases in all production figures (Table 2), it does not appear likely that such a regimen would be practical, despite any beneficial modification of the milk fat which might occur. Nevertheless, it is possible to change milk fat from a largely saturated to a largely unsaturated fat, as was evident in Figure 1.

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# EFFECT OF SOURCE OF NUTRIENTS AND NUTRITIONAL LEVEL OF THE COW ON DEVELOPMENT OF HYDROLYTIC RANCIDITY IN THE MILK [1]

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SUMMARY

Long-term continuous and short-term change-over feeding trials were used to determine the effects of the nutritional level of the cow and the inclusion of green feed in the ration on the susceptibility of the milk to spontaneous and agitation-induced lipolysis. In all trials, no effect of green feed or of nutritional level was found. Rather, the susceptibility of the milk to both types of lipolysis was a characteristic of the individual cow.

Cold milk, when aged, will show varying degrees of milk fat hydrolysis. This has been designated as spontaneous hydrolytic rancidity (11). Lipolytic activity in milk can be increased by altering the substrate through such means as shaking, agitation by air, temperature treatment, and homogenization. Lipolysis resulting from alteration of the substrate in milk has been designated as induced lipolysis (10).

Several investigations have been made of the effect of ration on the susceptibility of milk to spontaneous and induced hydrolytic rancidity. A decrease in the incidence of spontaneous rancidity has been attributed to the inclusion of green forage in the ration by some authors (1, 5, 10). Speer et al. (9), however, could not attribute any benefit to the inclusion of pasture. Gilmore et al. (2) report that variations in the susceptibility of milk to induced rancidity are closely related to the type of ration being fed, although no other details are given. On the other hand, no difference in the development of induced rancidity in the milk from cows on two different dry feeds (5), or from cows on dry feed and pasture (5, 9) was found.

In the studies cited, cows were fed normal herd rations that were probably nutritionally adequate. No information was found concerning the effect of feeding low-energy rations to the cow on the development of spontaneous and induced lipolysis in the milk.

The studies reported herein were designed to evaluate the effects of nutritional level and the inclusion of green feed in the ration on the development of spontaneous and agitation-induced hydrolytic rancidity in the milk fat.

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## EXPERIMENTAL PROCEDURE

Three feeding trials were carried out during the course of the study. Trials 1 and 2, of 7 and 9 wk duration, respectively, were designed to determine long-time effects of the various rations. In these trials, nine cows, balanced as to stage of lactation, were randomly assigned to three ration groups. The rations fed during Trials 1 and 2 are given in Table 1. Hay and silage ingredients were fed ad lib. No proximate analyses were made of these rations. However, Rations 1 and 2 are representative of rations commonly used in Alabama. Work at this Station (4) has shown Coastal Bermudagrass hay to be of low nutritive quality. This, with a low concentrate intake, would make Ration 3 a nutritionally inadequate one for lactating cows.

The short-time effects of nutritional level and of green feed on spontaneous and agitation-induced lipolysis of milk were studied in Trial 3. Six cows were assigned randomly to six rations according to a 6 × 6 Latin-square design. Rations were calculated using Morrison's standards (7) to supply 80, 100, and 120% of the recommended allowance of nutrients of each cow, based on body weight and milk production at the beginning of the experiment. The rations consisted of a grain concentrate, alfalfa hay, and corn silage, with and without green chopped alfalfa at each nutritional level. The cows were multiparous grade Holsteins, were 30 to 60 days post-partum, and were producing 50 to 60 lb of milk daily at the start of the experiment. Each cow was fed a different ration during each of the six ten-day periods (six days standardization and four days test).

TABLE 1

Ration ingredients used in feeding Trials 1 and 2

| Ration | Trial | Pasture | Hay | Silage | Concentrate (16% protein) |
|---|---|---|---|---|---|
| 1 | 1 | 2 hr/day on rye, wheat or fescue | Alfalfa | Millet | 1 lb/3 lb milk |
| | 2 | Cont. on CBG,[a] fescue, and millet | | | 1 lb/3 lb milk |
| 2 | 1 | | Alfalfa | Millet | 1 lb/3 lb milk |
| | 2 | | Alfalfa or CBG [a] | Alfalfa | 1 lb/3 lb milk |
| 3 | 1 | | CBG [a] | | 1 lb/day |
| | 2 | | CBG [a] | | 1 lb/day |

[a] Coastal Bermudagrass.

Intake of each ration component was measured and proximate analyses were done on a composite sample for each period. Because of variability in consumption and change in milk production of the cows, the TDN intake of the animals varied from the original calculated value. The extent of variation is given in Table 2.

Milk samples were taken from well-mixed milk in the weigh pail at the time of milking. One aliquot in a 50-ml screw cap tube was cooled immediately to 4 C in ice water and incubated for determination of spontaneous lipolysis. A second aliquot was agitated in a Waring Blendor under standard conditions, then transferred to a screw-cap tube, cooled to 4 C, and held for 24 hr for determination of agitation-induced lipolysis. At the end of the incubation period, all samples were heated in a water bath to 63 C for 15 min to stop further lipolytic activity. A third aliquot of each sample was analyzed for milk fat by the Babcock test and the specific gravity was determined using a Westphal balance. These values were used in calculation of acid degree values (ADV).[2]

Conditions of agitation and incubation for the various trials are shown in Table 3. Because of the variable conditions used, absolute values from the different trials are not comparable.

ADV's were determined on each sample, using the silica gel method of Harper et al. (3).

Samples were taken weekly at a PM milking during Trials 1 and 2. During Trial 3, samples were taken on alternate days from the AM and PM milking.

[2] Acid degree value equals ml of 1 N KOH required to neutralize the free fatty acids in 100 g of fat.

## RESULTS AND DISCUSSION

The average ADV's of milk from individual cows by ration during the preliminary period of Trials 1 and 2 are presented in Table 4.

TABLE 3

Agitation and incubation conditions in the determination of spontaneous and agitation-induced lipolysis

| | Spontaneous | Induced | |
|---|---|---|---|
| Trial | Incubation at 4 C | Agitation [a] | Incubation at 4 C |
| | *(hr)* | *(sec)* | *(hr)* |
| 1 | 24 | 10 | 24 |
| 2 | 48 | 2 | 24 |
| 3 | 72 | 30 [b] | 24 |

[a] 150 ml of sample agitated in a Waring Blendor jar.

[b] Speed of the Blendor was reduced by using a setting of 35 on a Powerstat type 116 variable transformer.

TABLE 2

Actual total digestible nutrient intake of cows on various rations as compared to calculated estimates (Trial 3)

| Calculated TDN [a] | Without green chopped alfalfa | | With green chopped alfalfa | |
|---|---|---|---|---|
| | Avg | Range | Avg | Range |
| | *(%)* | | | |
| 80 | 81.5 | 74.5– 88.0 | 85.3 | 75.0– 94.0 |
| 100 | 105.5 | 87.0–119.0 | 103.4 | 90.5–129.0 |
| 120 | 116.1 | 103.5–133.0 | 127.4 | 102.5–165.0 |

[a] TDN intake expressed as per cent of recommended allowance based on body weight and milk production.

TABLE 4
Average acid degree values of fat from milk of individual cows during preliminary periods of Trials 1 and 2

| Ration | Cow | Trial 1 | | Trial 2 | |
|---|---|---|---|---|---|
| | | Spontaneous | Induced | Spontaneous | Induced |
| 1 | 1 | 0.914 | 9.979 | 0.233 | 7.774 |
| | 2 | 0.905 | 12.876 | 0.043 | 3.406 |
| | 3 | 0.000 | 7.319 | 0.083 | 1.593 |
| | Mean | 0.606 | 10.058 | 0.120 | 4.258 |
| 2 | 1 | 0.555 | 6.383 | 0.000 | 3.355 |
| | 2 | 0.076 | 9.424 | 0.000 | 3.164 |
| | 3 | 0.540 | 10.984 | 0.057 | 8.714 |
| | Mean | 0.390 | 8.930 | 0.019 | 5.078 |
| 3 | 1 | 1.453 | 12.659 | 0.100 | 3.664 |
| | 2 | 0.757 | 11.731 | 0.281 | 4.709 |
| | 3 | 0.610 | 10.435 | 0.045 | 5.094 |
| | Mean | 0.940 | 11.607 | 0.142 | 4.489 |

Because of the large variation in ADV's among the individual cows, ADV's during the experimental period of Trials 1 and 2 were adjusted by covariance, to take into account the cow differences that existed during the preliminary period (8). Adjusted ADV's resulting from spontaneous lipolysis and agitation-induced lipolysis are shown in Figures 1 and 2. Differences in ADV's resulting from the various rations were not significant ($P > 0.05$) for either spontaneous or agitation-induced lipolysis. The ADV's resulting from spontaneous lipolysis of the milk fat from cows fed low nutritive ration (Ration 3) were higher ($P = 0.076$) than the others in Trial 1, but in Trial 2 there was no difference ($P > 0.25$).

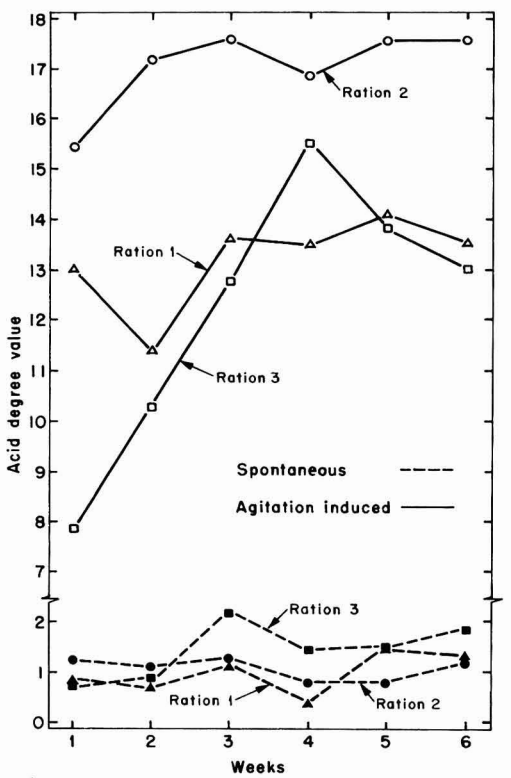


FIG. 1. Acid degree value (adjusted by covariance) of fat from milk of cows fed various rations resulting from spontaneous and agitation-induced lipolysis (Trial 1). (LSD, P = 0.05; spontaneous—1.151; agitation-induced—3.268).

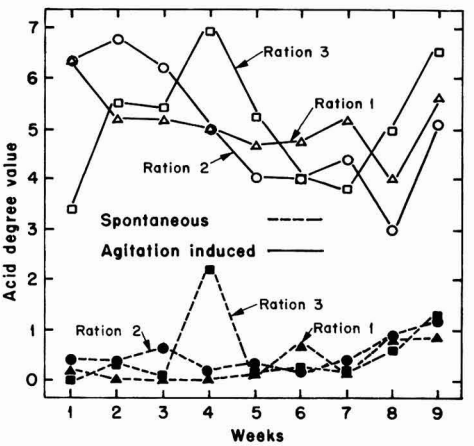


FIG. 2. Acid degree values (adjusted by covariance) of fat from milk of cows fed various rations resulting from spontaneous and agitation-induced lipolysis (Trial 2). (LSD, P = 0.05; spontaneous—0.687; agitation-induced—1.704).

For Trial 3, the average ADV's of the milk fat resulting from spontaneous and agitation induced-lipolysis are given in Table 5. The differences in ADV resulting from variation in TDN intake level and from feeding of green-chopped alfalfa are not significant ($P > 0.05$).

Several investigators (1, 5, 10) have reported

TABLE 5

Acid degree values of fat from milk of individual cows resulting from spontaneous and agitation-induced lipolysis as affected by total digestible nutrient level [a] and green feed

| Cow no. | Forage [c] | Acid degree values [b] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Spontaneous | | | | Agitation-induced | | | |
| | | Calculated TDN levels | | | Cow mean | Calculated TDN levels | | | Cow mean |
| | | 80% | 100% | 120% | | 80% | 100% | 120% | |
| 1 | 1 | 0.30 | 0.56 | 0.62 | 0.56 | 10.42 | 11.11 | 9.20 | 10.41 |
| | 2 | 0.65 | 0.47 | 0.76 | | 10.20 | 11.50 | 10.04 | |
| 2 | 1 | 0.35 | 0.28 | 0.81 | 0.39 | 8.85 | 8.50 | 9.91 | 7.76 |
| | 2 | 0.29 | 0.18 | 0.41 | | 8.09 | 6.44 | 4.80 | |
| 3 | 1 | 0.59 | 0.21 | 0.47 | 0.39 | 9.33 | 12.15 | 8.74 | 9.14 |
| | 2 | 0.58 | 0.45 | 0.29 | | 8.60 | 7.36 | 8.65 | |
| 4 | 1 | 0.64 | 0.84 | 0.39 | 0.49 | 9.24 | 6.62 | 7.49 | 7.82 |
| | 2 | 0.69 | 0.11 | 0.26 | | 8.84 | 8.12 | 6.60 | |
| 5 | 1 | 0.23 | 0.53 | 0.19 | 0.29 | 7.56 | 8.92 | 5.01 | 8.58 |
| | 2 | 0.16 | 0.26 | 0.39 | | 10.98 | 9.95 | 9.15 | |
| 6 | 1 | 0.76 | 0.43 | 0.39 | 0.44 | 12.47 | 12.63 | 11.96 | 11.02 |
| | 2 | 0.15 | 0.89 | 0.64 | | 8.97 | 6.79 | 13.30 | |
| Mean | 1 | 0.45 | 0.47 | 0.48 | 0.47 | 9.64 | 9.99 | 8.72 | 9.45 |
| | 2 | 0.42 | 0.33 | 0.46 | 0.40 | 9.28 | 8.36 | 8.75 | 8.80 |
| TDN Mean | | 0.44 | 0.40 | 0.47 | | 9.46 | 9.18 | 8.74 | |

[a] TDN level expressed as per cent of recommended allowance based on body weight and milk production.

[b] $S\bar{x}$: Spontaneous = 0.148; agitation-induced = 1.387.

[c] Forage 1—without green chopped alfalfa. Forage 2—with green chopped alfalfa.

a decrease in the incidence of spontaneous lipolysis resulting from the inclusion of green forage in the ration. In the trials reported here, all of the animals used showed a low rate of spontaneous lipolysis. These results indicate that the omission of green feed from the ration does not result in an increased incidence of spontaneous lipolysis. At the same time, they do not disprove the hypothesis that green forage may be beneficial in reducing high levels of spontaneous lipolysis. The lack of effect of green forage in the ration on the level of induced lipolysis in the milk fat is in agreement with the findings of others (5, 9).

Results of these trials indicate that there is no relationship between nutritive level of the cow and susceptibility of the milk to spontaneous or agitation-induced lipolysis.

As has been reported (1, 5, 6, 12), the milk from individual cows varied considerably in susceptibility to both spontaneous and agitation-induced lipolysis. The ADV's for individual cows on the various rations in Trial 3 are shown in Table 5. As can be seen from these data, the rations had less effect on the extent of lipolysis than did the individuality of the cow. Variations among the individuals within groups were greater than the variations among groups at different feeding levels.

Results of these trials show that under the feeding regimes studied neither the inclusion of green feed nor the nutritional level at which the cow is fed affects the susceptibility of the milk to spontaneous or agitation-induced lipolysis. Rather, the susceptibility of the milk to both types of lipolysis was a characteristic of the individual cow.

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# COMPARISON OF FATTY ACIDS FROM LIPID CLASSES OF SERUM LIPOPROTEINS AND OTHER LIPIDS IN THE BISON [1, 2]

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SUMMARY

High- ($D > 1.063$) and low-density ($D < 1.063$) lipoproteins, comprised 64 and 36%, respectively, of bison serum total lipids. Protein-bound nonesterified fatty acids accounted for less than 1%. High-density lipoproteins (HDL) contained comparatively greater concentrations of sterol esters, nonesterified fatty acids, and phospholipids. Liver lipids showed 53% triglycerides and 37% phospholipids. Rumen content lipids contained 4, 16, 17, 22, and 33%, respectively, of monoglycerides, triglycerides, sterols, phospholipids, and nonesterified fatty acids. Lipids in abomasal fluid showed 2% monoglycerides, 50% phospholipids, and 12% nonesterified fatty acids. HDL lipid classes resembled those of the low-density lipoproteins (LDL) in fatty acid composition, but triglycerides were more saturated and sterol esters more unsaturated in the latter. Oleate was the predominant unsaturated fatty acid in most of the lipid classes. Glycerides of whole serum and HDL; nonesterified fatty acids of whole serum, HDL, and LDL; and diglycerides of liver tissue were similar in fatty acid composition to the long-chain fatty acids of bison milk fat. Similarity to other ruminant species was noted in the fatty acid composition of rumen content total lipids, liver tissue lipid classes, and milk fat. However, fatty acid patterning of HDL and LDL lipid classes was species-specific.

Arterio-venous differences (25), transfer quotients (24), and absorption results during udder perfusion (11) have called attention to the utilization of blood lipids in ruminant milk fat synthesis. Triglycerides, phospholipids, cholesterol esters, and protein-bound nonesterified fatty acids have at one time or another been implicated as the major blood source of milk fatty acids (24). Separability of bovine serum lipids into two distinct lipoprotein groups suggested another frame of reference from which to interpret lipid utilization by the mammary gland (1, 3). In vitro lipid incorporation and exchange dynamics of the high-density lipoproteins (HDL) were suggestive of an endogenous transport function (1). This idea was reinforced by isotopic transfer from tripalmitin-1-$C^{14}$-labeled serum low-density lipoproteins (LDL) to the HDL during a 2-hr goat liver perfusion (4). Isotopic transfer from a tritiated triglyceride in the rumen to milk fat within 4 hr led to further speculation of a labile lipoprotein carrier of uncertain composition (7).

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The extramammary source(s) of milk long-chain fatty acids is still an uncertainty. Independent analyses of serum lipoproteins and other body lipids in various ruminants have not yet been correlated with mammary function in the same species to yield an integrative and meaningful whole. This paper attempts to emphasize such integration by analyzing similar products of intermediary metabolism in blood, body, and milk of the bison, and correlating intra- and inter-species similarities and differences. Lipids of whole serum, HDL, LDL, protein-bound nonesterified fatty acids, blood cells, liver tissue, rumen contents, abomasal fluid, and milk have been analyzed with regard to lipid class weights, distribution, and fatty acid composition. It is hoped that, by tracking down such variables, a more comprehensive estimation of milk fat precursors may be enabled within a context of ruminant lipid metabolism.

## METHODS

Blood was obtained by jugular venipuncture from two unanesthetized healthy bison heifers (Bison bison). They were on a diet of timothy hay ad lib., supplemented at 6 PM daily with approximately 1 lb of oats and a vitamin mixture. The blood was allowed to clot for 30 min at 10 C, then serum and blood cells were sepa-

rated by centrifugation at 550 × *g* for 30 min. Serum lipoprotein fractions of high- ($D > 1.063$) and low-density ($D < 1.063$) were isolated by differential ultracentrifugation in a 40.3 rotor, according to techniques described earlier (1). The serum was adjusted to a solvent density of 1.063 g/ml and spun at 105,000 × *g* for 16 hr. This allowed the low-density lipoproteins (LDL) to rise to the top of the ultracentrifuge tubes and be removed with a syringe and 20-gauge needle. The infranate was then changed to a solvent density of 1.21 g/ml and ultracentrifuged once again under the initial conditions. Lipoproteins of high-density (HDL) were subsequently recovered from the top of the tubes and the serum protein residue was obtained as a pellet at the bottom. Effectiveness of separation between the two major lipoprotein fractions was checked by horizontal paper electrophoresis (1).

Total lipids were extracted from whole serum, HDL, LDL, and lysed, homogenized blood cells (erythrocytic and leucocytic components) from both of the bison. Total lipids were also extracted from 200 g of combined aliquots of wet liver tissue, 350 ml of rumen contents, and 100 ml of abomasal fluid, all of which were obtained from the second test animal in the morning within 10 min following a lethal dose of sodium pentobarbital. All extractions were carried out at room temperature in a Waring Blendor at medium speed. (The blendor was washed thoroughly between samples.) Ethanol, ethyl ether, and petroleum ether (1:5:5 by volume) were added consecutively, spinning between additions for 30 sec. The cumulative $H_2O$-ethanolic-ethereal extract was decanted and filtered. Extraction procedures were repeated three times, combining the final extracts and adding excess anhydrous sodium sulfate and sodium chloride. Following overnight refrigeration at 10 C, the dehydrated extracts were filtered and evaporated to just dryness on a steam bath under O.P. nitrogen. If water or other impurities persisted, the extracts were washed with 0.5% saline, then re-extracted with ethyl and petroleum ether (1:5), refrigerated overnight with excess anhydrous sodium sulfate, filtered, and evaporated. This process seldom had to be repeated a third time.

Heat and acidification were avoided during the extraction processes, in an effort to minimize possible hydrolysis, trans-esterification, or oxidation of the lipids. Following solvent removal, total lipids were weighed and subjected to silicic acid column chromatography to isolate the major lipid classes (9, 17). Hydrocarbons, sterol esters, triglycerides, and nonesterified fatty acids, sterols, diglycerides, monoglycerides, and phospholipids were eluted, respectively, with petroleum ether 100%; ethyl ether in petroleum ether 1, 4, and 8, 15, 30%; ethyl ether, 100%; and methanol, 100%. Elution patterns were carefully followed, evaporating each 50-ml eluate immediately on a steam bath under O.P. nitrogen. Each lipid class was allowed to elute beyond the emergence of its unsaturated components before increasing the polarity of the eluant, with the exception of the triglycerides. To save time, and avoid the use of considerable quantities of 4% ethyl ether in petroleum ether, the saturated nonesterified fatty acids were allowed to elute with the unsaturated triglycerides. Later, both classes were completely combined, then completely separated on McCarthy and Duthie columns (17). Infrared spectra of all lipid classes were used to confirm the identifications made on the basis of chromatographic elution patterns.

Following gravimetric analysis, lipid classes, other than hydrocarbons and sterols, were transmethylated to convert their constituent fatty acids to methyl esters. This was accomplished by gently refluxing the lipids with 1% $H_2SO_4$ in methanol for 2 hr on a steam bath. To accommodate small sample size, the process was carried out in a 15- by 150-mm test tube with an inserted 40- by 80-mm glass funnel, containing a marble, acting as condenser for volatiles.

Methyl esters were identified by gas chromatography, using a Beckman GC-2A instrument. The coiled aluminum chromatography column was 10 ft long, with ¼-in. diameter, and contained 17% ethylene glycol succinate polyester coated on Gas-Chrom P, 80-100 mesh. The column temperature was 190 C, carrier gas was helium at an inlet pressure of 30 psi, and the detector cell current was 250 ma. Instrument standardizations were run with National Institutes of Health reference methyl ester mixtures of caprylate through stearate and palmitoleate through linolenate. Peak area percentages, calculated by triangulation, coincided with weight composition percentages within 1%. Duplicate runs showed a measurement error within 1%. However, reference esters butyrate and caproate, when included with the longer-chain methyl ester standards, required correction factors.

To facilitate certain inter-species lipid comparisons, two corollary studies were run. One examined the fatty acid composition of milk fat from an American bison and an Indian water buffalo. The bison milk fat, extracted (chloroform:methanol, 1:1) from cream fol-

lowing oxytocin administration, had been immediately sealed in a glass ampule. The water buffalo sample consisted of lyophilized whole milk. Both samples had been maintained in a deep freeze at −17 C since March, 1960, but showed no visual or olfactory evidence of oxidation. Total lipids were extracted from the water buffalo milk according to the technique described above. Infrared analysis of the two fat samples revealed triglyceride spectra with slight transisomerization. Conversion of constituent fatty acids to methyl esters and their identification by gas chromatography were carried out as described above.

The second corollary study determined the fatty acid composition of total lipids in rumen contents from a lactating goat, Jersey female yearling, and a Holstein steer yearling. The animals had been fed hay ad lib. and a concentrate mixture at 7 AM and 4 PM daily. Rumen content aliquots were removed through a fistula at 5-hr intervals from 8 AM to 12 MIDNIGHT. Infrared analysis of the fat, extracted as described above, indicated mixed lipids and nonesterified fatty acids, with some hydroxyl banding in each case.

## RESULTS

Lipid class representation in whole serum and blood cells was similar to that observed in cow, goat, and human blood (3, 4, 19) (Table 1). HDL contained 64% of the serum total lipids, as contrasted with 94 and 51%, respectively, in the cow and goat (3, 4). Weights of total lipids and lipid classes of the various blood components were very similar to those reported in the goat (4), with the one exception that bison HDL carry a greater absolute amount of phospholipid and, consequently, a heavier total lipid load. Protein-bound nonesterified fatty acids were present in trace amounts of less than 1%.

Liver tissue lipids were predominantly triglyceride and phospholipid. Goat and mouse studies have shown a similar distribution (4, 18).

Rumen contents and abomasal fluid, extracted immediately after collection, showed a small but significant proportion of monoglycerides. The absence of monoglycerides reported in sheep rumen contents (6), as well as the higher percentage (50%) of nonesterified fatty acids recovered (5), may be due to differences in

TABLE 1

Gravimetric analysis[a] of the lipid classes[b] from serum lipoproteins and other lipids in the bison

| Lipid class | Whole serum | | Serum lipoproteins D > 1.063 | | Serum lipoproteins D < 1.063 | | Blood cells | | Liver tissue[c] | | Rumen contents[c] | Abomasal fluid[c] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | (wt) | (%) | (wt) | (%) | (wt) | (%) | (wt) | (%) | (wt) | (%) | (%) | (%) |
| | | | | | Bison 1 | | | | | | | |
| HC | 5.0 | 3 | 3.2 | 3 | 3.3 | 5 | 8.4 | 3 | | | | |
| SE | 68 | 45 | 47 | 48 | 25 | 40 | 12 | 5 | | | | |
| TG | 27 | 18 | 13 | 13 | 13 | 21 | 10 | 4 | | | | |
| NEFA | 3.6 | 2 | 3.1 | 3 | 0.1 | 0.2 | 2.4 | 1 | | | | |
| S | 12 | 8 | 7.8 | 8 | 8.1 | 13 | 77 | 31 | | | | |
| DG | 2.3 | 2 | 1.8 | 2 | 1.1 | 2 | 6.9 | 3 | | | | |
| MG | 2.4 | 2 | 1.6 | 1 | 1.8 | 3 | 4.0 | 2 | | | | |
| PL | 30 | 20 | 21 | 22 | 10 | 16 | 127 | 51 | | | | |
| Total | 150 | | 99 | | 62 | | 248 | | | | | |
| | | | | | Bison 2 | | | | | | | |
| HC | 2.6 | 2 | 3.8 | 4 | 1 | 2 | 3.2 | 1 | 13 | 0.3 | 2 | 3 |
| SE | 62 | 50 | 59 | 57 | 19 | 38 | 6.3 | 3 | 85 | 2 | 6 | 4 |
| TG | 21 | 17 | 11 | 11 | 15 | 29 | 6.0 | 3 | 2,726 | 53 | 16 | 16 |
| NEFA | 3.7 | 3 | 2.7 | 3 | 0.6 | 1 | .... | .. | 109 | 2 | 33 | 12 |
| S | 9.3 | 8 | 8.4 | 8 | 3.3 | 7 | 69 | 30 | 202 | 4 | 17 | 13 |
| DG | 3.0 | 2 | 2.2 | 2 | 1.2 | 2 | 0.6 | 0.3 | 62 | 1 | .... | .... |
| MG | 2.9 | 2 | 1.8 | 2 | 1.7 | 3 | 1.9 | 0.8 | 41 | 0.8 | 4 | 2 |
| PL | 20 | 16 | 14 | 14 | 8.9 | 18 | 142 | 62 | 1,909 | 37 | 22 | 50 |
| Total | 125 | | 103 | | 51 | | 229 | | 5,147 | | | |

[a] Expressed as milligrams per 100 ml of serum or packed blood cells, milligrams per 100 g wet liver tissue, and per cent of rumen and abomasal lipids (391 and 327 mg/100 ml, respectively).

[b] HC = hydrocarbon, SE = sterol ester, TG = triglyceride, NEFA = nonesterified fatty acid, S = sterol, DG = diglyceride, MG = monoglyceride, PL = phospholipid.

[c] Samples of liver, rumen contents, and abomasal fluid were obtained only from the second test animal.

species, time interval between sample collection and extraction, extraction procedures, or handling of Hirsch and Ahrens columns.

Although well-represented in rumen content lipids, phospholipids were especially prominent in abomasal fluid. On the other hand, total lipids and nonesterified fatty acids showed abomasal decreases. Absorption of tripalmitin and palmitic acid by the isolated goat rumen has been reported by McCarthy (16).

To expedite intra- and inter-species comparisons, the fatty acid composition of total lipids from various parts of the bison is presented in Table 2. Similarities in fatty acid patterning were seen between whole serum and liver tissue, and between HDL and serum protein. The latter pair was the least saturated. Oleate was the predominant unsaturated fatty acid throughout the total lipids. Similar oleate prominence has been observed in the goat (4). In the cow, however, linoleate has been reported to be the major blood unsaturate. The fatty acid composition of total lipids from bovine lipoproteins, serum, and blood cells differed considerably from that of the bison (3).

The preponderance of stearate present in rumen content total lipids was not manifested in blood components or liver tissue. Its presence in the rumen, however, appears to be independent of time after eating and of ruminant species (Table 3). Intramucosal resynthesis of lipids has been demonstrated along the alimentary tract of the rat (12). Dehydrogenation, especially to the extent of one double bond, has been demonstrated in human liver (23).

Abomasal fluid showed a comparative decrease in stearate and a proportional increase in palmitate (Table 2). The possibility of palmitic acid formation from stearic acid has been suggested by Garton et al. on the basis of in vitro incubations of sheep rumen contents with various triglycerides (6).

Whole serum, HDL, LDL, and blood cells were grossly similar with regard to the fatty acid composition of the lipid classes (Table 4). However, differences could be distinguished between whole serum and HDL, on the one hand, and LDL and blood cells on the other. In whole serum and HDL, the sterol esters were com-

TABLE 2

Fatty acid composition [a] of total lipids from various sources in the bison

| Fatty acid | Whole serum | Serum lipoproteins | | Blood cells | Serum [b] protein | Liver tissue | Rumen contents | Abomasal fluid |
|---|---|---|---|---|---|---|---|---|
| | | D > 1.063 | D < 1.063 | | | | | |
| 14:0 | 2 | 3 | 3 | .... | 3 | 3 | 2 | 2 |
| 16:0 | 37 | 35 | 46 | 17 | 35 | 31 | 22 | 38 |
| 16:1 | 1 | 2 | 2 | .... | .. | 2 | .... | .. |
| 18:0 | 24 | 15 | 27 | 39 | 11 | 23 | 68 | 48 |
| 18:1 | 22 | 24 | 18 | 40 | 23 | 32 | 6 | 10 |
| 18:2 | 9 | 13 | 3 | 3 | 18 | 7 | 3 | 2 |
| 18:3 | 5 | 9 | 3 | .... | 10 | 4 | .... | .... |

[a] Expressed as weight per cent of the total major fatty acids. Fatty acids present in concentration less than 1% have been omitted. Values represent the mean of two test animals except in the case of liver, rumen contents, and abomasal fluid. These were obtained only from the second bison.

[b] Ultracentrifugal protein residue is associated with a small amount of nonesterified fatty acid.

TABLE 3

Fatty acid composition [a] of total lipids from rumen fluid sampled over time

| Fatty acid | Lactating goat | | | | Jersey female yearling | | | | Holstein steer yearling | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 8 AM | 1 PM | 6 PM | 12 MIDNIGHT | 8 AM | 1 PM | 6 PM | 12 MIDNIGHT | 8 AM | 1 PM | 6 PM | 12 MIDNIGHT |
| 14:0 | 6 | 7 | 6 | 2 | 9 | 3 | 2 | 4 | 3 | 4 | 3 | 1 |
| 16:0 | 26 | 26 | 27 | 26 | 25 | 24 | 24 | 23 | 26 | 26 | 23 | 26 |
| 18:0 | 51 | 54 | 42 | 58 | 55 | 66 | 55 | 62 | 67 | 61 | 60 | 63 |
| 18:1 | 8 | 10 | 16 | 11 | 6 | 4 | 10 | 9 | 4 | 9 | 12 | 8 |
| 18:2 | 9 | 3 | 9 | 3 | 5 | 3 | 9 | 2 | .. | .. | 2 | 2 |

[a] Expressed as weight per cent of the total long-chain fatty acids. Fatty acids present in concentration less than 1% have been omitted.

TABLE 4

Fatty acid composition [a] of the lipid classes [b] from whole serum, serum lipoproteins, and blood cells in the bison

| Fatty acid | Bison 1 | | | | | | Bison 2 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | SE | TG | NEFA | DG | MG | PL | SE | TG | NEFA | DG | MG | PL |
| | | | | | | Whole serum | | | | | | |
| 14:0 | 1 | 2 | 1 | 3 | 3 | .... | 1 | 5 | 4 | 2 | 3 | .... |
| 16:0 | 17 | 49 | 39 | 44 | 31 | 23 | 16 | 44 | 40 | 38 | 39 | 21 |
| 16:1 | 6 | 4 | .... | 5 | 23 | .... | 6 | .... | .... | 4 | 5 | 2 |
| 18:0 | 21 | 11 | 13 | 17 | 18 | 40 | 19 | 10 | 11 | 19 | 21 | 39 |
| 18:1 | 20 | 28 | 39 | 25 | 20 | 25 | 23 | 29 | 36 | 29 | 27 | 27 |
| 18:2 | 25 | 5 | 7 | 5 | 4 | 9 | 28 | 10 | 8 | 8 | 5 | 9 |
| 18:3 | 10 | 1 | 1 | 1 | 1 | 3 | 7 | 2 | 1 | .... | .... | 2 |
| | | | | | | High-density lipoproteins (D > 1.063) | | | | | | |
| 14:0 | 1 | 4 | 2 | 2 | .... | .... | 1 | 6 | 2 | 3 | 3 | 1 |
| 16:0 | 18 | 48 | 33 | 42 | 39 | 23 | 17 | 48 | 30 | 47 | 49 | 28 |
| 16:1 | 5 | .... | .... | 2 | .... | .... | 3 | .... | .... | .... | .... | .... |
| 18:0 | 27 | 5 | 16 | 17 | 19 | 47 | 24 | 3 | 19 | 15 | 19 | 53 |
| 18:1 | 23 | 32 | 38 | 30 | 30 | 21 | 24 | 29 | 37 | 29 | 24 | 16 |
| 18:2 | 19 | 9 | 8 | 7 | 12 | 7 | 25 | 11 | 10 | 6 | 5 | 2 |
| 18:3 | 7 | 2 | 3 | .... | .... | 2 | 6 | 3 | 2 | .... | .... | .... |
| | | | | | | Low-density lipoproteins (D < 1.063) | | | | | | |
| 14:0 | 2 | 5 | 3 | 2 | 3 | 2 | 1 | 7 | 8 | 4 | 3 | .... |
| 16:0 | 18 | 43 | 39 | 41 | 33 | 24 | 16 | 39 | 42 | 34 | 30 | 16 |
| 16:1 | 8 | 4 | .... | 1 | 21 | 1 | 7 | 2 | .... | 4 | 6 | 2 |
| 18:0 | 12 | 25 | 14 | 36 | 24 | 38 | 19 | 26 | 11 | 36 | 39 | 34 |
| 18:1 | 19 | 19 | 36 | 13 | 15 | 24 | 20 | 20 | 32 | 20 | 22 | 28 |
| 18:2 | 30 | 4 | 8 | 7 | 4 | 9 | 28 | 6 | 7 | 2 | .... | 16 |
| 18:3 | 11 | .... | .... | .... | .... | 2 | 9 | .... | .... | .... | .... | 4 |
| | | | | | | Blood cells | | | | | | |
| 14:0 | 4 | 6 | 5 | 6 | 7 | .... | 2 | 3 | 6 | 5 | 4 | .... |
| 16:0 | 19 | 44 | 30 | 42 | 46 | 8 | 14 | 43 | 25 | 38 | 39 | 9 |
| 16:1 | 10 | .... | 6 | 3 | .... | 2 | 7 | 1 | 2 | 1 | 3 | 1 |
| 18:0 | 3 | 21 | 24 | 23 | 32 | 29 | 21 | 24 | 32 | 27 | 28 | 28 |
| 18:1 | 18 | 25 | 33 | 20 | 15 | 46 | 30 | 27 | 26 | 26 | 26 | 47 |
| 18:2 | 34 | 4 | 2 | 6 | .... | 12 | 16 | 2 | 9 | 3 | .... | 12 |
| 18:3 | 12 | .... | .... | .... | .... | 3 | 10 | .... | .... | .... | .... | 3 |

[a] Expressed as weight per cent of the total major fatty acids. Fatty acids present in concentration less than 1% have been omitted.

[b] SE = sterol ester, TG = triglyceride, NEFA = nonesterified fatty acid, DG = diglyceride, MG = monoglyceride, PL = phospholipid.

paratively more saturated, containing greater proportions of stearate and less linoleate. Glycerides were much less saturated, due primarily to lower stearate levels.

LDL monoglycerides contained an unusually large proportion of palmitoleate. Monoglycerides and phospholipids of the blood cells differed markedly from those of the other blood components in degree of saturation.

In the cow, HDL were exceedingly unsaturated due to the predominance of linoleate in the sterol esters (3). Phospholipid fatty acid distribution was similar to that of the bison, except that linoleate rather than oleate was the major unsaturate. The LDL lipid classes, except phospholipids, showed a great degree of saturation. Although triglycerides in both lipoprotein groups were essentially identical in fatty acid composition, the LDL triglycerides tended to be more saturated.

Lipid classes between HDL and LDL in the goat showed much similarity in fatty acid composition (4). Triglycerides, however, were considerably more saturated in the LDL. Oleate was the major unsaturated fatty acid. In the bison, cow, and goat, triglycerides of the LDL tended to be more saturated and contained more stearate than those of the HDL (3, 4).

In liver tissue and abomasal fluid, sterol esters were similar in fatty acid composition (Table 5). They showed a much greater degree of saturation than the sterol esters of the blood components, but were less saturated than those of rumen contents. Liver tissue glycerides resembled those of the HDL; phospholipid fatty acids were similar to those of whole serum, HDL, and LDL. Fatty acid patterning in all

TABLE 5

Fatty acid composition [a] of the lipid classes [b] from liver tissue, rumen contents, and abomasal fluid in the bison

| Fatty acid | Liver tissue | | | | | | Rumen contents | | | | | Abomasal fluid | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | SE | TG | NEFA | DG | MG | PL | SE | TG | NEFA | MG | PL | SE | TG | NEFA | MG | PL |
| 14:0 | 4 | 8 | 2 | 4 | 1 | 1 | 3 | 2 | .... | 10 | 7 | 2 | 3 | 2 | 8 | 2 |
| 16:0 | 28 | 33 | 27 | 40 | 36 | 16 | 41 | 22 | 15 | 42 | 45 | 25 | 46 | 19 | 62 | 42 |
| 16:1 | 4 | 5 | .... | 2 | 2 | 1 | .... | .... | .... | .... | .... | 3 | 1 | .... | .... | .... |
| 18:0 | 38 | 2 | .... | 17 | 12 | 49 | 43 | 47 | 85 | 12 | 13 | 28 | 35 | 66 | 14 | 33 |
| 18:1 | 21 | 48 | 71 | 31 | 25 | 23 | 9 | 3 | .... | 7 | 19 | 26 | 4 | 11 | 13 | 16 |
| 18:2 | 5 | 2 | .... | 5 | 18 | 7 | 4 | 22 | .... | 16 | 10 | 11 | 10 | 2 | 3 | 5 |
| 18:3 | .... | 2 | .... | 1 | 6 | 3 | .... | 4 | .... | 13 | 6 | 5 | 1 | .... | .... | 2 |

[a] Expressed as weight per cent of the total major fatty acids. Fatty acids present in concentration less than 1% have been ommitted.

[b] HC = hydrocarbon, SE = sterol ester, TG = triglyceride, NEFA = nonesterified fatty acid, S = sterol, DG = diglyceride, MG = monoglyceride, PL = phospholipid. Diglycerides were not observed in rumen contents or abomasal fluid.

of the liver lipid classes was consonant with that seen in the goat (4).

Sterol esters and nonesterified fatty acids of rumen contents showed the palmitate-stearate, high-saturation doublet demonstrated routinely in rumen content total lipids (Tables 3 and 5). Triglycerides, monoglycerides, and phospholipids, however, were comparatively unsaturated.

In abomasal fluid, a saturation shift evinced increased proportions of oleate and linoleate in sterol esters and nonesterified fatty acids, decreased linoleate in the other lipid classes. Fatty acid patterning of the phospholipids was similar to that of the LDL triglycerides. An alimentary source of bovine LDL has been suggested (1).

The milk fat from bison and Indian water buffalo was very similar in fatty acid composition (Table 6). Very little linoleate or linolenate, short-chain fatty acids, and the characteristic stearate:oleate ratio were in evidence. The low butyrate value in bison milk fat was thought due to loss during a lengthy storage period undergone by the original sample maintained in fat form. Aside from this butyrate value, the general fatty acid composition of both bison and water buffalo milk fat was very similar to that reported by other investigators for the cow, goat, sheep, and water buffalo (10, 22).

## DISCUSSION

Various rationales may be used in accounting for the inter-species similarity in ruminant milk fatty acid composition. As in most biological systems, the final explanation will probably be multidimensional. However, at present it may be fruitful to interpret lipid utilization in the mammary gland from two points of view.

One view sees the mammary gland as a functionally autonomous fat-synthesizing system. In it a genetically selective absorption combines with in situ alteration to determine (within wide limits) the composition of milk fat. This view has been recently emphasized by intracisternal infusion studies of the mammary gland. These showed in situ glycerol synthesis (14) and fatty acid esterification into milk lipid classes (13, 15, 20, 21). Two enzyme systems, one synthesizing long- and short-chain fatty acids, and the other long-chain fatty acids, have further been described in microsomal and supernatant mammary cell fractions (8). Such systems may explain the seeming discrepancy between infusion esterification and the naturally occurring lack of esterification of the short-chain fatty acids into milk cholesterol esters and phospholipids.

A second point of view emphasizes mammary

TABLE 6

Fatty acid composition [a] of milk fat from the bison and Indian water buffalo

| Fatty acid | Bison bison | Bubalus bubalus |
|---|---|---|
| 4:0 | 0.6 [b] | 2.1 |
| 6:0 | 1.6 | 3.0 |
| 8:0 | 1.6 | 1.3 |
| 10:0 | 3.6 | 2.3 |
| 12:0 | 3.9 | 3.3 |
| 14:0 | 10.5 | 13.2 |
| 16:0 | 35.0 | 36.7 |
| 16:1 | 1.1 | 0.9 |
| 18:0 | 13.6 | 13.2 |
| 18:1 | 26.6 | 21.9 |
| 18:2 | 1.1 | 1.0 |
| 18:3 | 0.8 | 1.1 |

[a] Expressed as weight per cent of the major fatty acids. Fatty acids present in concentrations of less than 0.5% have been omitted.

[b] Deep-freeze storage of the bison milk fat since 1960 may account for the low butyric acid value.

utilization of preformed milk fat precursors from the blood (11, 24, 25). It equates the long-chain fatty acid composition of ruminant milk fat with that of an extramammary lipid of low concentration, complex association, and rapid turnover. The present paper has attempted to empirically characterize such a hypothetical construct on the basis of intra- and inter-species lipid comparisons. Although no lipid component can be ruled out as a possible contributor to milk fat, certain ones in the bison showed a palmitate:oleate:stearate ratio closely resembling that of milk fat. These included the glycerides from whole serum and HDL; nonesterified fatty acids from whole serum, HDL, and LDL; and the diglycerides from liver tissue. However, fatty acid comparisons of lipids from the bison, Indian water buffalo, goat, and cow revealed inter-species similarities only in rumen content total lipids, liver tissue lipid classes, and milk fat. The major serum lipoproteins of the bison, cow, and goat appeared to be species-specific.

To account, then, for the similarity seen in ruminant milk fat composition, one may be led to a further delineation of the original bifurcated hypothesis. There may exist: 1) a preformed milk fat precursor in blood even smaller than that carried by the HDL, LDL, blood cells, and protein or 2) a dynamic lipid conversion capability in mammary tissue.

Because correlative studies can point out content similarities but only guess at process relationships, a more definitive evaluation of lipoprotein triglycerides and nonesterified fatty acids, as milk fat precursors, is at present being carried out via organ perfusion techniques.

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# REGULATION OF FEED INTAKE IN DAIRY COWS. I. CHANGE IN IMPORTANCE OF PHYSICAL AND PHYSIOLOGICAL FACTORS WITH INCREASING DIGESTIBILITY [1]

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SUMMARY

Voluntary dry feed intake and dry matter digestibility were determined in 114 trials with lactating dairy cows. The rations ranged between 52 and 80% in digestibility and cow weights ranged between 625 and 1,457 lb. In a multiple regression analysis digestibility (D), fecal dry matter per 1,000 lb body weight per day (F) and body weight (W) accounted for the variation in feed intake between 52 and 66% digestibility, provided the rations were mostly roughage. $R = 0.997$ ($P < 0.01$). The terms: $W^{0.99}$, $D^{1.53}$, and $F^{1.01}$ expressing these relationships were essentially those predicted empirically if capacity limited feed intake. With high-roughage rations between 67 and 80% dry matter digestibility, intake decreased with increasing digestibility after adjusting for body weight and productive energy. In this region, intake was directly related to the 0.73 power of the body weight at peak lactation.

It was concluded that physical and physiological factors regulating feed intake change in importance with increasing digestibility. At low digestibility they were: Body weight (reflecting roughage capacity), undigested residue per unit body weight per day (reflecting rate of passage), and dry matter digestibility. At higher digestibilities intake appeared to be dependent on metabolic size, production, and digestibility.

Restrictions on feed intake, whether they arise inherently from chemical and physical characteristics of the feed or the anatomy and physiology of the cow, represent the most obvious practical limitations on milk production. McCullough (20) and Huffman (14) have discussed the usually high correlation of milk production and nutrient intake in dairy cows.

A large body of data bearing on the factors leading to satiety in dairy cows has been reviewed by Fissmer (11). He concluded that no satisfactory measure for predicting feed intake had been developed. More recently, Blaxter (3) generalized that the amount of feed consumed, measured in terms of dry matter, increases with increasing concentration of net energy in the ration. He also concluded that criteria were not available which explained in practice the differences in voluntary feed intake of cows. Krüeger and Schulze (18) have developed a method for calculating the satiety units of feeding stuffs. Recent concepts of the regulation of voluntary feed intake in ruminants were reviewed and ably discussed by Balch and Campling (2).

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Adolph (1) stated that food intake is one of the best regulated animal functions. Fourteen different variables which affect the balance of energy utilization are listed by Kleiber (17). Mather (22) has noted seven other factors that affect the total roughage intake in dairy cattle. Recently, Dowden and Jacobson (10) have shown that injections of acetate and propionate drastically reduced feed intake. This number of variables obviously precludes precise measurement of each factor as it affects the regulation of feed intake in practice. However, the problems may be greatly lessened in the case of ruminants under many conditions. Crampton (9) showed the relationship between voluntary intake and digestible nutrient content. In a careful study of the regulation of feed intake in sheep, Blaxter et al. (4) found that, within the limits of the quality of forage used, the amount of feed taken in by sheep is determined by the capacity of their digestive tract, and physical factors; that is, digestibility of feed and rate of passage through the digestive tract, rather than physiological factors, regulate appetite. The digestibility of their forages ranged between 44.7 and 74.2% of the dry matter consumed. Earlier, Lehmann (19) had observed in Germany that feed intake was such that the amount of undigested organic matter was the same for all feeds used. Recently,

McCullough (21) has reported that dry matter digestibility was a highly significant factor influencing dry matter intake of direct-cut silage in dairy cows. Reid (29) has thoroughly reviewed the relationships among forage quality, digestible nutrients, and milk production.

As noted by Blaxter et al. (4), the positive relationship between feed intake and digestibility found in sheep and cattle, and the negative influence of undigested residue on eating capacity, are contrary to the situation found in several nonruminant species. In the nonruminant, physiological factors including rising heat load, relief of hypoglycemia, and tissue dehydration appear to effect satiety (5). Dilution of the diet with indigestible materials is of less importance (1, 13, 25). For example, Adolph (1) diluted the diets of rats with kaolin, indigestible cellulose, or water and observed that rats on diluted diets increased their total food intake to the point where the digestible nutrient intake remained constant for all groups.

It should not be assumed that the results of Blaxter et al. (4) apply to ruminants under all environmental conditions. Greenhalgh and Runcie (12) found no causative relationship between feed intake and digestibility. In addition, Kleiber (17) has used the data of Ittner et al. (15) obtained in the Central Valley of California to illustrate how the rising heat load that resulted from an increased calorigenic effect attributed to hay was closely related to a diminished digestible nutrient intake of steers.

In this study, a series of multiple regression analyses of digestibility data was used to quantify certain physical and physiological factors considered to determine directly or indirectly the voluntary feed intake of milking cows. In this way an effort was made to answer the question, Do the data embody the principle that appetite in the 114 milking-cow trials was primarily regulated by physical factors, or, if both physical and physiological factors regulated appetite then at what level of digestibility does the change from one to the other occur? It was postulated that if capacity were limiting feed intake, dry matter intake would be proportional to the body weight or, if physiological occurrences were limiting feed intake, dry matter intake would be proportional to the metabolic size in accordince with Kleiber's law (17).

## EXPERIMENTAL PROCEDURES

Apparent dry matter digestibility and nitrogen balances were determined in cows on various experimental regimes for collection periods of five or seven days. A minimum preliminary period of 14 days on the ration to be studied was allowed. However, in many cases the preliminary period was as long as 8 wk, since the digestion trials were done in conjunction with feeding trials.

Dry matter was determined by oven drying overnight at 100 C, except that of silage, determined by toluene distillation. The nitrogen content of the feed, feces, urine, and milk was determined, using the Kjeldahl procedure. In all cases fresh or refrigerated samples were used. Urine samples were preserved with hydrochloric acid or thymol. Milk fat was determined on one-day composite milk samples.

Feed was sampled for analysis daily when weighed into the feed mangers of individual cows. Refused feed was weighed daily and composited for sampling. The cows were weighed previous to the digestion trials.

Details of the methods used for collecting feces and urine, milking, and feeding in the stalls usually occupied by the cows in the milking barn have been published previously (7).

In the calculation of estimated productive energy from the chemical data and milk produced, gain or loss in body fat was disregarded for the five- or seven-day trial, since carbon balances were not available. The formulas of Overman and Gaines (24) were used to estimate the chemical energy content of milk in kilocalories. Productive energy of retained protein was estimated as grams of nitrogen retained $\times$ 6.25 $\times$ 5.65 kilocalories.

*High-roughage rations.* Results from several combined feeding and digestion trials carried out during the period 1956 through 1961 (6-8, 26, 27) were compiled and analyzed statistically. All rations containing mostly forage, or others in which the combined content of cellulose and hemicellulose exceeded 50% of the organic matter, were classified as high-roughage rations. Types of forages used were: Freshly chopped and ensiled legume grass, alfalfa hay, and alfalfa-timothy hay. The stage of forage maturity varied widely among the experiments, resulting in a wide range of digestion coefficients. Flaked soybean hulls were used with hay in one experiment and were considered roughage because of their high fiber content (6, 28). The level of grain concentrate fed ranged between 0 and 46% of the dry weight of the total ration and was composed of ground shelled corn, ground whole oats, or soybean oil meal, singly or in combination. The rations are listed in Table 1 along with summaries of the mean and ranges for dry matter digestibility, body weight, 4% FCM, nitrogen retained, per cent roughage in the ration, and daily feed intake.

TABLE 1

Means and ranges are shown of dry matter digestibility, body weight, production, nitrogen retention, and feed intake of dairy cows fed various rations

| Type of ration | No. of trials | Dry matter digested | Body weight | 4% FCM | Nitrogen retained | Roughage in ration [a] | Feed intake |
|---|---|---|---|---|---|---|---|
| | | *(%)* | *(lb)* | *(lb/day)* | *(g/day)* | *(%)* | *(lb/day)* |
| Chopped legume-grass forage | | 68 | 859 | 38 | 48 | | 27 |
| 1. With grain | 34 | (59, 76) [b] | (659, 1,105) | (29, 55) | (−25, 97) | 77 | (23, 34) |
| | | 62 | 842 | 29 | 25 | | 22 |
| 2. Without grain | 14 | (55, 69) | (702, 930) | (19, 38) | (−20, 68) | 100 | (18, 25) |
| Ensiled legume-grass forage | | 62 | 898 | 29 | 11 | | 23 |
| 1. With grain | 36 | (52, 72) | (698, 1,272) | (15, 55) | (−25. 90) | 75 | (16, 34) |
| | | 55 | 732 | 23 | −2 | | 17 |
| 2. Without grain | 6 | (53, 58) | (661, 808) | (16, 31) | (−31, 27) | 100 | (15, 21) |
| Timothy hay and grain | | 67 | 926 | 32 | 7 | | 28 |
| | 12 | (62, 73) | (748, 1,451) | (20, 49) | (−49, 46) | 70 | (23, 42) |
| Flaked soybean hulls with limited hay | | 76 | 1,147 | 42 | 64 | | 33 |
| | 12 | (73, 80) | (982, 1,457) | (18, 64) | ( 3,128) | 81 | (27, 41) |
| Alfalfa hay and corn silage | | 69 | 957 | 47 | 31 | | 29 |
| | 20 | (65, 72) | (625, 1,354) | (30, 71) | (−30, 67) | [c] | (19, 43) |

[a] The per cent of total ration dry matter furnished as roughage; the remainder was grain-concentrate.

[b] Range shown in parentheses.

[c] Not calculated because the corn silage contained an undetermined amount of grain.

The daily ration was provided in two feedings of equal size. Maximum voluntary intake was further encouraged by feeding 5 to 15% more than the daily measured roughage intake.

Conditions established for including data in this analysis were: (a) That dry matter coefficients of digestibility should range between 50 and 80; (b) that both Holstein and Jersey cows would be included to provide a wide range in body weight; (c) that the cows were allowed to eat freely to satisfy their appetites at all times during the digestion trials and, (d) that two consecutive trials on the same type of ration were available. If more than two consecutive digestion and feed intake trials had been completed on the same ration, the pair of trials selected was chosen by employing Snedecor's (30) table of random numbers.

A series of coefficients of correlation and multiple correlation was calculated for the measured predicting variables, for subsequent usage as a measure of the closeness of fit of the various regression planes. All possible combinations of the predicting variables were inserted stepwise in the multiple linear regression analysis. From this, the proportion of total variation in dry matter intake attributable to regression was calculated as $R^2$ to obtain the most meaningful combinations. Measured variables used were: Per cent dry matter digested (D), body weight (W), the sum of estimated chemical energy in milk and retained protein ($E_{m+p}$), per cent roughage by weight in the ration (R), and the order of trials within animal pairs (T). Daily dry matter intake (I) was considered subsequent to these variables and, therefore, dependent upon them. The individual cow data were treated statistically as a population without regard to original experimental groupings. Common logarithms of all values were employed to obtain linear functions between dry matter digestibility or body weight and dry matter intake.

The variables for which the best fit of the regression was obtained were entered in a least-square multiple regression analysis to obtain prediction equations and partial regression coefficients. The model used was that described for fixed variables in a multivariate population (30). The model for fixed variables was considered realistic here. While there is some randomness in the variables, the cows were chosen to provide a particular range in lactation (one to six months) and body weight, and the rations used were selected for differences in digestibility.

The relationship between digested dry matter intake (DI) and body weight was examined separately at peak lactation and for all stages of lactation for cows eating rations above 67% in dry matter digestibility. As part of this analysis, data from single trials of all cows, 24 in number, in the second and third month of lactation were used for regressing log DI on log W.

*Further studies at peak lactation.* The ration used in this experiment consisted of: Alfalfa hay ad libitum, 16 to 30 lb of corn silage, and from 10 to 16 lb of grain concentrate containing 16% protein. Twenty cows were started on this ration three days after calving. Seven-day digestion trials were carried out during the period between the 30th and 60th days of their lactation. The relationships between dry matter intake and body weight, productive energy and digestibility were examined by carrying out a multiple regression of log dry matter intake (I) on log W, log $E_{m+p}$, and log D. Finally, the relationship between digested dry matter intake and body weight was obtained in a separate regression analysis of log DI on log W.

## RESULTS AND DISCUSSION

The results relating dry matter digestibility to feed intake disregarding other variables are shown in the scattergram, Figure 1. Generally

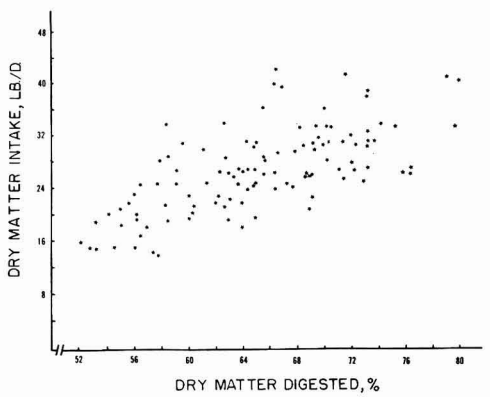


FIG. 1. Showing variations observed in dry matter intake of cows fed rations varying in digestibility from 52 to 80%. Linear regression equation was: $Y = 0.67 \times -17$; the standard deviation equals $\pm$ 5.8 lb where X = per cent dry matter digested and Y = dry matter intake in pounds.

speaking, there was an increase in dry matter intake with increasing percentage of dry matter digested. Although the linear regression was found to be statistically significant, the results, as shown in Figure 1, provide no new physiological information. They confirm previous observations that dry matter intake increases with increasing concentration of digestible nutrients in the feed of ruminants (3, 4).

*Multiple regression analysis of results with high-roughage rations.* Factors included as predicting variables were order of trials within pairs, per cent of dry matter digested, body weight, per cent roughage in the ration, and the productive energy in milk and retained protein. A part of the list of variables correlated singly and in all possible combinations with feed intake is shown in Table 2, opposite the percentage of total variation in feed intake attributable to regression. The correlation coefficient for the variable, order of trials within pairs, was numerically trivial and statistically insignificant.

TABLE 2

Fractions of total variation in dry matter intake attributable to regression when predicting variables were entered stepwise in a multiple linear regression (11 of 127 possible combinations)

| Variables entered | Variation attributable to regression |
|---|---|
| | *(%)* |
| Order of trials *(T)* | 0.14 |
| Per cent roughage in ration *(R)* | 11.1 |
| Per cent dry matter digested *(D)* | 44.3 |
| Body weight *(W)* | 31.8 |
| Productive energy ($E_{m+p}$) | 57.5 |
| D + R | 48.1 |
| D + W | 58.0 |
| D + $E_{m+p}$ | 62.8 |
| D + W + R | 58.0 |
| D + W + $E_{m+p}$ | 69.7 |
| D + W + $E_{m+p}$ + R | 69.7 |

Although log feed intake regressed on log per cent roughage in the ration resulted in a significant correlation coefficient, per cent roughage became obscured as a meaningful predicting variable when other variables, dry matter digested in particular, were entered stepwise in the multiple linear regression, Table 2. A corollary to this is that within the limits of these results (0 to 46% grain in the ration), most of the effects of changes in the proportion of grain-concentrate in the ration on feed intake of dairy cows was elicited through a change in dry matter digestibility. This is not to say that the proportion of grain-concentrate failed to affect the efficiency of animal energy utilization.

The best fit of the regression was obtained when the predicting variables W, $E_{m+p}$, and D were used as measured by a significant increase in the variation attributable to regression over that when any two of these variables are entered, Table 2. Highly significant partial regression coefficients were observed for log feed intake regressed on log body weight, log $E_{m+p}$, and log dry matter digestibility, Table 3. The exponential expression for the multiple linear regression equation was $I = 0.046\ W^{0.37}\ D^{0.75}\ E^{0.32}{}_{m+p}$. The exponent, 0.37, relating body weight to dry matter intake, was significantly

TABLE 3

Values of partial regression coefficients for log dry matter intake regressed on log of dry matter digested, body weight, per cent roughage in ration, and estimated productive energy, $E_{m+p}$

| Variable | Partial regression coefficients | Significance of linear regression |
|---|---|---|
| Per cent dry matter digested | 0.751 | $P < 0.01$ |
| Body weight | 0.369 | $P < 0.01$ |
| Per cent roughage | −0.011 | N.S. |
| $E_{m+p}$ | 0.316 | $P < 0.01$ |

different from the generally accepted exponent 0.73 (4, 17), or 1.00 if it were a function of capacity of the digestive tract. This suggests the failure of the variables to predict clear-cut relationships of physiological occurrences, even though statistically significant values were obtained, and the need for more critical mathematical evaluation. In particular, highly significant correlations between some predicting variables limited the value of the model used. For example, the following correlation coefficients were obtained for: D × $E_{m+p}$, r = 0.345; D × W, r = 0.042; and W × $E_{m+p}$, r = 0.422.

The unsuitability of treating the total group of data as a single sample of linear partial regressions was further demonstrated by graphing the three-dimensional projection of dry matter intake versus per cent digestibility and $E_{m+p}$ while holding body weight constant at 1,400 lb. The projection is shown from the maximum view in Figure 2 and covers only that area bounded by the limits of the data. The strong correlation between $E_{m+p}$ and I may be observed by comparing the surface of the projection with the side view. Digestible dry matter intake tended to level out with increasing digestibility, with a point of inflection at about 66% dry matter digested.

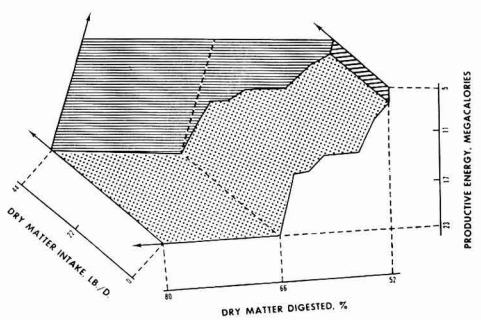


FIG. 2. Three dimensional projection relating per cent dry matter digested, dry matter intake, and estimated energy in milk and retained protein of dairy cows. The drawing shows only the maximal view for a constant body weight of 1,400 lb.

*Digestible dry matter intake adjusted for differences in metabolic size and productive energy.* The tendency for per cent dry matter digested to decrease in importance as a variable limiting dry matter intake at the higher percentages suggested that the quantity of digested dry matter intake was limited by physiological conditions other than the amount of fill in the digestive tract. Kleiber (17) had concluded that digestible energy intake was proportional to metabolic size in cows of similar genetic capacity to produce milk. In a second multiple regression analysis, therefore, per cent dry matter digested, metabolic size, and $E_{m+p}$ were used as predicting variables. The graph of the digestible dry matter intake versus per cent digestibility after adjusting the cow means for differences in metabolic size and productive energy indicated that the average adjusted digestible dry matter intake leveled out and approached a straight line between 66 and 80% digestibility, Figure 3. Also, the adjusted mean

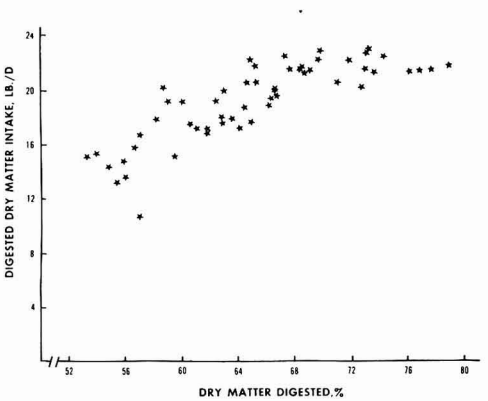


FIG. 3. Showing diffferences in daily digestible dry matter intake of cows fed rations ranging in digestibility from 52 to 80% after adjusting by analysis of covariance for variations in metabolic size ($W^{0.73}$) and the estimated energy content of milk produced plus retained protein.

dry matter intakes for coefficients of digestibility above 66 were significantly different from those below 66.

It was tentatively postulated that, up to approximately 66% dry matter digested, the rate of passage from and the amount of undigested materials in the digestive tract determined feed intake as Blaxter et al. (4) showed. Above 66%, the results coincided with observations

made on nonruminant species (1). This was considered justification for arbitrarily regrouping the data into a low-digestibility group with a range in digestion coefficients from 52.1 to 66.7 and a high-digestibility group with coefficients between 66.7 and 80.

*Factors determining voluntary dry matter intake of milking cows fed rations between 52.1 and 66.7% dry matter digestibility.* The wide variations remaining in digestible dry matter intake may be seen in Figure 3. Blaxter et al. (4) found that the feed intake of sheep was regulated by the rate of passage of indigestible residue through digestive tracts of limited capacity. The best measure of the rate of passage of undigested residue made in this experiment was the 24-hr fecal dry matter excretions. To equate for differences in body size, daily fecal dry matter output must be related to some function of body weight. In this case, division by body weight itself seemed appropriate, since it was assumed that the digestive tract filled to maximum volume represented a constant proportion of each cow's weight, and volume and weight are related by the proportionality constant, one. Thus, in cases where digestive capacity is limiting and per cent dry matter digested is a measured predicting variable, the remaining restrictions on feed intake were postulated to be undigested residue per unit body weight per day and body size itself. The undigested residue in this case is analogous to Lehmann's (19) ballast. Deviations in this variable should occur when, for any reason, the rate of passage increases or decreases or variations in anatomical structure cause the capacity for undigested residue to be larger or smaller than predicted by a simple volume-weight relationship. In our sample of 66 cow-digestion trials these were expected to occur at random.

The regression coefficients resulting from regressing log of dry matter intakes on log per cent digestibility, body weight, and fecal dry matter per 1,000 lb are shown in Table 4, along with the logarithmic equations and multiple correlation coefficients. These three variables accounted for essentially all of the variation in dry matter intake. This finding strongly supports the contention that within this range of digestibility and types of rations fed, physical factors determined appetite.

The average daily production of fecal dry matter was 10.7 lb. After subtracting fecal ash, undigested organic matter was 9.4 lb/1,000 lb of body weight, which compares favorably to 9.5 lb calculated from the data of Lehmann (19) for cattle. Moreover, the exponents relating body weight and undigested residue to feed intake were 0.99 for body weight and 1.01 for fecal dry matter, Table 4. Neither of these is significantly different from one.

TABLE 4

Multiple regression analysis showed that three predicting variables covered most of the variation in feed intake among dairy cows digesting 52 to 66% of their dry matter intake

| Variable | Partial regression coefficient | Standard partial regression coefficient |
|---|---|---|
| Digestibility *(D)* | 1.53 | 4.41 |
| Fecal dry matter/1,000 lb *(F)* | 1.01 | 7.10 |
| Body weight *(W)* | 0.99 | 5.57 |
| Multiple correlation coefficient, $R = 0.997$; $R^2 = 0.995$ | | |
| Regression equation: $\text{Log } I = 1.53 \log D + 1.01 \log F + 0.99 \log W - 5.296$ | | |

The logarithmic curve resulting from plotting total dry matter intake versus per cent digestibility, while holding body weight and undigested residue constant at 1,000 and 10.7 lb, respectively, is shown in Figure 4. The extension (broken-line) of this curve represents mathematical extrapolation to 80% dry matter digested. The adjusted data fall essentially on the empirical curve obtained from solving the formula, $\text{Intake} = \frac{\text{undigested dry matter}}{\text{per cent undigested}}$. Thus, if dry matter digestibility and live weight are

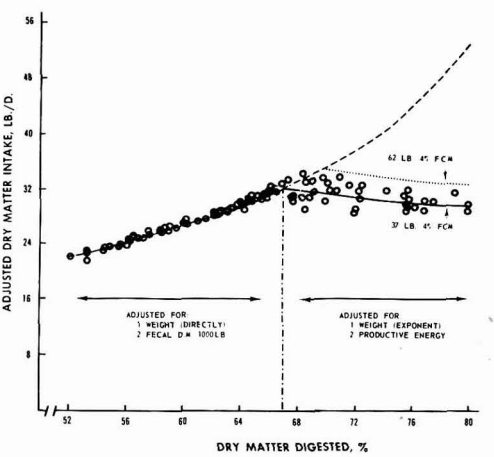


FIG. 4. Showing the relationship of dry matter digestibility to adjusted feed intake at two levels of milk production. Adjustments were proportional to weight below 66.7% dry matter digested and weight raised to the 0.51 power above 66.7% digested.

known [or, in the case of the former, if it can be estimated by in vitro digestion procedures (16)], the average feed intake for a herd of milking cows may be predicted with considerable precision in the low range of digestibility. The equation is $I = \frac{W}{1{,}000} \cdot \frac{10.7}{F}$ where I is the intake in pounds per day, W equals body weight, and F equals per cent undigested. The regression equation has less physiological meaning. The exponential form was $I = 0.54WD^{1.63} \times 10^{-6}$ where I = digestible dry matter intake in lb/day, W = weight in pounds, and D is the dry matter digestion coefficient. Finally, the remainder of the variation among individuals affecting feed intake may be differences in rate of passage of undigested residue. Comparisons of the standard partial regression coefficients showed this variable to be greatest in importance. Its contribution approximates 42% of the total variation. This is commensurate with results of Moore and Winter (23), showing wide variations among individuals with respect to rate of passage of undigested residue and Stone et al. (31), who found that differences among individuals contributed slightly more than half of the variation in roughage capacity of dairy cows.

The arbitrarily selected digestion coefficient, 66.7, should not be construed as a fixed maximum point where physical factors cease to limit feed intake. As discussed in the following section, this is a variable point with respect to per cent digestibility influenced by the metabolic size and level of milk production.

*Factors related to the voluntary dry matter intake of milking cows fed rations between 66.7 and 80% digestibility.* Results in this range of digestibility were obtained with cows fed rations high in flaked soybean hull content or early-cut green-chopped or ensiled forage. Counting the flaked soybean hulls as a roughage on the assumption that they require ruminal digestion to be converted to metabolically useful compounds, the per cent grain-concentrate in these rations ranged from 0 to 42%. The highest digestion coefficients were obtained when cows were fed soybran flakes, Table 1.

In contrast to the highly significant regression of feed intake on rate of passage of fecal dry matter/1,000 lb of body weight/24 hr, that was found with values from cows with digestion coefficients below 66.7%, with higher digestibilities the regression coefficient was negative and statistically insignificant. It was previously noted that the average digestible dry matter intake remained constant and was presumably independent of per cent dry matter digested when the coefficient exceeded 66%. It was, therefore, concluded that within the limits of the data from the high digestibility group of cows, volume of their digestive tract was not restricting the quantity of feed eaten. An alternative postulate was that animal energy utilization (for production and maintenance) encompassed most of the variables determining appetite. Also, at the higher digestibilities, digestible nutrient intake followed a pattern predictable from Kleiber's (17) law with respect to metabolic size and productive energy.

The relationship between dry matter intake and body weight was determined by carrying out separate regression analysis of log I on log W, first for cows at all stages of lactation and then for cows only in the second and third month of lactation. The exponents relating feed intake to body weight for cows in peak lactation were the same as the generally accepted value of 0.73. The data for all stages of lactation yielded a lower value, 0.593, which was expected, Table 5.

Because a much greater part of the total variation in feed intake could be accounted for, a multiple regression was calculated for log dry matter intake on log dry matter digestibility, productive energy, and body weight to obtain prediction equations, Table 5. The exponential form of the equation for the cows on the high-roughage rations was $I = 3.4\ W^{0.61}\ E^{0.25}{}_{m+p}\ D^{-0.16}$ and for the cows on corn silage and hay at peak lactation, $I = 30\ W^{0.62}\ E^{0.27}{}_{m+p}\ D^{-1.19}$. After adjusting for productive energy and body size by covariance, the relationship of total dry matter intake to digestibility is shown in the right side of Figure 4 for cows on the high-roughage rations adjusted to two levels of milk production. The mean adjusted dry matter intake declined more sharply in the cows fed the higher grain ration; that is, corn silage with hay as indicated by a larger negative exponent for D, –1.19 compared to –0.46, in the regression equation, Table 5. Because digestibility is inversely proportional to total dry matter intake, the value obtained from empirical calculations, that is, digestibility raised to $-1.00$ power, is intermediate between the results observed for the high-roughage and the higher grain ration. The empirical calculation assumes a constant level of digestible nutrient intake.

Using the 114 digestion trials of the cows eating high-roughage rations, a highly significant correlation coefficient, $r = 0.811$, was obtained between digestible dry matter intake and 4% FCM. This corroborates the findings

TABLE 5

Relationship between digested dry matter intake and body weight in separate regression analyses and between total dry matter intake and per cent dry matter digested, body weight, and productive energy in multiple regression analyses of observations from cows digesting 67 to 80% of their dry matter

| Variables in regression | Regression coefficient | Fit of regression, $R^2$ |
|---|---|---|
| I. Log digested d.m. on log weight | | |
| a. High roughage—peak lactation (24)[a] | 0.735 ± 0.144 | 0.060 |
| b. High grain—peak lactation (20) | 0.733 ± 0.109 | 0.074 |
| c. High roughage—throughout lactation (42) | 0.593 ± 0.071 | 0.063 |
| II. Multiple regression analysis with high-roughage rations (42) | | |
| a. Log dry matter intake on: | | 0.833 |
| 1. Per cent d.m. digested | −0.461 [b] | |
| 2. Body weight | 0.513 [b] | |
| 3. Productive energy, $E_{m+p}$ | 0.251 [b] | |
| Regression equation: Log I = 0.5536 − 0.461 log D + 0.513 log W + 0.251 log $E_{m+p}$ | | |
| III. Multiple regression analysis with corn silage and high grain (20) | | |
| a. Log dry matter intake on: | | 0.809 |
| 1. Per cent d.m. digested | −1.194 [b] | |
| 2. Body weight | 0.620 [b] | |
| 3. Productive energy, $E_{m+p}$ | 0.268 [b] | |
| Regression equation: Log I = 1.484 − 1.194 log D + 0.620 log W + 0.268 log $E_{m+p}$ | | |

[a] Number in parentheses indicates numbers of cows.

[b] Partial regression coefficients.

of previous investigators (20). However, the preceding analyses evinced that at low digestibilities variations in milk yield arose from variations in feed intake. At high levels of digestibility the reverse condition was true; that is, milk production was a determinant of feed intake. The point at which this change in level of feed intake occurred for two levels of milk production is shown in Figure 4 as the intercept of two logarithmic curves in the high digestibility range with the result from the low-digestibility group. Thus, the point among this series of increasing digestion coefficients at which physical limitations on eating capacity vanished and the influence of production became dominant varied with body size and production.

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# EFFECT OF SUPPLEMENTAL FEEDING OF COWS ON PASTURE ON MILK COMPOSITION AND YIELD

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SUMMARY

Thirty-five cows were used to study the effects on milk composition and production of supplementing medium-quality pasture with ground-shelled corn or corn silage. Rations were: 1) Corn; 2) corn plus silage; 3) corn (1 lb/6 lb milk) plus silage; and 4) silage. Except for corn in Ration 3, feeds were fed ad libitum for 3 hr twice daily. Trial A compared Rations 1 and 4 and employed a reversal design with 40-day treatments. Trial B was a continuous study with 12-wk treatments.

In Trial A, significant ration effects were noted for SNF, protein and fat in milk, and milk yields. Values for cows on corn and silage, respectively, were: 8.65, 3.22, 3.05%, and 39.9 lb; 8.44, 3.11, 3.65%, and 35.8 lb.

In Trial B, persistency of production was over 80% for all groups. Cows on grain and silage had highest milk yields. SNF decreased as level of corn decreased (9.16, 8.92, 8.66, and 8.42% for consecutive groups). Milk protein accounted for most of the change in SNF. Fat in milk was lower on high-corn rations (2.53, 3.23, 3.50, and 3.59% for consecutive groups). Corn silage alleviated milk fat depression noted on high-corn rations. Treatment effects on milk composition were not complete until the 6th to 8th wk of treatment.

Feeding high levels of energy to dairy cows has generally resulted in an increase in SNF in milk (principally the protein fraction) when compared with rations of normal or subnormal energy content (3, 6, 7, 10, 11, 14, 16). However, some studies have reported no difference in SNF and protein in milk from rations which varied widely in energy (2, 4). Concurrent with the increase in milk protein, there has often been noted a decrease in the fat content of the milk (3, 16). Several investigations have shown an increase in protein from feeding high levels of energy, but no change in fat (6, 11, 14). These changes in milk composition (increased protein and decreased fat) have usually been more pronounced when forage intake was very low (3, 9, 11).

In many areas, pastures supply a large portion of the forage dairy cows consume. However, very little is known on the effect of energy supplementation to cows on pasture on the major constituents of milk. Such knowledge would seem important, particularly because of the problem of low-fat milk frequently reported during the grazing season.

The purpose of this study was to investigate the effect upon milk composition and yields of varying the level and source of energy in the ration of lactating dairy cows grazing medium-quality bluegrass pasture. Of further interest was the time required for the changes in milk composition induced by ration to occur.

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## EXPERIMENTAL PROCEDURE

The study was divided into two trials. In Trial A (summer, 1961), 11 lactating Holstein cows in their fifth month of lactation were allotted to two groups (five and six per group). A simple reversal-type of design was used with two 40-day treatment periods. During the first period (6/22 through 7/31), one group of cows received all of the ground-shelled corn they would eat for 3 hr twice daily (2 hr prior to, and 1 hr during each milking), whereas the other group was allowed high-quality corn silage (31.1% dry matter with a grain content of about 50%) on the same basis. Treatments were switched during the second period (8/1 through 9/9). Cows were weighed for three consecutive days just prior to and at the end of treatment periods. At 12-day intervals, a daily composite of milk was sampled for composition analyses. Fat was determined by the Babcock method. A modified AOAC method (1) as adapted by Stone et al. (18) was used for determination of total solids, and total protein was analyzed according to the Kofranyi Direct Steam Distillation Method (12), as modified by Stone et al. (18). Per cent solids-not-fat and lactose-mineral were determined by

difference. Milk production and composition data are presented for the last 30 days of each treatment period (allowing ten days adjustment).

In Trial B (summer, 1962) 24 lactating Holstein cows in their third to fifth month of lactation were used. Cows were allotted to six outcome groups (four per group) on the basis of age, stage of lactation, and production during a 21-day standardization period. During standardization all cows were on pasture and received a daily allowance of 40 lb of corn silage. Additionally, a commercial concentrate (16% C.P.) was fed at the rate of 1 lb per 3.5 lb of milk produced.

One cow from each outcome group was then assigned at random to one of the following treatment groups: 1) Ground-shelled corn; 2) ground-shelled corn plus corn silage; 3) ground-shelled corn (1 lb per 6 lb milk produced during standardization) and corn silage; and 4) corn silage. All feeds were fed ad libitum for 3 hr twice daily (2 hr prior to, and 1 hr during milking) except for the grain in Group 3. Additionally, all cows were fed 2 lb soybean meal (44% crude protein) per day.

Pasture quality and grazing schedules were similar to Trial A. Treatment period was for 86 days (6/7 through 8/31) which included 15 days of adjustment to rations. Cows were weighed for three consecutive days at the beginning and end of treatment and at biweekly intervals during the trial. Daily composite milk samples were taken twice during the preliminary period and at ten-day intervals thereafter. Composition analyses of milk were similar to Trial A.

On the last day of treatment, a sample of the rumen contents from three cows in each of Groups 1 and 4 and from three additional cows consuming a normal ration of hay (ad libitum) and commercial concentrate (1 lb per 3.5 lb milk produced) was taken by stomach pump approximately 5 hr after the morning feeding. A 10-ml aliquot of the sample was immediately mixed with a 2% solution of $H_2SO_4$. Rumen samples were stored at 40 F until analyzed for relative proportions of volatile fatty acids, according to the method described by Bruno and Moore (5).

The method of Snedecor (17) was used for statistical analyses of data and significance of difference between treatment means was tested according to Duncan (8).

## RESULTS AND DISCUSSION

Pastures during both trials were of medium quality. Continuous grazing was employed and sufficient land was available to insure a liberal supply of forage during all experimental periods.

In Trial A, milk yields, SNF and its components (protein and lactose-mineral) were significantly higher for the ground corn ration (Table 1). Similar effects on milk composition have been noted in other studies where high levels of energy were fed (10, 11, 16). Concurrent with the increase in SNF, there was noted a significant decrease in fat content of milk. Milk fat depression has also been reported previously, particularly in rations containing large amounts of grain and very little forage (3, 9). No difference was noted in production of FCM or weight change of animals, despite the large differences in energy intake between the two rations. Because changes in milk composition among cows consuming high levels of grain were not complete after 40 days on treatment, a continuous-type study with a 12-wk treatment period was planned for the following year.

Supplemental TDN per pound of FCM, estimated according to Morrison (13), amounted to 0.60 and 0.30 lb for the grain and silage treatments, respectively. It is postulated that much of this difference was due to a lower intake of pasture by cows on high grain.

In Trial B, a normal decline in production was noted among all groups. Cows receiving both silage and grain maintained the highest milk yields (Table 2). The most efficient production, from a standpoint of supplemental

TABLE 1

Intake, milk production, weight gains, and milk composition of grazing cows fed supplemental corn or corn silage (Trial A)

| Suppl. feed | Intake | Milk | FCM | ADG | Fat | Prot. | L-M | SNF | TS |
|---|---|---|---|---|---|---|---|---|---|
| | *(lb/day)* | | | *(lb)* | *(%)* | | | | |
| Ground corn | 25.6 | 39.9** | 34.2 | 0 | 3.05 | 3.22* | 5.46* | 8.68** | 11.73 |
| Corn silage [a] | 46.4 | 35.8 | 33.7 | 0.2 | 3.65** | 3.11 | 5.34 | 8.45 | 12.07* |

[a] 31.1% dry matter.
* Significantly higher ($P < .05$).
** Significantly higher ($P < .01$).

TABLE 2

Effect of various supplemental feeding systems on intake, production, and body weight (Trial B)[a]

| | Group | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| Intake *(lb/day)* | | | | |
| Ground corn | 24.0 | 22.8 | 6.9 | |
| Corn silage [b] | | 24.1 | 46.1 | 54.1 |
| Soybean meal | 2.0 | 2.0 | 2.0 | 2.0 |
| Milk yields *(lb/day)* | | | | |
| Standardization | 44.5 | 43.7 | 43.9 | 43.7 |
| Treatment | 36.6 | 39.5 | 38.0 | 35.0 |
| % of Standardization [c] | 80.4ab | 90.4a | 86.6ab | 80.1b |
| Avg daily gain *(lb)* | 1.64a | 1.43a | 0.58b | 0.64b |

[a] Avg for 71-day period.
[b] 29.9% dry matter.
[b] Groups not sharing a common letter are significantly different ($P < .05$).

TDN, was again obtained from cows fed only corn silage. Supplemental TDN per pound of FCM for Groups 1 through 4, respectively, was 0.73, 0.70, 0.46, and 0.37 lb. Substantial increases in body weight were noted for all groups; however, animals on the high-grain rations gained at a much faster rate than those in groups where grain was either limited or excluded. The greater gains accounted for some of the additional energy consumed by these groups. It is again postulated that intake of pasture was lower for cows consuming liberal quantities of grain.

The effects of ration on milk composition in Trial B were similar, but of greater magnitude than those noted in Trial A (Table 3). Maximum depression of fat content of milk among cows receiving high levels of grain was shown only after 6 wk on treatment, and greatest differences in milk protein (between Groups 1 and 4) were shown after about 7 to 8 wk on treatment (Figure 1).

As has been noted previously (3), the sensitivity for dectecting small differences in milk production and composition was greater in the reversal-type study; however, the relatively long period of time required for the maximum change in milk composition to occur would seem to make a continuous-type trial more useful in studies where the effect of ration on milk constituents is of prime interest.

Cows in Group 2, which received both ground corn and corn silage ad libitum, consumed only slightly less corn than cows on corn alone (22.8 vs. 24.0 lb per day). The small amount of silage in this ration (24.1 lb) seemed to prevent partially the depression in the fat content of milk, as was noted among cows in Group 1.

Analyses for volatile fatty acids in rumen contents of cows in Group 1 revealed a much lower proportion of acetate and higher proportion of propionate than was shown for cows receiving only silage or a normal hay-grain ration (Table 4). Similar effects, but of lesser magnitude, were reported previously for lactating cows on high-concentrate, low-forage rations (3, 9). These data also tend to support those of English workers (15), in which it was shown that intraruminal infusion of propionic acid resulted in an increase in the per cent of

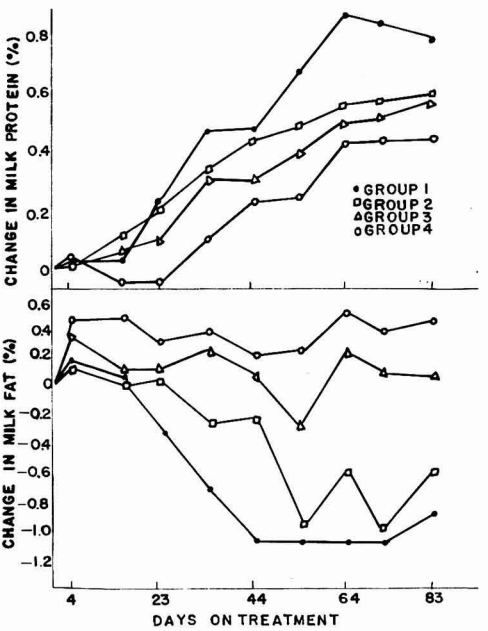


FIG. 1. Average change from standardization in milk protein and fat of cows on different systems of pasture supplementation (Trial B).

TABLE 3

Effect of various supplemental feeding systems on milk composition (Trial B)[a]

| | Group [b] | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| Fat | 2.53(−.82[c])a | 3.23(−.35)ab | 3.50(+.08)bc | 3.59(+.28)c |
| Protein | 3.45(+.57)a | 3.31(+.41)ab | 3.19(+.33)bc | 2.97(+.22)c |
| Lactose-mineral | 5.71(−.06)a | 5.61(−.09)a | 5.47(−.11)a | 5.45(−.27)a |
| SNF | 9.16(+.55)a | 8.92(+.35)ab | 8.66(+.22)bc | 8.42(0.0)c |
| Total solids | 11.69(−.29)a | 12.15(−.03)ab | 12.16(+.32)b | 12.01(+.23)b |

[a] Avg for 71-day period.
[c] Groups not sharing a common letter are significantly different ($P < .05$).
[c] Avg change from standardization period. Statistical treatment of all composition data is based on these values.

TABLE 4

Mean molar percentages of rumen volatile fatty acids (Trial B)

| Group [a] | $C_2$ | $C_3$ | $C_4$ | $C_5$ | $C_2$:$C_3$ |
|---|---|---|---|---|---|
| | *(% of total)* | | | | |
| 1 | 52.8a | 33.1a | 9.3a | 3.9a | 1.60a |
| 4 | 64.1b | 18.7b | 13.2a | 4.0a | 3.43b |
| Hay-grain | 65.5b | 20.4b | 10.4a | 3.7a | 3.21b |

[a] Groups not sharing a common letter are significantly different ($P < 0.1$).

protein and SNF in milk and a decrease in milk fat.

Further studies are needed to define more clearly the effects of various factors such as concentrate-to-forage ratios, sources of concentrates and forages, and energy levels, on the major milk constituents. The physiological mechanisms involved in such changes should also be further investigated.

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# INFLUENCE OF AGE OF RADIOACTIVE FALLOUT MATERIAL UPON ITS DEPOSITION IN MILK AND VERTICAL DISTRIBUTION IN SOIL

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SUMMARY

The delay period between the liberation of radiostrontium and its appearance in milk, soil, and grass was studied. Milk samples showed consistently higher S.U.$^{90}$ and Strontium$^{90}$ values in October than in August, 1961, and the presence of Strontium$^{89}$ was evidence of fresh fallout, proving that the delay period was less than two months. The soil samples taken from the site where the cows grazed showed no increase of Strontium$^{90}$ and no evidence of Strontium$^{89}$. In grass, however, there was a sharp increase of both isotopes from September to November, 1961, the simultaneous rise suggesting recent pollution. The effective delay period, therefore, for fallout to be incorporated into grass was less than two months. That there was no increase in either isotopes in the soil suggests that Strontium$^{89}$ and Strontium$^{90}$ entered the vegetation by direct routes.

There are numerous factors which may accelerate or slow down the entry of radioactive isotopes into the human organism. Knowledge of air currents, rainfall, seasonal effects, soil composition, folial uptake, and the rate of vertical movement within the soil are but a few of the factors necessary if we are to predict the future levels of fission products in plants, animals, and man.

In a previous report (8), milk samples from Jersey and Holstein cows were analyzed for Strontium$^{90}$ and calcium and strontium unit values calculated during the spring and summer of 1961. Results showed that the S.U. values ($\mu\mu$c Strontium$^{90}$ per gram of calcium) were significantly higher in the spring (April-May, 1961) than in the samples (July-August, 1961). This difference may have been due to a higher calcium intake during pasture feeding in the summer (9) or because during the spring months the cows were fed silage and hay grown in the summer of 1960, when the Strontium$^{90}$ fallout in Ontario was relatively higher than in 1961. The difference in the fallout rate between the spring and summer periods probably explained the higher levels of Strontium$^{90}$ in the spring milk samples. It was also shown that the S.U. values were somewhat higher in Holstein than in Jersey milk samples during both spring and summer seasons.

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This paper describes a further investigation using the same cows, fed and pastured under the same conditions. Milk samples were obtained 6 wk after the first announced resumption of an atmospheric nuclear detonation in October, 1961, approximately two months after the collection of summer samples from July 17 to August 18. The data obtained have been examined to seek evidence whether Strontium$^{89}$, Strontium$^{90}$, and S.U.$^{90}$ values of milk increased from the summer to the fall sampling periods, and whether an increase in the amount of atmospheric Strontium$^{89}$ and Strontium$^{90}$ was similarly reflected in the isotope content of the milk sample of the two breeds of cows.

The present investigation is also concerned with the Strontium$^{89}$, Strontium$^{90}$, and calcium levels of the grass of the pasture land where the experimental animals grazed, and the vertical distribution of these alkali-earth metals in soil, 4 and 12 wk after resumption of nuclear tests.

## MATERIALS AND METHODS

*Milk.* Four Jersey and four Holstein cows were selected for this study. The same cows were used in a previous investigation (8). No efforts were made to select the cows at random; hence, all statements in this paper should be interpreted with reference to the eight particular cows in this study. The Jersey cows ranged in production from approximately 8,000 to

11,000 lb of milk per lactation and the Holsteins ranged from 10,000 to 18,000 lb of milk per lactation. Milk was again collected in October, 1961, 6 wk after the resumption of nuclear testing, while the cows were still on the same pasture as in our previous study (8). All cows were more than two months past their freshening date at the time of the April collection and still in full production in October. This was important, because calcium and phosphate values of milk fall during the first 5 wk and rise again in the last 15 wk of lactation (9). The milk samples collected represented 35 milkings. Saturday and Sunday milkings were not sampled, due to scheduling problems at the barn. A composite sample of each cow's milk was prepared by accumulating 1% of each of her milkings throughout the sampling period. Two liters of milk were necessary for each analysis. Duplicate analyses were performed for each cow in the summer, but only for Holsteins in October because the 1% of the milk yield of the Jersey cows was insufficient for two samples during the period available.

The Strontium$^{90}$ content of the milk samples was determined by the method of Grummitt (7) and Brown et al. (5). Two methods were used to calculate the Strontium$^{89}$ activities. In the first method, the separated strontium was counted after a storage period of 17 days, to allow for the development of an equilibrium condition with the Yttrium$^{90}$ daughter product. Following this, Yttrium$^{90}$ was separated by the method of Grummitt et al. (7), and the Strontium$^{90}$ content was calculated from the Yttrium$^{90}$ count. The count due to Strontium$^{89}$ was calculated by subtracting Strontium$^{90}$ and Yttrium$^{90}$ activities from the equilibrium count. The second method depended on measuring the decay rate of the equilibrated strontium source three to four times at intervals of 6-8 wk. In both instances corrections were made to obtain the Strontium$^{89}$ activities at the time of collection. Calcium was analyzed using a Unicam SP-900 flame spectrophotometer.

*Grass and soil.* Strontium$^{89}$ and Strontium$^{90}$ determinations in soil and grass are lengthy and time-consuming procedures. For this reason, the number of sampling sites necessary was determined and the samples were pooled, as described below. All samples were obtained on September 15, 1961 (after the resumption of atmospheric testing) and again on November 26, 1961 (approximately 12 wk after the recommencement of atmospheric nuclear testing).

The pasture site was an area of level land, 533 yards in length and 525 yards in width. The pasture was ploughed and sown with a grass-clover-alfalfa mixture in 1960. The pasture soil was Guelph Loam, a well-drained soil derived from loam-textured till. This type of soil has good depth, is relatively stone-free, and is neutral in reaction. A random sample of 12 plots was selected; each of the sample plots measured one square yard and was selected in this way: The whole pasture site was first divided into 533 bands of one yard width and a random sample of four of these bands taken. Next, this same plot was divided into 525 bands of one yard width at right angles to the previous strip and three of these bands selected at random. The two sets of bands were overlapping in 12 plots of each of one yard square. The grass from each of these 12 plots was cut with a mower about 1 in. from the ground, to obtain the vegetation on which a cow would normally graze. A 6-in.-deep core of soil with a diameter of 2.5 in. was taken at random within each one-square-yard area, where the grass had been removed. The surface soil—the top 2 in. containing the roots of the vegetation—was removed and kept separately, and the remaining 4-in. core was also retained (subsurface soil). To reduce the number of determinations necessary, it was decided to pool some of the samples. Twelve sample units were paired at random to obtain six subsamples each of grass, surface-soil, and subsurface-soil samples (2-6 in.). Thus, there were six determinations made for each of the herbage, surface-soil, and subsurface-soil samples. The six results can, therefore, be considered as six independent unbiased estimates of the radioactive contamination of the samples.

Strontium$^{90}$ in soil was determined by fusing 100-g samples with sodium hydroxide and sodium carbonate (11). Corrections were made for decay, counter efficiency, and background counts. The lower limit of detection with the Philips beta counter was 4 $\mu\mu$c. The determination of Strontium$^{89}$ in soil is beset with analytical difficulties, because of the relatively low Strontium$^{89}$ levels, and the presence of activities other than strontium in the samples. For this reason, the strontium carbonate samples were repeatedly counted at suitable intervals and the Strontium$^{89}$ decay curve carefully followed.

Grass samples obtained from a square yard of area were ashed in the presence of added strontium carrier and the ash was refluxed with 6 N hydrochloric acid for 2 hr. The leachate was filtered and diluted to 1 N hydrochloric acid. This was passed through a Dowex 50 ion exchange column and strontium was recovered,

using the method of Grummitt (7). The Strontium$^{90}$ and Strontium$^{89}$ content was calculated as described above for milk. Calcium was determined with a Unicam SP-900 flame spectrophotometer.

## RESULTS

Estimates of Strontium$^{90}$ and Strontium$^{89}$ for surface soil (2 in. deep) and subsurface soil taken of 2-6-in. depth, on September 15 and November 26, respectively, are given in Table 1.

TABLE 1

$Sr^{90}$ $\mu\mu c$/100 g sample in surface soil and subsurface soil

| | September, 1961 | | November, 1961 | |
|---|---|---|---|---|
| Sample no. | Surface soil | Subsurface soil | Surface soil | Subsurface soil |
| I | 8.6 | 8.0 | 9.0 | 6.5 |
| II | 11.2 | 3.6 | 11.7 | 6.1 |
| III | 11.2 | 4.9 | .... | 4.9 |
| IV | 9.8 | 6.8 | 7.6 | 4.6 |
| V | 11.5 | 4.7 | 9.2 | 7.4 |
| VI | 4.0 | .... | 6.4 | 6.3 |
| Mean | 9.4 | 5.6 | 8.8 | 6.0 |

The mean values give the best estimates of the contamination. A similar average estimate could have been obtained by pooling the 12 samples and making just one determination for each type of sample. The advantage of the present method is that it makes it possible to determine whether November averages differed significantly from those of September. The values of Strontium$^{90}$ are expressed in $\mu\mu c$/100 g dry soil. The specific activity of Strontium$^{90}$ with respect to calcium (S.U.) is not considered to be a meaningful unit, because it does not take into consideration the nonuniform nature of Strontium$^{90}$ distribution and the sharp variations which occur with depth in soils (4). A "t" test of the means (Strontium$^{90}$ $\mu\mu c$/100 g soil) showed no significant difference from September to November in soil samples. In both periods, however, there was a significant difference between the subsurface-soil (2-6 in.) and surface-soil samples, the latter containing almost twice the activity per unit weight. In none of the soil samples was Strontium$^{89}$ detected.

The levels of Strontium$^{90}$, Strontium$^{89}$, and calcium in grass are summarized in Table 2. S.U. values have been calculated for both strontium isotopes. Strontium$^{90}$ values increased from an average of 2.4 $\mu\mu c$/g ash in September to 4.0 $\mu\mu c$/g ash in November. S.U.$^{90}$ levels rose from 35 to 70. During both periods there was Strontium$^{89}$ present, in the September samples an average of 33 $\mu\mu c$/g ash and in November 293 $\mu\mu$/g ash. S.U.$^{89}$ values increased from 467 to 5,142 during this period. The "t" test was applied to test the differences between the September and November samples. The difference was not significant for calcium, but it was significant for Strontium$^{90}$, S.U.$^{90}$, Strontium$^{89}$, and S.U.$^{89}$, indicating that Strontium$^{89}$ and Strontium $^{90}$ levels, as well as their ratio to calcium, increased at the sampling site between September and November.

The levels for Strontium$^{89}$, Strontium$^{90}$, and calcium in the milk of individual Jersey and Holstein cows are summarized in Tables 3 and 4, respectively. An analysis of variance was performed for Strontium$^{90}$, S.U.$^{90}$, and calcium levels (Table 5). It appears that S.U.$^{90}$ levels and absolute Strontium$^{90}$ activities are consistently and significantly higher in October than in the July-August period (over-all time-effect significant). Within each breed not every cow was affected to the same extent (time-by-cow within breed effect significant). As in our previous report (8) S.U.$^{90}$ levels were significantly higher in Holstein than in Jersey milk. This difference was primarily due to higher calcium concentration in Jersey milk.

TABLE 2

Levels of Strontium$^{89}$, Strontium$^{90}$, Calcium, S.U.$^{89}$, and S.U.$^{90}$ in grass samples

| | September, 1961 | | | | | November, 1961 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Ca g/ g ash | $Sr^{89}$ $\mu\mu c$/ g ash | S.U.$^{89}$ | $Sr^{90}$ $\mu\mu c$/ g ash | S.U.$^{90}$ | Ca g/ g ash | $Sr^{89}$ $\mu\mu c$/ g ash | S.U.$^{89}$ | $Sr^{90}$ $\mu\mu c$/ g ash | S.U.$^{90}$ |
| I | 0.116 | 51 | 438 | 2.7 | 23 | 0.073 | 277 | 3,801 | 3.4 | 46 |
| II | 0.051 | 25 | 484 | 2.1 | 40 | 0.043 | 375 | 8,709 | 5.4 | 125 |
| III | 0.075 | 34 | 450 | 2.5 | 33 | 0.050 | 142 | 2,841 | 4.7 | 93 |
| IV | 0.061 | 28 | 462 | 2.8 | 45 | 0.063 | 333 | 5,278 | 4.4 | 70 |
| V | 0.067 | 36 | 540 | 1.6 | 24 | 0.072 | 311 | 4,322 | 4.8 | 67 |
| VI | 0.061 | 26 | 428 | 2.7 | 44 | 0.054 | 319 | 5,901 | 1.1 | 21 |
| Mean | 0.072 | 33 | 467 | 2.4 | 35 | 0.059 | 293 | 5,142 | 4.0 | 70 |

TABLE 3

Calcium, Strontium$^{90}$, Strontium$^{89}$, S.U.$^{90}$, and S.U.$^{89}$ levels in Holstein milk

| Sample no. | July-August, 1961 | | | October, 1961 | | | | |
|---|---|---|---|---|---|---|---|---|
| | Ca g/ liter | $Sr^{90}$ $\mu\mu c$/ liter | S.U.$^{90}$ | Ca g/ liter | $Sr^{90}$ $\mu\mu c$/ liter | S.U.$^{90}$ | $Sr^{89}$ $\mu\mu c$/ liter | S.U.$^{89}$ |
| 3 A | 1.35 | 2.15 | 1.60 | 1.21 | 2.20 | 1.82 | 39.47 | 32.62 |
| B | ..... | ..... | ..... | 1.14 | 2.28 | 2.00 | 33.32 | 29.23 |
| Avg | 1.35 | 2.15 | 1.60 | 1.175 | 2.240 | 1.910 | 36.395 | 30.925 |
| 5 A | 1.25 | 4.40 | 3.52 | 1.23 | 3.23 | 2.62 | 69.41 | 56.43 |
| B | 1.28 | 4.10 | 3.20 | 1.22 | 3.27 | 2.68 | 62.24 | 51.02 |
| Avg | 1.265 | 4.250 | 3.360 | 1.225 | 3.250 | 2.650 | 65.825 | 53.725 |
| 19 A | 1.25 | 1.63 | 1.31 | 1.22 | 3.10 | 2.54 | 32.15 | 26.35 |
| B | 1.30 | 2.38 | 1.83 | ..... | ..... | ..... | ..... | ..... |
| Avg | 1.275 | 2.005 | 1.570 | 1.22 | 3.10 | 2.54 | 32.15 | 26.35 |
| 29 A | 1.19 | 1.41 | 1.18 | 0.94 | 2.51 | 2.68 | 39.60 | 42.13 |
| B | 1.19 | 1.78 | 1.49 | 1.02 | 2.02 | 1.98 | 39.36 | 38.59 |
| Avg | 1.190 | 1.595 | 1.335 | 0.980 | 2.265 | 2.330 | 39.480 | 40.360 |
| Mean | 1.270 | 2.500 | 1.966 | 1.150 | 2.714 | 2.358 | 43.463 | 37.840 |

TABLE 4

Calcium, Strontium$^{90}$, Strontium$^{89}$, S.U.$^{90}$, and S.U.$^{89}$ levels in Jersey milk

| Sample no. | July-August, 1961 | | | October, 1961 | | | | |
|---|---|---|---|---|---|---|---|---|
| | Ca g/ liter | $Sr^{90}$ $\mu\mu c$/ liter | S.U.$^{90}$ | Ca g/ liter | $Sr^{90}$ $\mu\mu c$/ liter | S.U.$^{90}$ | $Sr^{89}$ $\mu\mu c$/ liter | S.U.$^{89}$ |
| 61 A | 1.35 | 2.37 | 1.75 | 1.37 | 2.64 | 1.93 | 46.2 | 33.7 |
| B | 1.48 | 3.10 | 2.09 | ..... | ..... | ..... | ..... | ..... |
| Avg | 1.415 | 2.735 | 1.92 | 1.37 | 2.64 | 1.93 | 46.2 | 33.7 |
| 68 A | 1.51 | 1.83 | 1.21 | 1.33 | 2.44 | 1.83 | 67.5 | 50.6 |
| B | 1.48 | 2.72 | 1.84 | ..... | ..... | ..... | ..... | .. |
| Avg | 1.495 | 2.275 | 1.525 | 1.33 | 2.44 | 1.83 | 67.5 | 50.6 |
| 71 A | 1.44 | 2.32 | 1.61 | 1.41 | 3.19 | 2.26 | 66.8 | 47.4 |
| B | 1.48 | 1.87 | 1.26 | ..... | ..... | ..... | ..... | ..... |
| Avg | 1.460 | 2.095 | 1.435 | 1.41 | 3.19 | 2.26 | 66.8 | 47.4 |
| 77 A | 1.42 | 2.49 | 1.75 | 1.41 | 2.72 | 1.93 | 63.3 | 44.9 |
| B | 1.38 | 2.18 | 1.58 | ..... | ..... | ..... | ..... | ..... |
| Avg | 1.400 | 2.335 | 1.665 | 1.41 | 2.72 | 1.93 | 63.3 | 44.9 |
| Mean | 1.442 | 2.360 | 1.636 | 1.380 | 2.747 | 1.987 | 60.95 | 44.15 |

## DISCUSSION

*Grass and soil.* In the migration of contaminants along the ecological chain, soil plays an important role, serving both as a site of concentration and a place where the contaminant may be fixed in a way rendering it inaccessible to vegetation. For this reason, the levels of Strontium$^{90}$ and Strontium$^{89}$ in soil are of great importance.

In our studies, Strontium$^{90}$ activities in soil did not increase from September to November, 1961. Both surface and subsurface soil samples (2-6 in.) stayed essentially unchanged in this period. This suggests that the period between the liberation of fission products into the stratosphere and their appearance in soil in measurable quantities was in excess of two months.

Results for grass are expressed per gram ash weight. The time required for the entry of strontium isotopes into grass was less than two months. This is shown by the significant rise of Strontium$^{90}$ in November grass samples as compared with those obtained in September. That this increase was, in fact, due to fresh fallout is supported by the simultaneous increase of Strontium$^{89}$ in the November grass samples, a sign of recent pollution. It is of interest to note that there was no appreciable difference in the distribution of rainfall in the months prior to the two sampling periods. During the periods when sampling took place the amount of precipitation was also similar (2.2 in.) in September as well as in November.

TABLE 5

Analysis of variance for Jersey and Holstein milk

| Effect | Degrees of freedom | Mean squares | F | Significance |
|---|---|---|---|---|
| Ca g/l | | | | |
| Interbreed difference | 1 | 0.330 | 183.39 | ** |
| Over-all time-effect | 1 | 0.065 | 36.00 | ** |
| Intrabreed difference | 6 | 0.014 | 7.78 | ** |
| Time by breed effect | 1 | 0.004 | 2.22 | |
| Time by cow within breed effect | 6 | 0.007 | 3.61 | * |
| Error | 10 | 0.002 | | |
| Total | 25 | | | |
| $Sr^{90}$ $\mu\mu c/l$ | | | | |
| Interbreed difference | 1 | 0.03 | 0.19 | |
| Over-all time-effect | 1 | 0.72 | 5.23 | * |
| Intrabreed difference | 6 | 1.34 | 9.75 | ** |
| Time by breed effect | 1 | 0.06 | 0.41 | |
| Time by cow within breed effect | 6 | 0.54 | 3.93 | * |
| Error | 10 | 0.14 | | |
| Total | 25 | | | |
| $S.U.^{90}$ | | | | |
| Interbreed difference | 1 | 0.98 | 11.82 | * |
| Over-all time-effect | 1 | 1.10 | 13.30 | * |
| Intrabreed difference | 6 | 0.69 | 8.27 | * |
| Time by breed effect | 1 | 0 | 0.04 | |
| Time by cow within breed effect | 6 | 0.38 | 4.56 | * |
| Error | 10 | 0.08 | | |
| Total | 25 | | | |

* Indicates significance at the 5% level.
** Indicates significance at the 1% level.
Note: Entries in this table have been calculated from the original unrounded measurements.

It was suggested (12) that fallout may be incorporated into the vegetation by several routes; such as by absorption through the roots from soil, by which previously deposited cumulative fallout enters the plants, and by direct contamination of plants. This may occur by at least three different mechanisms: a) foliar absorption, which refers to the direct absorption of isotopes through plant stem and leaf surfaces; b) floral absorption, which describes the incorporation of material through developing of influorescences of plants; c) plant-base absorption—absorption of material which has been previously carried down to the basal parts of plants, enrolled leaves, and shallow roots.

The simultaneous increase in $Strontium^{90}$ and $Strontium^{89}$ levels of grass in November as compared with September, and the lack of concurrent increase in the soil samples, suggest that the strontium isotopes entered the vegetation primarily by direct routes.

Although the soil was ploughed as recently as 1960, the top 2 in. contained more $Strontium^{90}$ in all samples than the succeeding 4 in. This is in agreement with other published results (1, 2, 6), and with the results of Kulp (10), who found that $Strontium^{90}$ concentration decreases exponentially with depth in all types of soils.

*Milk.* An analysis of variance was performed for $Strontium^{90}$ $S.U.^{90}$ levels and calcium. For this purpose the missing observations in Tables 3 and 4 were filled in by duplicating the A measurements of the same cell and, following this, the total variance was partitioned according to three main effects and their interactions. The first effect is the over-all time-effect, due to the difference between contamination conditions in the July-August period, and in October, 1961—6 wk after the resumption of atmospheric nuclear testing. The second effect is the inter-breed difference, which shows whether there is a consistent difference in $S.U.^{90}$ and $Strontium^{90}$ activities between the Jersey and Holstein milk tested. The third main effect is the intrabreed difference, which measures the difference between the milk of individual cows of the same breed. The other effects are interactions of the three above-mentioned effects; these are the time by breed effect and the time by cow within breed effect.

A. *Interbreed and intrabreed difference.* In a previous report (8), we found that the average $S.U.^{90}$ values were higher in the Holstein

milk than in milk obtained from the Jersey cows tested. Our present analyses show that in the October samples there was a similar difference between milk of the two breeds, as represented by the eight selected cows. The mean S.U.$^{90}$ values were 2.358 for Holstein milk as compared with 1.987 for Jersey samples. This significant difference between the milk samples of the two breeds of cows was most likely due to the consistently higher levels of calcium in Jersey milk in October, 1961 (1.380 g/liter for Jersey, and 1.150 g/liter for Holstein milk). The intrabreed differences continued to be significant for both Strontium$^{90}$ and S.U.$^{90}$ levels, indicating that the four cows within each of the breeds differ greatly. It is difficult to explain why there is no interbreed difference in Strontium$^{89}$ levels, although it is possible that the error of the determinations was too great for small differences to show up.

B. *Effect of renewed testing.* The comparison of milk analyses in August and October, 1961, shows that Strontium$^{90}$ levels increased significantly from August to October and that the S.U.$^{90}$ values were also significantly higher in the samples taken after nuclear testing was resumed (over-all time-effect significant). There was no significant difference in Strontium$^{90}$ levels and S.U.$^{90}$ values between the two breeds tested (time by breed effect not significant). The levels of calcium, however, were lower in the October samples; thus, the rise in S.U.$^{90}$ levels was also due to a fall in calcium concentration.

The increase in Strontium$^{90}$ in the October milk samples was simultaneous with the appearance of Strontium$^{89}$—proving that the rise was due to fresh fallout. Thus, the delay period between the injection of nuclear debris into the stratosphere and its appearance in milk was not more than 6 wk.

Anderson et al. (3) studied the effect of fallout rate and accumulated fallout on Strontium$^{90}$ activities in milk. They found that there was a high correlation between the activity levels of milk and the amounts of monthly deposition. In their studies, milk activities were most affected by the deposition in the month preceding milk production and also were affected, but to a lesser degree, by the amount deposited in the current month. They developed a formula (relevant for the Felinfach area) relating Strontium$^{90}$ activities in milk to fallout rates and to accumulated fallout. This empirical formula was very successful in predicting Strontium$^{90}$ levels.

Our milk samples, obtained approximately 6 wk after the resumption of atmospheric nuclear testing, show a considerable rise in Strontium$^{89}$ and Strontium$^{90}$ activities in both breeds tested. This increase could not have been due to an increase in cumulative soil levels, because there was no detectable rise in the Strontium$^{90}$ concentration of soil. Thus, it was probably the increased deposition rate of the preceding 6 wk which affected the significant rise in the activity levels of the milk of both breeds of cows.

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# MAMMARY GLAND GROWTH AND THE INITIATION OF LACTATION IN DAIRY GOATS [1]

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SUMMARY

The efficacy of estradiol, oxytocin, and bovine somatotrophin to initiate lactation in goats with artificially induced mammary gland growth was tested. In a second experiment, the amount of mammary gland growth resulting from the oral administration of diethylstilbestrol (DES) and 6-methyl-17 acetoprogesterone (MAP) was determined.

In the first experiment, the milk production of goats previously treated for 120 days with estradiol and progesterone was not increased by the injections of either estradiol, oxytocin, or bovine somatotrophin. In the second experiment, no marked milk secretion occurred in goats fed DES or MAP for 150 days until a dose of 1.0 mg estradiol had been injected daily for a 7-day period.

The oral administration of DES or MAP alone, or a combination of the two, resulted in some mammary gland growth and milk production, but the production was much lower than that resulting from pregnancy. The combination of both compounds was no more effective than either one alone. The feeding of MAP to goats for the last 60 days of pregnancy resulted in higher levels of milk production for the first 4 wk of lactation, but the difference was not statistically significant ($P > 0.10$). Thereafter, the milk production was approximately equal in the treated and the normal pregnant groups. The correlation between previous milk production and total alveolar surface area was 0.935.

Meites and Turner have theorized that estrogen is the primary stimulator of prolactin secretion [see review by Meites (8)] and consequently have used large doses of estrogen to initiate lactation in goats and cattle previously treated with estrogen and progesterone. Benson et al. (1) found that triggering doses of estrogen failed to have a significant lactogenic effect on milk yield after goats had received estrogen and progesterone injections for 150 days. Meites (8) considered the dose of estrogen employed by Benson et al. (1) for mammary gland growth sufficiently high in relation to progesterone to initiate lactation without use of additional estrogen.

Most of the work on the artificial development of mammary gland growth has been done with injected hormones. If the artificial development of the mammary gland and the initiation of milk secretion is to be employed under practical conditions, it would seem that oral compounds should prove useful and several oral progestational compounds have recently become available.

The present paper reports the results of two experiments. The first was designed to determine the effect of various hormones on the initiation of milk secretion of goats primed with estrogen and progesterone. The second experiment was designed to determine the extent of mammary gland growth and milk production after oral administration of DES and MAP.

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## EXPERIMENTAL PROCEDURE

*Experiment 1.* Twenty-seven mature dairy goats having had one or two previous lactations were injected with estradiol and progesterone at the rate of 3.5 mg estradiol and 490 mg progesterone weekly. This was injected subcutaneously three times a week for 120 days. Thirteen of the goats had been ovariectomized previously. At the end of the 120-day injection period, the goats were assigned to four groups. Approximately equal numbers of ovariectomized and intact goats were assigned to

each group. One group received no additional hormone, the goats in the second group received 0.5 mg estradiol daily, the goats in the third group were injected with 50 USP oxytocin [3] daily after the morning milking, and goats in the fourth group received 5 mg bovine somatotrophin [4] daily. The injections were continued for ten days. Twice-daily milking was begun at the end of the 120-day injection period. Daily milk weights were obtained for 8 wk.

*Experiment 2.* Forty goats were divided into five groups of eight each. Groups I and II each contained four intact goats used in Experiment I and four intact yearling does; Groups III, IV, and V each contained four ovariectomized does, one intact doe used in Experiment I, and three intact yearling does.

Goats in Groups I and II were bred and Group I served as a control group. The goats in Group II received 18 mg MAP [5] daily, starting on the 91st day of pregnancy and continuing to the day of kidding. The goats in Group III received 2 mg diethylstilbestrol (DES) daily for 105 days and 3 mg daily for an additional 45 days. Goats in Group IV received 18 mg MAP daily. The goats in Group V received a combination of DES and MAP at the same dose levels as those in Groups III and IV. The DES and MAP were mixed with soybean oil meal and this was fed over the grain mix once a day. No difficulty was encountered with palatability after the first three or four days.

At the end of the 150-day feeding period, twice-daily milking of the goats in Groups III, IV, and V was started. The milk yield of the goats during the first week was very low in comparison to those in Experiment 1; therefore, 1.0 mg estradiol was injected daily for seven days, starting on the eighth day of milking. Milking was continued for 56 days. At that time the goats were sacrificed and one-half of each udder was fixed for measuring the total alveolar surface area, as outlined previously (11). During the last four milkings the yield of each half was recorded separately to determine the per cent of the milk produced by each half.

[3] Armour's P.O.P.

[4] Calbiochem, Grade B.

[5] 6-Methyl-17 acetoprogesterone, kindly supplied by R. E. Zimbelman, The Upjohn Company, Kalamazoo, Michigan.

## RESULTS

*Experiment 1.* Weekly milk production of the goats treated with estrogen and progesterone and given supplementary doses of hormones is given in Table 1. An analysis of variance indicated that differences in milk production due to treatments were not significant ($P > 0.05$). The analysis also showed that there was no difference in response of the ovariectomized and the intact goats. It will be noted that the average weekly milk production for the somatotrophin-treated goats was somewhat lower than that of the three other groups. One goat in this group failed to respond to the treatment; milking was continued for 2 wk with no response in milk production. The milk production for the three other groups was rather close for the first 3 wk, but thereafter the estradiol and oxytocin-treated groups maintained or increased their production, whereas that of the control group decreased.

*Experiment 2.* Weekly milk production of the goats in Experiment 2 is given in Table 2. Data for the ovariectomized and intact goats are given separately. Only the data on the goats completing the experiment are given in Table 2. Three goats assigned to Group II were not pregnant; one goat each in Groups III and V did not respond to treatment after

TABLE 1

Weekly milk production (lb) of goats primed with estradiol and progesterone and given supplementary doses of hormones for ten days

| Supplementary dose | No. goats | Weeks | | | | | | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | |
| None | 7 | 2.7 | 2.6 | 3.1 | 2.4 | 2.1 | 2.2 | 2.1 | 1.9 | 19.1 |
| Estradiol (0.5 mg/day) | 8 | 3.1 | 3.2 | 3.8 | 4.6 | 4.8 | 4.3 | 4.1 | 3.9 | 31.8 |
| Oxytocin (50 IU/day) | 7 | 3.4 | 3.4 | 4.1 | 4.3 | 4.2 | 4.1 | 4.2 | 3.9 | 31.6 |
| Somatotrophin (5 mg/day) | 5 | 1.5 | 1.8 | 1.0 | 1.0 | 1.1 | 0.9 | 0.8 | 0.6 | 8.7 |

TABLE 2

Weekly milk production (lb) of goats in Experiment 2

| Group | Ovarectomized or intact [a] | No. goats | Weeks 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|
| I Pregnant | Intact | 8 | 24.1 | 26.7 | 25.9 | 25.6 | 26.2 | 25.8 | 23.8 | 22.8 | 200.9 |
| II Pregnant + MAP | Intact | 5 | 28.1 | 29.5 | 32.0 | 30.6 | 27.0 | 25.3 | 23.6 | 23.1 | 219.2 |
| III DES | Ovarectomized | 4 | 0 | 0.4 | 2.7 | 3.5 | 4.6 | 5.3 | 5.5 | 4.6 | 26.6 |
| | Intact | 2 | 0 | 0.3 | 1.1 | 1.6 | 1.9 | 2.1 | 3.1 | 2.5 | 12.6 |
| | Average | | 0 | 0.3 | 2.2 | 2.9 | 3.7 | 4.2 | 4.7 | 3.9 | 21.9 |
| IV MAP | Ovarectomized | 3 | 0 | 0.1 | 0.9 | 2.1 | 2.5 | 3.8 | 3.5 | 3.7 | 16.6 |
| | Intact | 4 | 0.2 | 1.7 | 3.5 | 5.7 | 6.1 | 6.3 | 6.1 | 6.3 | 35.9 |
| | Average | | 0.1 | 1.0 | 2.4 | 4.1 | 4.5 | 5.0 | 5.0 | 5.2 | 27.3 |
| V DES + MAP | Ovarectomized | 4 | 0.1 | 0.7 | 2.4 | 3.8 | 3.8 | 3.7 | 4.7 | 4.2 | 23.4 |
| | Intact | 2 | 0.1 | 1.5 | 2.7 | 4.3 | 4.5 | 5.7 | 4.3 | 5.1 | 27.2 |
| | Average | | 0.1 | 1.0 | 2.5 | 4.0 | 4.0 | 4.4 | 4.9 | 4.5 | 24.4 |

[a] Indicates whether the goats were ovariectomized or had intact ovaries at the start of the experiment.

3 wk of milking; one goat in Group III was pregnant; and one goat in Groups IV and V died during the experiment.

In Table 2, it can be seen that the animals receiving MAP during the last 60 days of pregnancy had higher weekly milk production during the first 4 wk than the control group. This difference, however, was not statistically significant ($P > 0.10$). At the fifth week, they were nearly the same and remained about the same thereafter.

No significant differences occurred in milk production between the groups of animals with artificially induced mammary glands. In all cases, milk production during the first week was very close to zero and did not increase greatly until the supplementary dose of estradiol was used. This is in contrast to the milk production of goats in Group I, where the milk production of all groups was quite high the first week, even without the use of a supplementary dose of estradiol.

The histological observations on the goat udders removed at 8 wk of milking are given in Table 3. Analysis of variance indicated that there were no significant differences in any of the measurements between the ovariectomized and the intact goats; therefore, these measurements were combined for Groups III, IV, and V. In Table 3, it will be noted that the previous milk yield and the total alveolar surface area of the pregnant groups were significantly higher ($P < 0.01$) than those of the other groups. The increase in alveolar surface area was due to the significant increases in the weight of the removed mammary gland and the number of alveoli per area of tissue, as shown by the porosity index. No significant differences

TABLE 3

Histological observations of mammary glands of goats in Experiment 2

| Treatment | No. goats | Milk from excised half—28 days | Wt of excised half | Porosity index | Alveolar surface area | Asymmetry index |
|---|---|---|---|---|---|---|
| | | (*lb*) | (*g*) | | ($m^2$) | |
| Pregnant | 8 | $50.0^a$ | $424^c$ | $208^c$ | $8.8^a$ | 54 |
| Pregnant + MAP | 5 | $50.6^a$ | $467^c$ | $222^c$ | $10.7^a$ | 53 |
| DES | 6 | $9.4^b$ | $117^d$ | $160^d$ | $2.5^b$ | 57 |
| MAP | 7 | $10.1^b$ | $187^d$ | $152^d$ | $3.0^b$ | 65 |
| DES +MAP | 6 | $8.5^b$ | $137^d$ | $148^d$ | $1.7^b$ | 52 |

[a] $>$ [b], $P < 0.01$; [c] $>$ [d], $P < 0.05$; within each measurement. Observations within each measurement having the same superscript are not statistically different, $P > 0.05$.

occurred in the asymmetry index; however, the asymmetry was somewhat higher in goats receiving the individual compounds in comparison to a combination of the two or pregnancy. One goat in the DES group showed abnormal folding of the alveoli as reported by Cowie et al. (3); however, no other histological abnormalities were observed in any of the other goats.

The correlation coefficient between previous milk production and the total alveolar surface was 0.935, in close agreement to that found in two previous studies (10, 11).

## DISCUSSION

Levels of estrogen and progesterone used in Experiment 1 are the same as those used by Benson et al. (2) when they tested the effect of triggering doses of estrogen on the initiation of milk secretion. In the work by Benson et al. (2) hexoestrol was used, and the goats were injected for 150 days, whereas in the present study estradiol was used and the injection period was only 120 days. Results of Experiment 1 are in agreement with those of Benson et al. (2), whereas in Experiment 2 it appears that the estradiol was involved in the initiation of milk secretion. Positive proof of this is not available because of the lack of control animals not given the supplementary doses. This lends support to the belief of Meites (8), that the dose of estrogen in relation to progesterone used by Benson (2) and reported in Experiment 1 was sufficiently large to initiate lactation without additional estrogen. The amount of estrogenic activity entering the blood stream from DES feeding is probably much less than that entering from the subcutaneous injection of 0.5 mg estradiol. Mixner et al. (9) found that DES andministered orally was about 1% as effective in inhibiting lactation in goats compared to its effect when administered subcutaneously. Lewis and Turner (7) found that the dose of DES needed to produce mammary development in male mice was six times higher when administered orally when compared to subcutaneous injections. Folley and Malpress (5) report that less than 10% of orally administered estrogens are utilized by the body. The amount of MAP used was calculated at a level of 0.2 mg per lb of body weight daily. An average weight of 90 lb was estimated. Average weight of the goats in Groups III, IV, and V was 77 lb; thus, the average daily MAP intake was 0.23 mg per pound of body weight. This level is slightly below the level per pound of body weight required to inhibit ovulation and estrus in the bovine [reviewed by Hansel (6)]. Inspection of the data indicated that there was no association between the weight of the goat and the amount of milk produced or the amount of alveolar surface area in Groups III, IV, and V.

In the previous study (11), it had been found that goats injected with bovine somatotrophin along with estradiol and progesterone to induce mammary gland growth started their milk production at a higher level than the other treatment groups. Bovine somatotrophin had no beneficial effect in this trial.

From results in Tables 2 and 3, it can be seen that the production of the goats with artificially induced mammary gland growth was considerably lower than that of the pregnancy groups. Production for the groups was also somewhat lower than that reported earlier (11) for goats injected with estrogen and progesterone for 150 days, but was comparable to that produced by the estradiol- and oxytocin-treated groups in Experiment 1. If the induction of mammary grand growth and the initiation of lactation is to become practicable for sterile and virgin animals, considerably more work must be done to determine the optimum level of feeding of both compounds.

It is interesting to note that three of the goats produced considerable mammary gland growth on MAP alone without a major source of estrogen. This is in agreement with the reports indicating that suitable doses of progesterone given alone to ovariectomized guinea pigs (2), rats, mice, and monkeys [reviewed by Folley (4)] will produce some mammary gland growth.

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# EFFECTS OF OXYTOCIN UPON THE REPRODUCTIVE TRACT OF THE LACTATING BOVINE [1]

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SUMMARY

Twenty-four lactating, multiparous Holstein and Jersey cows were used in the study. Twelve cows were injected with 200 and 150 USP units, respectively, of oxytocin (P.O.P.) for 42 days; the other 12 cows received nothing and served as controls. Average intervals from parturition to uterine involution were 41.0 and 42.7 days; to ovulation, 27.5 and 24.7 days; to first estrus, 41.3 and 40.9 days, for the treated and untreated groups, respectively. Daily injections of 400 USP units of P.O.P. from Day 0-4, inclusive, of the estrous cycle had no effect upon the langth of diestrus in 13 multiparous, lactating Holstein cows. Conception rates were not affected by treatments.

Most studies on the control of reproductive cycles in farm animals have centered around the use of gonadal hormones or their derivatives. However, studies by Hays and VanDemark (5) and Marion et al. (6) have suggested an association between the release of oxytocin and gonadotrophic secretion. Armstrong and Hansel (1) and Hansel et al. (3, 4) have reported that injections of oxytocin were effective in shortening diestrus in dairy heifers. Clapp (2) and Wiltbank and Cook (7) have reported that the noncycling post-partum interval was longer for nursed cows than for cows milked twice daily.

This study is an attempt to evaluate the effect of oxytocin on interval variation from parturition to uterine involution, first ovulation, and first estrus, as well as the conception rates for oxytocin-treated cows.

## MATERIALS AND METHODS

During the 1961-62 fall and winter calving season, 24 multiparous Holstein and Jersey cows which had calved normally were alternately assigned to treated or untreated status. The eight treated Holsteins received 200 USP units of oxytocin (P.O.P.), and the four treated Jerseys each received 150 USP units P.O.P. daily for 42 days following parturition. The daily doses were divided into two equal subcutaneous injections at approximately 12-hr intervals following the AM and PM milkings.

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Weekly rectal palpations of the cervix, uterus, and ovaries were started 10 to 16 days post-partum and continued until uterine involution was judged to be complete, ovulation had occurred as evidenced by a new corpus luteum, and estrus was observed. Uterine involution was judged to be complete when the uterine horns had returned to or near the pelvic region and had returned to normal tone and consistency.

In 1962-63, 13 Holstein cows, 42 to 73 days post-partum, were injected subcutaneously with 400 USP units of P.O.P. daily for five days, starting with the day of estrus, and then bred at the first estrus subsequent to treatment. Cows were examined for pregnancy at 28 to 35 days post-breeding.

## RESULTS AND DISCUSSION

Averages and mean squares for intervals from parturition to uterine involution, ovulation, and estrus are given in Table 1. Treatment with oxytocin did not significantly hasten involution. It might be theorized that contractions of smooth muscle fibers in the myometrium as a result of the action of oxytocin would shorten the interval to involution. However, the results indicate that the effect of oxytocin on the uterus during the post-partum interval to uterine involution is negligible.

The interval from parturition to ovulation was not significantly altered due to treatment. But, the interval variation among treated Holsteins was significantly greater than for the untreated group ($P < .05$). The range for the treated group was 13.5-58.5 days; for the untreated, 10.5-29.5 days. The ovulations in-

TABLE 1

Intervals from parturition to uterine involution, ovulation, and estrus in oxytocin-treated and untreated dairy cows

| | | Treated [a] | | | Untreated | | |
|---|---|---|---|---|---|---|---|
| | Interval from parturition | No. cows | Avg | M.S. | No. cows | Avg | M.S. |
| Both breeds combined | Uterine involution | 12 | 41.0 | 322 | 12 | 42.7 | 211 |
| Holsteins | Uterine involution | 8 | 41.6 | 357 | 8 | 42.3 | 158 |
| Jerseys | Uterine involution | 4 | 39.8 | 122 | 4 | 43.5 | 151 |
| Both breeds combined | Ovulation | 12 | 27.5 | 213 | 12 | 24.7 | 174 |
| Holsteins | Ovulation | 8 | 27.5 | 199* | 8 | 19.5 | 31 |
| Jerseys | Ovulation | 4 | 27.5 | 317 | 4 | 35.0 | 353 |
| Both breeds combined | Estrus | 12 | 41.3 | 722 | 12 | 40.9 | 611 |
| Holsteins | Estrus | 8 | 49.5 | 889 | 8 | 45.4 | 753 |
| Jerseys | Estrus | 4 | 25.0 | 38 | 4 | 32.0 | 325 |

[a] Holsteins received 200 USP units daily; Jerseys 150 USP units of oxytocin.
* Significantly ($P < .05$) different from untreated.

cluded a high incidence of silent heats; the average intervals to ovulation were 27.5 and 24.7 days for the treated and untreated groups, respectively. Average intervals to first estrus were 41.3 and 40.9 days, respectively.

Oxytocin per se seems to have no significant effect upon the interval from parturition to estrus. That estrus is delayed in nursed cows has been reported by Clapp (2) and Wiltbank and Cook (7). Nursing apparently stimulates the hypothalamus to secrete and release oxytocin. At the same time, gonadotrophins are released as an indirect response to the nursing stimulus. Pituitary gonadotrophins, alone or in combination with oxytocin, may be the hormones responsible for delaying estrus in nursed cows, rather than oxytocin itself. In this study three of the treated Holsteins and all four treated Jerseys exhibited estrus during the 42-day treatment period. Conception rates based upon pregnancy diagnosis were 84.6 and 88.8% for the treated and untreated groups, respectively.

Table 2 shows the effect of injecting lactating cows with oxytocin on Days 0-4 inclusive, where Day 0 was the day of estrus. Three of the cows apparently skipped one estrual period as they came back into estrus in 42, 44, and 47 days, respectively. The other ten cows had estrous cycles of 19-23 days. The conception rate for the 13 cows was 86.6%. Rectal palpation on the day of estrus and again five days later confirmed that all cows ovulated and that a corpus luteum formed at the ovulation site. Some of the corpora lutea were small, which is in agreement with reports from other studies (1, 4). Hansel and Wagner (4) were successful in shortening diestrus in eight out of 12 cows, using injections of 200 USP units of oxytocin twice daily on Days 1-6 inclusive. In this study, using the same dosage levels, twice-daily injections were started 24 hr earlier and continued for five days. We noticed the same reduction in daily milk yield and observed milk dripping from the ends of the teats between milkings, as previously reported by Hansel and Wagner (4). Milk yields returned to pretreatment levels following end of treatment. But, in contrast to their work, no cows returned to estrus in less than 19 days. However, this does parallel their findings with Holstein heifers, as they reported that injections of 100 USP units of oxytocin on Days 0-2 and 0-4 were ineffective in shortening the estrous cycle. If precocious estrus is dependent upon the degree to which luteal inhibition is accomplished,

TABLE 2

Cows treated with 400 USP units of oxytocin for five days starting on the day of estrus

| Cow no. | Days from calving to start of treatment | Days from beginning of treatment to estrus | Services per conception |
|---|---|---|---|
| 185 | 63 | 19 | 1 |
| 131 | 67 | 20 | 1 |
| 160 | 73 | 21 | 1 |
| 193 | 71 | 47 | 2 |
| 130 | 56 | 20 | 1 |
| 186 | 66 | 20 | 1 |
| 21 | 42 | 42 | 1 |
| 151 | 60 | 19 | 2 |
| 77 | 55 | 21 | 1 |
| 91 | 69 | 23 | 1 |
| 112 | 57 | 23 | 1 |
| 179 | 73 | 44 | 1 |
| 217 | 69 | 22 | 1 |
| Mean | 63.2 | 26.2 | 1.15[a] |

[a] Conception rate = 86.6%.

then it would appear that injections of oxytocin started after ovulation are more effective in inhibiting the formation of normal corpora lutea. Oxytocin does not directly affect lengths of diestrus. Apparently, it exerts its influence through inhibiting gonadotrophic secretions from the pituitary complex, either directly or indirectly through an associated neurohumoral substance.

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# PROGESTOGEN TREATMENT IN RELATION TO TIME OF INSEMINATION AND ITS EFFECT ON THE BREEDING EFFICIENCY OF DAIRY COWS [1]

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SUMMARY

The effect of routine therapy with 500 mg of progesterone caproate (PC) alone or in combination with 0.5 mg of estradiol valerate (PC + EV) on breeding efficiency was determined with 594 Holstein and 282 Guernsey female reproductive cycles. In three controlled trials the drug was injected intramuscularly at 0, 2, 10, or 14 days after artificial insemination on the first and (if necessary) the second insemination during a reproductive cycle.

In the first trial, when PC was administered at zero day, breeding efficiency values were 20.7% lower for Holsteins and 13.5% lower for Guernseys than for the controls. When PC was administered two days after insemination, the breeding efficiency of the Holsteins was almost equal (3.1% lower) to that of the controls, though it was markedly lower (15%) for the Guernseys. Such early treatments showed a detrimental effect on breeding efficiency.

When PC was administered alone or in combination with EV at ten days in Trial II to both breeds and at 14 days in Trial III to Holsteins, breeding efficiency values were only slightly below those of the control group, except for the PC + EV group in Trial II, in which the Holsteins averaged 17.7% below their controls. No beneficial effects were seen in any of the treatment groups.

Other comparative analyses of the data yielded no conclusive evidence that the treatments were causing prolonged injurious effects on the reproductive system.

Infertility in bovine females may have many causes, though embryo mortality may be associated most frequently with this deficiency. With repeat-breeder cows embryo mortality was found to be accountable for up to 65% of all fertilizations (10, 15); with efficient cows it may be as low as 10% (2), whereas in a random population it has been estimated to account for about 15% (10) of the insemination failures. These estimates would rank embryonic mortality as the predominant factor of low conception.

Embryo mortality has often been ascribed to an inadequate intrauterine environment, possibly resulting from a deficiency or imbalance of ovarian hormones. Progesterone alone or in combination with estrogen has been used successfully in maintaining pregnancy in ovariectomized cattle (3, 4, 7, 9, 11, 14). On the other hand, treatment of intact females with these hormones has produced varying results in relation to fetal mortality. Several workers (1, 5, 16) reported increased conception rates with progesterone in repeat-breeder cows; others (6, 8) reported improved breeding efficiencies in normal cows, which usually conceived on the first or second service; still others (13) reported detrimental results. Trials with hormones such as chorionic gonadotropins (17) and estradiol (12) did not improve the conception rates.

The present study was initiated to determine

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whether routine treatments with a long-acting progestogen alone or in combination with estrogen given at different time intervals after the day of insemination would improve the conception rate of dairy cows.

## METHODS AND MATERIALS

Three trials were conducted from October, 1958, to November, 1962, each utilizing Holstein and Guernsey females of breeding age in the New Jersey Agricultural Experiment Station's herd located at Sussex. In addition, Holstein and Guernsey females of breeding age in the South Carolina Agricultural Experiment Station's herd located at Clemson were used in Trial II and part of the Clemson Holstein herd was used in Trial III. A total of 876 Holstein and Guernsey first-service cycles was utilized: 142 in Trial I, 459 in Trial II, and 275 in Trial III.

Treatment consisted of 500 mg of progesterone caproate[2] (PC) alone or in combination with 0.5 mg of estradiol valerate (PC + EV), administered intramuscularly at 0, 2, 10, or 14 days after artificial insemination on the first and (if necessary) the second service. In all trials the animals were randomized in trios: Trial I, control, PC at 0 day, and PC at 2 days; Trial II, control, PC at 10 days, and PC + EV at 10 days; Trial III, control, PC at 14 days, and PC + EV at 14 days.

Animals not returning to heat were checked for pregnancy by rectal palpation about 70 days from the last service. Conception rates were calculated for first and second services; breeding efficiency for the two services combined was calculated by dividing total conceptions by total services. Since conception rates were generally consistent with breeding efficiency values, only the latter are presented. Treatment effects were evaluated by Chi-square analysis of breeding-efficiency values. In an attempt to determine whether there occurred any lasting effect of the treatments on the reproductive system, analyses were made in regard to the intervals from first insemination to conception and to the percentages of animals disposed from the herd for infertility reasons.

[2] 17-α-hydroxyprogesterone-17-n-caproate (Delalutin, E. R. Squibb & Sons, Division of Olin Mathieson Chemical Corp.).

## RESULTS AND DISCUSSION

Since breeding efficiency values of the Holstein and Guernsey control groups differed by 10 to 15%, results were analyzed within breeds.

The breeding efficiency values in Trial I (Table 1) for the PC group treated on zero day were considerably lower than the values for the control groups for both Holsteins (20.7% lower) and Guernseys (13.5% lower).

TABLE 1

Effect of hormonal treatments on the breeding efficiency of Holstein and Guernsey females in terms of combined first- and second-service conception rates

| Group | Treatment PC | EV | Interval from insemination | Holsteins Services | Breeding efficiency | Chi-square | Guernseys Services | Breeding efficiency | Chi-square |
|---|---|---|---|---|---|---|---|---|---|
| | *(mg)* | *(mg)* | *(days)* | *(no.)* | *(%)* | | *(no.)* | *(%)* | |
| | | | | Trial I | | | | | |
| 1 Control | | | .... | 48 | 52.1 | 2.56[b] (1 vs. 2) | 26 | 38.5 | <1.0 |
| 2 PC[c] | 500 | | 0 | 51 | 31.4 | 1.94[b] (2 vs. 3) | 24 | 25.0 | <1.0 |
| 3 PC | 500 | | 2 | 49 | 49.0 | 0.05 (1 vs. 3) | 34 | 23.5 | <1.0 |
| | | | | Trial II | | | | | |
| 4 Control | | | .... | 105 | 59.0 | 1.08 (4 vs. 5) | 144 | 39.6 | <1.0 |
| 5 PC | 500 | | 10 | 107 | 48.6 | 0.67 (5 vs. 6) | 93 | 38.7 | <1.0 |
| 6 PC + EV[d] | 500 | 0.5 | 10 | 121 | 41.3 | 3.56[a] (4 vs. 6) | 132 | 40.9 | <1.0 |
| | | | | Trial III | | | | | |
| 7 Control | | | .... | 140 | 47.8 | <1.0 | | | |
| 8 PC | 500 | | 14 | 145 | 46.9 | <1.0 | | | |
| 9 PC + EV | 500 | 0.5 | 14 | 137 | 46.0 | <1.0 | | | |

[a] $P < 0.10$.
[b] $P < 0.20$.
[c] Progesterone caproate (17-α-hydroxyprogesterone-17-n-caproate).
[d] Progesterone caproate + estradiol valerate.

The difference by Chi-square for the Holsteins was reliable at the 20% probability level, which for breeding data may well indicate a real difference. With the limited numbers involved, the difference for the Guernseys was not great enough to give a high Chi-square value. It was apparent that this treatment was far from beneficial and warranted an early termination in both breeds. The PC treatment administered at two days gave very different results in the two breeds, the Holsteins being only 3.1% lower than the respective control groups, whereas the Guernseys were 15.0% lower.

When PC administration was delayed to the tenth day in Trial II (Table 1) the breeding efficiency values for both breeds were only slightly below the control values. It was thought that the addition of estrogen to the progestogen at 1:1,000 (PC + EV) might increase the effectiveness of the progestogen; however, as can be seen in Table 1, the result was the opposite for the Holsteins, with a marked reduction in the breeding efficiency of 17.7% ($P < 0.10$) below the control group. Breeding efficiency for the Guernseys with PC + EV treatment was essentially the same as that of the control group. The exceptionally high breeding efficiency of the Holstein control group accentuated somewhat the comparisons for both treatments in Trial II.

The further delay of treatment administration to the 14th day in Trial III resulted in breeding-efficiency values very similar to the control value. The PC + EV treatment did not appear to be detrimental in this trial when compared among herds (Table 1); when compared within herds, however, there was an inexplicable inconsistency with the treatment group, inferior to the control group in the New Jersey herd and superior to the control group in the South Carolina herd. All other within-herd comparisons were consistent with each other.

It was quite apparent for both breeds that PC treatment on the day of insemination was detrimental to conception, possibly due to an upset of the ovarian hormone balance by the added progestogen at a time when the animal was still in the estrous phase of the cycle. Stott and Williams (13) reported similar results when progesterone was given 12 hr after insemination. Delay of the PC treatment until two days after insemination continued to interfere with conception among the Guernsey breed, whereas the Holsteins were unaffected. These findings do not agree with those of Johnson (6), and Johnson et al. (8), who have reported marked improvement in breeding efficiency following progestogen treatment of normal cows on the second day after insemination.

When PC treatment was further delayed to 10 and 14 days after insemination there was no effect, beneficial or detrimental, on conception. Addition of estrogen to the progesterone had no effect on the Guernseys and a detrimental effect on the Holsteins when administered on the tenth day, and no effect on Holsteins when administered on the fourteenth day. Recent work by Hawk (3), who attempted to maintain pregnancy in both first-service and repeat-breeder ovariectomized cattle with progesterone alone or in combination with estrogen, indicated that an ovarian hormone imbalance or deficiency was not solely responsible for embryo fatality in repeat-breeder cattle. The problem might possibly involve some metabolic failure in connection with hormone utilization by the uterus.

Analysis of the intervals from first service to conception (Table 2) revealed a highly significantly greater ($P < 0.01$) mean interval length for the treatment groups in Trial I than for the control group.

In Trials II and III no significant differences in conception intervals could be found between treatment groups. The absence of such differences might be attributed to an increase in the interval length of the control groups rather than to a decrease in the treatment groups. With the random selection of animals for treatments, it is possible, though doubtful, that this could indicate a carryover of treatment effects to the next reproductive cycle.

Chi-square analyses of disposal percentages (Table 2) revealed that in Trial I the number of treated Holsteins removed for infertility were not significantly different from the one in the control group. However, significantly more animals ($P < 0.05$) were removed from the group treated with PC on Day Zero than from the group treated with PC on Day Two, indicating a possible harmful effect on the cow's reproductive system when treatments were given during estrum. Another indication of a possible harmful effect could be found among Guernseys in Trial II, where significantly more PC-treated animals ($P < 0.05$) were removed for infertility than control animals. In addition, only a slightly harmful effect was indicated among the Guernseys in Trial II and among the Holsteins in Trial III when treated with PC + EV. Since these results were of inconsistent nature and based on relatively small numbers, it left considerable doubt as to whether the differences proved real and repeatable.

TABLE 2

Analyses of intervals from date of first insemination to conception, and of disposals for infertility

| Treatment | | Conception rate | | | | Infertility disposals | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Analysis of variance | | Animals in trial | | Analysis | |
| Breed group | Interval from insemination | Total conceptions | Mean interval | F | Comparison | Total | Disposed | Chi-square | Comparison |
| | *(days)* | *(no.)* | *(days)* | | | *(no.)* | *(%)* | | |
| | | | | | Trial I | | | | |
| Holstein | | | | | | | | | |
| 1 Control | .... | 25 | 9.48 | 4.92** | (Among 1, 2, + 3) | 27 | 7.41 | 1.73 | (1 vs. 2) |
| 2 PC[c] | 0 | 22 | 36.18 | 7.97** | (1 vs. 2 + 3) | 29 | 20.69 | 4.09* | (2 vs. 3) |
| 3 PC | 2 | 31 | 24.97 | 1.87 | (2 vs. 3) | 32 | 3.12 | 0.54 | (1 vs. 3) |
| Guernsey | | | | | | | | | |
| 1 Control | .... | 12 | 24.50 | 40.84** | (Among 1, 2, + 3) | 15 | 13.33 | 0.21 | (1 vs. 2) |
| 2 PC | 0 | 11 | 39.91 | 65.64** | (1 vs. 2 + 3) | 13 | 7.69 | 0.40 | (2 vs. 3) |
| 3 PC | 2 | 15 | 52.73 | 16.04** | (2 vs. 3) | 19 | 15.79 | 0.03 | (1 vs. 3) |
| | | | | | Trial II | | | | |
| Holstein | | | | | | | | | |
| 4 Control | .... | 37 | 38.68 | 1.32 | (Among 4, 5, + 6) | 39 | 2.56 | 1.08 | (4 vs. 5) |
| 5 PC | 10 | 40 | 24.52 | | | 42 | 0.00 | 3.23[a] | (5 vs. 6) |
| 6 PC + EV[d] | 10 | 34 | 37.79 | | | 39 | 7.69 | 1.00 | (4 vs. 6) |
| Guernsey | | | | | | | | | |
| 4 Control | .... | 50 | 39.36 | 0.18 | | 52 | 1.92 | 4.25* | (4 vs. 5) |
| 5 PC | 10 | 24 | 44.96 | | | 29 | 13.79 | 0.65 | (5 vs. 6) |
| 6 PC + EV | 10 | 45 | 44.29 | | | 51 | 7.84 | 1.86[b] | (4 vs. 6) |
| | | | | | Trial III | | | | |
| Holstein | | | | | | | | | |
| 7 Control | .... | 64 | 30.12 | 1.15 | (Among 7, 8, + 9) | 66 | 3.03 | 0.00 | (7 vs. 8) |
| 8 PC | 14 | 64 | 34.78 | | | 66 | 3.03 | 2.12[b] | (8 vs. 9) |
| 9 PC + EV | 14 | 58 | 42.48 | | | 64 | 9.38 | 2.12[b] | (7 vs. 9) |

* $P < 0.05$.
** $P < 0.01$.
[a] $P < 0.10$.
[b] $P < 0.20$.
[c] Progesterone caproate (17-α-hydroxyprogesterone-17-n-caproate).
[d] Progesterone caproate + estradiol valerate.

## CONCLUSIONS

The hormone treatments as outlined were detrimental to breeding efficiency when administered near the time of insemination. The delay of treatment administration to 10 or 14 days from insemination, while not markedly detrimental, was not beneficial and of no value in improving breeding efficiency under the conditions of this study.

There was slight, inconsistent evidence that some of the treatments could have caused prolonged injury to the reproductive system of some cows.

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# BOVINE SEMEN METABOLISM. VI. COMPARATIVE EFFECTS OF INITIAL FRUCTOSE LEVEL AND INCUBATION TEMPERATURE ON FRUCTOLYSIS AND SPERM MOTILITY [1]

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SUMMARY

Forty-four semen ejaculates from 12 bulls were used in a 3 × 3 factorial experiment to study the effects of three initial fructose levels (1.25, 1.96, and 4.81 mg/ml) and three incubation temperatures (27, 37, and 47 C) over a 3-hr incubation period on fructolysis and motility.

Increases in initial fructose levels and in the incubation temperatures caused increases in fructolysis in all instances with the exception of the highest initial fructose level at the highest incubation temperature. Motility responses were similar, except that they were depressed markedly at the high initial fructose level with all incubation temperatures. Thus, depression of motility anticipated and preceded the depression in fructolysis. Hyperstimulation of the sperm may have interfered with the recycling of ATP-ase. It is also possible that structural changes in the sperm proteins (denaturation?) may have led to paralysis of the contractile mechanism before actual cell death.

Spermatozoa motility and metabolic activity are markedly affected in vitro by changes in their environment. Recent reports (3, 4) have indicated that increases in the initial fructose concentration resulted in increased fructose utilization during 20, 60, and 120 min of incubation at 37 C. An increase in rate of fructolysis also occurred when the temperature of incubation was increased from 27 to 37 to 47 C (5). A decline in the rate of fructolysis was noted during the third hour of a 3-hr incubation period at 47 C. This decline was postulated to be due to the exhaustion of fructose in the substrate.

This study was designed to further examine the interaction of incubation temperature and fructose substrate concentration upon fructolysis and motility of bull spermatozoa.

## MATERIALS AND METHODS

Forty-four semen samples were collected by use of an artificial vagina from 12 Holstein and Guernsey bulls located at the Dairy Research Center of the New Jersey Agricultural Experiment Station. Immediately after collection the spermatozoa concentration of each sample was determined turbidimetrically with a Klett-Summerson photometer and the motile spermatozoa estimated to the closest 5%.

A 3 × 3 factorial experiment was arranged with three initial fructose levels and three incubation temperatures. The initial fructose concentrations were adjusted approximately to 1, 2, or 4 mg/ml after the addition of 0.5 ml of semen to 2.0 ml of 2.9% solution of sodium citrate dihydrate. Subsamples from each fructose level were incubated in water baths at 27, 37, and 47 C. Initially and after 1, 2, and 3 hr of incubation, the per cent of motile spermatozoa was estimated and a 0.5-ml sample of diluted semen was deproteinized with 3.5 ml of 10% trichloroacetic acid and prepared for fructose assay.

Fructose determinations were according to the method of Roe (7), as modified by Erb (1, 2). Fructose utilization was expressed as milligrams of fructose utilized by $10^9$ spermatozoa in a given period of time. Analysis of variance was calculated according to the procedures of Snedecor (8).

## RESULTS AND DISCUSSION

The mean spermatozoa concentration of the semen samples was $1.194 \times 10^9$ per ml, with a mean initial motility of 74.8% and a mean initial fructose concentration of 6.25 mg/ml.

Treatment means for fructolysis and motility are presented in Tables 1 and 2, respectively.

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TABLE 1

Effect of initial fructose level and incubation temperature on the mean fructose utilization

| Fructose level in incubating fluid | Incubation temperature | Fructose utilization per $10^9$ sperm in minutes | | | |
|---|---|---|---|---|---|
| | | 0-60 | 60-120 | 120-180 | 0-180 |
| *(mg/ml)* | *(C)* | *(mg)* | | | |
| A. All treatments | | | | | |
| 1.29 | 27 | 0.69 | 0.28 | 0.25 | 1.23 |
| 1.26 | 37 | 0.77 | 0.50 | 0.39 | 1.66 |
| 1.21 | 47 | 1.78 | 0.73 | 0.50 | 3.00 |
| 2.00 | 27 | 1.50 | 1.20 | 0.48 | 3.18 |
| 1.97 | 37 | 1.62 | 1.21 | 0.86 | 3.69 |
| 1.92 | 47 | 2.02 | 1.50 | 1.23 | 4.75 |
| 4.86 | 27 | 2.26 | 1.26 | 0.54 | 4.06 |
| 4.86 | 37 | 2.72 | 1.60 | 1.01 | 5.32 |
| 4.72 | 47 | 2.32 | 1.30 | 0.67 | 4.30 |
| B. Across temperatures | | | | | |
| 1.25 | Combined | 1.08 | .50 | .38 | 1.96 |
| 1.96 | Combined | 1.71 | 1.30 | .86 | 3.87 |
| 4.81 | Combined | 2.43 | 1.39 | .74 | 4.56 |
| C. Across fructose levels | | | | | |
| 2.72 | 27 | 1.48 | .91 | .42 | 2.82 |
| 2.70 | 37 | 1.70 | 1.10 | .75 | 3.55 |
| 2.62 | 47 | 2.04 | 1.18 | .80 | 4.02 |
| D. Over-all means | | 1.74 | 1.06 | .66 | 3.46 |

TABLE 2

Effect of fructose level and incubation temperature on the mean percentage motility

| Fructose level in incubation fluid | Incubation temperature | Motility | | | |
|---|---|---|---|---|---|
| | | Initially | After 1 hr | After 2 hr | After 3 hr |
| *(mg/ml)* | *(C)* | *(%)* | | | |
| A. All treatments | | | | | |
| 1.29 | 27 | 74.8 | 35.6 | 31.8 | 26.3 |
| 1.26 | 37 | 74.8 | 39.3 | 35.2 | 30.7 |
| 1.21 | 47 | 74.8 | 41.6 | 38.5 | 33.1 |
| 2.00 | 27 | 74.8 | 44.0 | 41.1 | 32.3 |
| 1.97 | 37 | 74.8 | 48.1 | 46.5 | 37.7 |
| 1.91 | 47 | 74.8 | 49.3 | 47.6 | 40.7 |
| 4.86 | 27 | 74.8 | 20.0 | 5.3 | 0.5 |
| 4.85 | 37 | 74.8 | 23.6 | 9.1 | 1.1 |
| 4.71 | 47 | 74.8 | 18.5 | 7.6 | 1.3 |
| B. Across temperatures | | | | | |
| 1.25 | Combined | 74.8 | 38.8 | 35.2 | 30.0 |
| 1.96 | Combined | 74.8 | 47.1 | 45.1 | 36.9 |
| 4.81 | Combined | 74.8 | 20.7 | 7.3 | 1.0 |
| C. Across fructose levels | | | | | |
| 2.72 | 27 | 74.8 | 33.2 | 26.1 | 19.7 |
| 2.70 | 37 | 74.8 | 37.0 | 30.3 | 23.2 |
| 2.62 | 47 | 74.8 | 36.5 | 36.8 | 25.0 |

An examination of Table 1 and the statistical analysis indicated that there were significant differences in fructose utilization, due to initial fructose levels and incubation temperature, increases in both instances being generally stimulatory. The exception to this trend was at the highest fructose level (4.72 mg/ml) and at the highest incubation temperature (47 C). Here, at all incubation times, fructolysis was depressed below that of the corresponding 37 C temperature; and at the 2- and 3-hr incubation periods, below that of the medium fructose level (1.92 mg/ml) and the 47 C temperature. This was the cause of significant interactions between fructose substrate levels and incubation temperatures. These results confirm and extend the results of Freund et al. (3-5).

Motility of spermatozoa was only moderately affected by temperature changes (Table 2), higher temperatures resulting in higher motility estimates. The middle level of fructose concentration (1.96 mg/ml) stimulated motility at all

temperatures over the corresponding low level of fructose (1.25 mg/ml). The high level of fructose (4.81 mg/ml) markedly depressed motility below that of either of the other two fructose levels. Thus, the high level of fructose and the higher incubation temperature depressed the motility of spermatozoa sooner and more severely than it did the fructolysis. An analysis of variance of the motility data showed highly significant effects of initial fructose levels, temperature of incubation, time of incubation, and the interactions of fructose level with both the time and temperature of incubation.

The depression in fructolysis and motility at the higher fructose and higher temperatures was not due to substrate exhaustion. The effect of the high fructose level when combined with a high temperature could be a hyperstimulatory action. This possibly caused enough interference with the recycling of ATP-ase, resulting in a reduction in both fructolysis and motility (6). Also these effects conceivably could cause structural changes in the sperm proteins (denaturation?) leading to paralysis of the contractile mechanism before actual cell death.

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# TECHNICAL NOTES

## EFFECTS ON PRODUCT QUALITY OF CROSS-CIRCULATION AIR DRYING OF WHOLE MILK FOAM

Cross-circulation drying of whole milk foam was studied at the Engineering and Development Laboratory of the Eastern Utilization Research and Development Division, Agricultural Research Service, United States Department of Agriculture, as part of a broad program directed toward the development of a commercially feasible process for preparing beverage-quality dry whole milk of rapid dispersibility and adequate shelf life. A beverage quality product is one possessing flavor similar to flash-pasteurized fluid milk and is free of the characteristic cooked flavor of present dry milks. Techniques for drying whole milk foam under vacuum and some properties of this product have been reported (1, 4, 5, 9). The spray drying of whole milk foam is also under study in this program (6). In addition, cross-circulation drying of whole milk foam was begun at the Eastern Division in 1959 after Morgan et al. (8) of the Western Division had devised a new method which they called foam-mat drying. In the course of their work with a wide variety of foodstuffs, they prepared dry whole milk of good dispersibility. Their method involved addition of a stabilizing agent to milk concentrates, preparation of a foam by incorporating air or an inert gas, application of this foam to a tray or a moving belt and exposing it to a hot air stream until dry. Since good dispersibility was one of the important goals of our work, a special dryer was built at the Eastern Division and a continuous unit was set up to study the mechanism of drying and to provide information needed to adapt drying conditions and techniques to obtain the best product quality. The purpose of this paper is to report the effects of drying conditions on the quality of the resulting product.

### EXPERIMENTAL PROCEDURES

Figure 1 is a flow sheet of the pilot plant used in these studies. Concentrate of 40 to 46% solids made from whole milk flash-pasteurized at 162 F for 16½ sec was chilled to below 50 F. Nitrogen was then dispersed in the concentrate by means of a gas-liquid mixer to yield a foam of 0.4 g/cc density. It was found that a stabilizer was not required for the preparation of suitable foams. The foam was applied in the form of ropes to the belt of a continuous, cross-circulation air dryer. Rope diameters ranged from 0.05- to 0.10-in. Air conditions studied were: velocity from 240 to 710 ft/min, dry-bulb temperature from 112 to 232 F, and relative humidity from 1 to 24%.

Product quality was measured by the Solubility Index Test of the American Dry Milk Institute (3), and by an organoleptic scoring test similar to the one adopted by the American Dairy Science Association (2). The taste panel consisted of 8 to 11 trained judges. The final flavor score reported for each sample was the panel's arithmetic average score for that sample.

### RESULTS

*Drying.* A comprehensive report on the engineering aspects of the drying study may be found elsewhere, along with supporting experimental evidence (7). Findings pertinent to this report are: (a) Drying time between two given moisture contents varied as the square of the rope diameter. (b) Foam temperature during drying was an important variable affecting drying rate. (c) Relative humidity had no effect on drying rate, but only on the final moisture content that could be attained. (d) Air velocity had a slight effect on drying rate which could be explained on the basis of its effect on foam temperature.

*Flavor.* Products prepared using a wide range of drying temperatures and drying times were presented to a trained taste panel for flavor evaluation. In all cases these products were found to be unsatisfactory, due to the development of an oxidized off-flavor (Table 1), although results indicate some improvement in flavor with decrease in drying time.

TABLE 1

Flavor evaluations

| Total drying time | Dry-bulb air temp | Avg score of fresh whole milk standard | Avg score of sample | Most objectionable flavor |
|---|---|---|---|---|
| *(min)* | *(F)* | | | |
| 6.0 | 143 | 38.8 | 28.5 | Oxidized |
| 5.1 | 121 | 39.5 | 28.1 | Oxidized |
| 4.6 | 174 | 39.5 | 30.0 | Oxidized |
| 3.4 | 174 | 39.5 | 32.0 | Oxidized |
| 3.2 | 209 | 39.5 | 35.5 | Oxidized |
| 1.8 | 209 | 39.3 | 34.4 | Oxidized |
| 1.3 | 209 | 39.3 | 35.7 | Oxidized |
| 1.1 | 209 | 39.3 | 35.1 | Oxidized |
| 1.0 | 232 | 39.0 | 31.5 | Oxidized |
| 0.92 | 232 | 39.0 | 32.3 | Oxidized |

*Heat damage.* The ADMI Solubility Index Test was used as a measure of the heat damage to the product. Experiments were made to determine the effect of dry-bulb temperature and drying time on the solubility index. Results are given in Table 2. They show that the dry-bulb temperature must be restricted to meet the standards of solubility index, that this temperature limit is about 232 F with ropes of 0.05-in. diameter, and that it is lower with thicker ropes.

TABLE 2
Heat damage due to drying

| Dry-bulb air temp | Rope diameter | Drying time | Moisture | Solubility index |
|---|---|---|---|---|
| *(F)* | *(in.)* | *(min)* | *(%)* | *(ml)* |
| 174 | 0.05 | 2.1 | 3.47 | Trace |
| 174 | 0.075 | 3.0 | 3.53 | Trace |
| 174 | 0.10 | 6.3 | 2.82 | Trace |
| 209 | 0.048 | 1.8 | 3.13 | 0.1 |
| 209 | 0.048 | 1.3 | 3.36 | Trace |
| 209 | 0.05 | 2.2 | 2.47 | 0.3 |
| 209 | 0.075 | 3.2 | 3.52 | 0.6 |
| 209 | 0.10 | 5.47 | 3.73 | 0.4 |
| 209 | 0.10 | 3.3 | 4.55 | 1.0 |
| 232 | 0.048 | 1.0 | 3.05 | 0.4 |
| 232 | 0.048 | 0.92 | 3.57 | 0.5 |

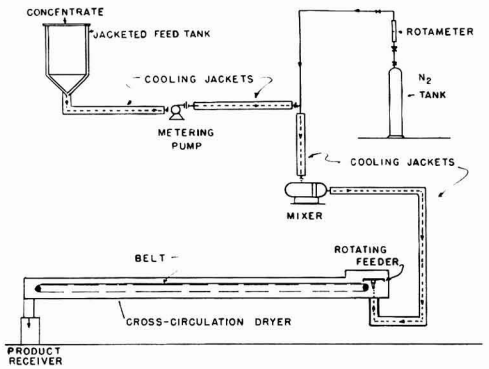


FIG. 1. Flow sheet of the pilot plant used to study the cross-circulation air drying of whole milk foams.

## DISCUSSION

The main limitation in applying this process to whole milk is the development of an oxidized off-flavor. Results of this work show that less oxidized off-flavor is developed as drying time is decreased by employing higher dry-bulb temperatures. However, dry-bulb temperature has an upper limit because of heat damage, as evidenced by solubility index. Even when drying at the upper limit, sufficient oxidative defect is developed to render the product unsatisfactory when dried in air.

It is common practice when drying food products to lower the dry-bulb temperature as drying proceeds to reduce heat-induced deteriorative reactions. In this case, the chief problem was the development of an oxidized off-flavor shown to increase as the dry-bulb temperature was lowered (drying time increased). Thus, temperature lowering as drying proceeds did not offer a solution to the oxidation problem.

There are other means that may be considered for overcoming oxidative deterioration during processing of milk. Heat-treating the fluid milk prior to spray drying is commonly employed to inhibit fat oxidation. However, this treatment imparts a decided cooked flavor to the product, inconsistent with the objectives of this work. Moreover, other work (5) has shown that heat treatment impairs dispersibility of the dry milk on storage. Drying in an inert gas atmosphere would inhibit oxidative deterioration, but would add substantially to the cost and complexity of the operation.

Although drying time can be decreased by the use of rope diameters smaller than the 0.05-in. used in this work, it would be difficult to approach the short time of exposure to oxidation possible in spray drying. Spray drying is cheap and, with the recent development of spray drying of foamed concentrates (6), improved dispersibilities can be had. Moreover, dry whole milk of excellent initial flavor and dispersibility can be made by the continuous vacuum drying of foamed concentrates (1). Through pilot-plant research the cost of the vacuum process is now approximating profitability. Work on cross-circulation drying has been discontinued, as it offers little prospect of yielding a product of the desired properties at a reasonable cost.

## ACKNOWLEDGMENT

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# A SIMPLE METHOD FOR ESTIMATING CURD FINES IN COTTAGE CHEESE WHEY[1]

During the manufacture of Cottage cheese, shattering of the curd detracts from the appearance of the product marketed. Unless curd fines are recovered from the whey and wash water, loss in yield may be substantial.

Several reports (1–4, 6, 7) have shown that some of the most important factors which affect the amount of shattering during the cooking and washing of Cottage cheese curd are: 1. Quality and solids content of skimmilk used; 2. type and activity of culture used; 3. firmness of coagulum and acidity at cutting; 4. method of cutting and cooking the curd; 5. type and rate of agitation used.

Kosikowski (5) reported a method of measuring curd uniformity for Creamed Cottage cheese. Using a series of sieves, he separated the curd into several size ranges. By comparing weights of fine curd to uniform desired curd, degree of curd breakage was established.

For research on the cooking of Cottage cheese curd, a simple and rapid semiquantitative method to determine curd fines in the Cottage cheese whey was needed. A procedure using calibrated centrifuge tubes for measuring the quantity of curd fines was developed. With the exception of calibrated centrifuge tubes, all of the equipment required for the method reported in this paper is normally available in the dairy plant laboratory.

## EXPERIMENTAL PROCEDURE

When Cottage cheese was cooked to the desired firmness, the agitator was stopped and the strainer inserted in the outlet of the cheese vat. Sufficient whey was permitted to drain to remove the curd trapped in the valve outlet by the strainer. Following this, a pint sample of whey was collected. The whey mixed well and 30 ml was tranferred to each of two 30-ml conical-bottom graduated centrifuge tubes. The tubes containing the whey were centrifuged 5 min. at 870 rpm in a Babcock centrifuge. The centrifuge cups were lined with cloth or paper to accommodate the special centrifuge tubes. The volume of curd fines, to the nearest 0.1 ml, was read from the graduated centrifuge tube.

The dry weight of curd fines from duplicate 30-ml samples of several lots of whey was determined by pouring off the whey and transferring the curd fines to weighed Mojonnier solids dishes. Two ml of distilled water were added to transfer curd fines to the solids dish and to help spread the curd fines in an even layer in the dish. After this, the water was evaporated on a Mojonnier solids plate, the sample in the dish placed in the Mojonnier vacuum oven for 10 min, tempered for 10 min to room temperature in a desiccator, and weighed.

## RESULTS AND DECISION

Figure 1 is a photograph of three completed tests illustrating the appearance of the test and the type of centrifuge tube used. Sample 1 (0.5 ml), Sample 2 (0.8 ml), and Sample 3 (2.7 ml) are Cottage cheese whey from commercial dairy plants and represent losses of 7.4, 11.9, and 40.1 lb of curd per 100 gal of whey. These figures were calculated using the weights of dried curd fines in 30 ml of whey. These weights were converted to the weights of curd fines with 80% moisture that would be contained in 100 gal of the whey sample. The

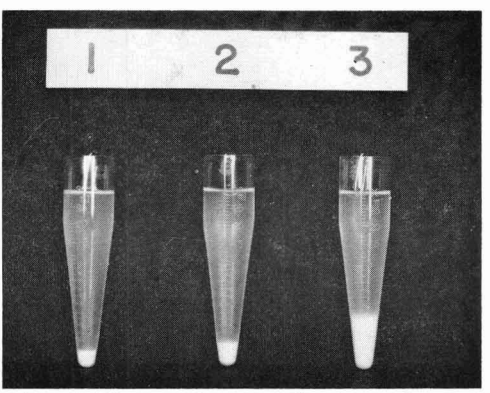


FIG. 1. Photograph of completed test for curd fines on three different samples of Cottage cheese whey.

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same procedure was used in determining the points plotted in Figure 2. The above samples were taken from vats of Cottage cheese when curd breakage was a problem. Results of determining the amounts of curd fines on whey from over 30 vats of Cottage cheese indicate that 0.4 ml or less of curd fines per 30 ml sample of whey is desirable.

Figure 2 relates the volume of curd fines in 30 ml of whey to the pounds of curd fines per 100 gal of whey. The pounds of curd lost per 100 gal of whey can be read directly from the graph when test results are available.

In Table 1, results are presented for quadruplicate determinations on seven lots of whey rounded to the nearest 0.1 ml of curd fines. Variations between duplicate tests were 0.1 ml or less in all cases. Also included in Table 1 are the average dry weights of curd fines for the four determinations on each lot of whey. A direct relationship exists between volume of curd and weight of dry curd fines obtained. This information was used to prepare the graph shown in Figure 2.

Other than the centrifuge tubes, the equipment needed is available in most dairy plant laboratories. The test is a useful research technique to demonstrate loss of curd in Cottage cheese whey due to curd breakage. It also serves as a simple procedure for daily checks on curd breakage in commercial Cottage cheese operations.

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TABLE 1

Volume and dry weight of curd fines in different lots of cottage cheese whey [a]

| Lot no. | Volume of curd fines | Dry weight of curd fines |
|---|---|---|
| | *(ml)* | *(g)* |
| 1 | 0.3 | .0364 |
| 2 | 0.5 | .0644 |
| 3 | 0.6 | .0705 |
| 4 | 0.8 | .0884 |
| 5 | 1.5 | .1785 |
| 6 | 1.8 | .2185 |
| 7 | 2.0 | .2449 |

[a] Average of four determinations on each lot of whey.

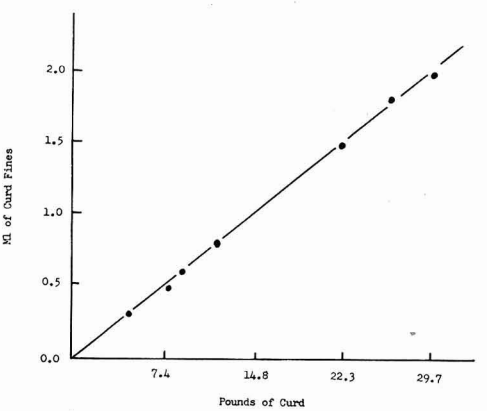


FIG. 2. Comparison of ml of curd fines in 30-ml aliquots of whey to lb of curd fines lost in 100 gal of whey.

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## ELIMINATION OF 2-METHYL-4-CHLOROPHENOXYACETIC ACID AND 4-(2-METHYL-4-CHLOROPHENOXYBUTYRIC) ACID IN THE URINE FROM COWS

The herbicides 2-methyl-4-chlorophenoxyacetic acid (MCP) and 4-(2-methyl-4-chlorophenoxybutyric) acid (MCPB) are used for broadleaf weed control in forage and other crops. It is, therefore, essential to know the mode of metabolism of these compounds when ingested by cows as herbicide residues on forage.

Using electron affinity gas chromatography, the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) was found in the urine of Holstein steers fed either 2,4-D or 4-(2,4-dichlorophenoxybutyric) acid [4-(2,4-DB)] (3). In the work reported, MCP was found in the urine of animals fed MCP or MCPB.

EXPERIMENTAL PROCEDURE

Two Holstein cows were catheterized and fed 2.5 and 5 ppm (based on a 50-lb daily ration) of MCPB on November 1, 1963. The pure herbicide in absolute ethyl alcohol was mixed with the grain. A Holstein steer was fed 5 ppm (based on a daily ration of 50 lb) of MCP on October 8, 1963. The pure herbicide in absolute ethyl alcohol was similarly mixed with the grain. The total urine samples from all of the animals were weighed and collected the day before feeding (control sample) and daily for six days after feeding the herbicides.

Method of analysis consisted of blending 25 g of the well-mixed, daily total urine sample with 70 ml of distilled acetone and 1 ml of 85% phosphoric acid for 2 min. The mixture was filtered and the filtrate made to a total volume of 100 ml with acetone. A 4-ml portion of the filtrate was transferred to a 100-ml beaker and the acetone evaporated with air. The remainder of the procedure involving extraction of interferences into diethyl ether at pH 7.7, nitration and methylation of the herbicides, and electron affinity gas chromatography of the nitrated ester derivatives is identical to the procedure described previously (1). Nitration produced the sensitive electron capturing five and six nitro derivatives of MCP and MCPB methyl esters. The un-nitrated esters would not have had the requisite sensitivity for this study.

RESULTS AND DISCUSSION

Figure 1 shows chromatograms of (A) urine from the MCP-fed steer three days after feeding and (B) the prefeeding control urine sample. The peak appearing after approximately 11 min represented one of the nitrated MCP methyl ester derivatives. The peak for the other nitrated derivative appeared after 8 min, but was obscured by other early-appearing interferences. It was necessary to manually zero the recorder pen just before the appearance of the 11-min peak, to allow it to be observed. The height of either peak (appearing when nitrated and methylated MCP standard was injected) was linear with MCP concentration. The recoveries of 0.4 ppm of MCP added to urine were 106, 75, 93, and 119%. The method was sensitive to about 0.1 ppm of MCP.

Table 1 lists the daily concentrations and the total quantities of MCP excreted in the urine (probably as the sodium salt) and the total daily urine production. No MCP was detectable in the urine of either of the MCPB-fed animals after the first day. The MCP-fed steer showed MCP in its urine up to four days following feeding. The lower urine production of this animal probably accounts for this difference.

The total amount of MCP fed to the steer was 113.5 mg. The total amount found in the urine was 140.8 mg. Considering experimental error, this may well indicate complete elimination of MCP in the urine of the animal.

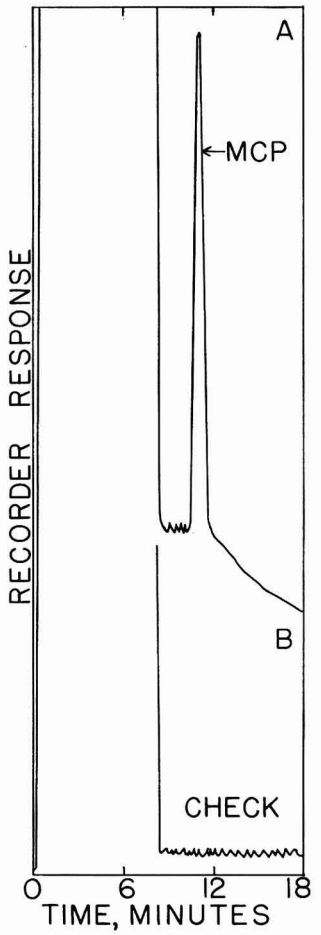


FIG. 1. Chromatograms of (A) urine from the MCP-fed steer three days after feeding and (B) prefeeding control urine.

The total amounts of MCPB fed to the 2.5 and 5 ppm fed cows were 56.75 and 113.5 mg, respectively. The total amounts of MCP eliminated represent 5.25 and 9.64 mg of MCPB equivalent, respectively. These values represent, respectively, 9.2 and 7.2% conversion of MCPB to MCP. In the study reported earlier (3), 7.3% of the 4-(2,4-DB) was converted to 2,4-D in a steer. MCPB was also found to convert to MCP on bean leaves, presumably by beta oxidation (2).

The MCPB-fed cows consistently showed (based on repeated analyses) the characteristic nitrated MCPB methyl ester peaks appearing after 16 and 48 min (1) in their first day's urine. Based on the height of the 16-min peak, the 2.5 and 5 ppm level animals showed respective MCPB concentrations of 0.35 ppm (4.56 mg total) and 0.55 ppm (8.37 mg total).

TABLE 1
MCP found in the urine and daily urine production

| Cow fed 2.5 ppm MCPB | |
|---|---|
| | First Day |
| Urine (*kg*) | 13.05 |
| MCP (ppm) | 0.35 |
| Total MCP (*mg*) | 4.56 |
| **Cow fed 5.0 ppm MCPB** | |
| | First Day |
| Urine (*kg*) | 15.22 |
| MCP (ppm) | 0.55 |
| Total MCP (*mg*) | 8.37 |

| Steer fed 5.0 ppm MCP | | | | |
|---|---|---|---|---|
| | First day | Second day | Third day | Fourth day |
| Urine (*kg*) | 4.38 | 4.95 | 4.86 | 6.33 |
| MCP (ppm) | 1.90 | 3.60 | 22.40 | 0.95 |
| Total MCP *(mg)* | 8.30 | 17.80 | 108.80 | 5.90 |

However, recovery of MCPB added to urine was not satisfactory, and these results are, therefore, not listed in Table 1.

Retention times for the observed MCP (and MCPB) peaks were identical with the pure standards. In several cases, addition of the herbicides to the urine sample simply increased the height of the respectively observed peaks, thereby indicating further that the compounds were identical.

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## OAT PASTURE FOR LACTATING COWS [1]

Several reports (1, 2, 4, 7, 13) have indicated that green oat forage stimulates milk production. Copeland (1) found that cows produced 4 to 5.5 lb more milk daily when grazing oats compared to periods when oats were not grazed. McClymont (8) reported a drop in milk fat test from 4.3 to 2.6% within a week following the start of grazing oat pasture. The animals were changed to another type of roughage and the milk fat test rapidly returned to normal. Several workers (2, 3, 8, 10) have studied the effects of various supplements on the composition of milk produced by cows grazing oat pasture. Generally, little or no benefit was realized from supplementing the rations of cows grazing oats with high-fiber or bulky ingredients (2, 3, 10, 13).

On the dry matter basis immature oat forage was reported to contain 12 to 33% crude protein (2, 7, 9, 10), 20 to 33% crude fiber, and 32 to 40% nitrogen-free extract (3, 9, 10). Hawkins and Autrey (4) determined the digestibility of the dry matter of harvested immature oat forage to be 79.4%.

The apparent high quality of oat pasture appeared to warrant additional study, especially relative to its value as the complete ration for lactating cows. This experiment was conducted to evaluate oat pasture in terms of its composition, palatability, digestibility, and its effect on the lactation curve and milk composition. A secondary objective was to compare methods of estimating pasture intake and digestibility.

[1] Published with the approval of the Director of the Arkansas Agricultural Experiment Station, Fayetteville.

### EXPERIMENTAL PROCEDURE

Five Holstein cows were pastured continuously, except for about 2 hr twice daily when removed for milking. Four of the cows had been lactating four or five months when placed on experiment. The other cow had been lactating eight months and was used only in the digestion-intake phase of the study.

Cows grazed fall-seeded oats, starting March 27, for a 1-wk transition period followed by six weekly experimental periods. Except for mineral supplements available on pasture, no additional feeds were allowed.

At the start of the grazing season there was a good stand of oats about 6 to 12 in. high, with relatively few weeds. The available forage was regulated by using put-and-take cows at intervals to keep the growth to an average of about ten in. tall. Grazing tended to keep the plants vegetative; however, some heading was observed during the last 2 wk of the experiment.

Digestibility was estimated by three methods: the chromogen formula of Reid et al. (11); from the intake formula of Lancaster (6), based on fecal nitrogen plus fecal dry matter output data; and the chromogen ratio (feces: forage) method (12). Chromogens were determined by the method of Reid et al. (12). Fecal dry matter output was determined, using chromic oxide as an indicator (16). Chromic oxide was analyzed by the method of Schürch et al. (15). Intake of forage was estimated from the digestibility and intake data (16). The first fecal collection period was preceded by a 1-wk preliminary grazing period. During this period cows received 8 g of chromic oxide daily in gelatin capsules. Grab samples of feces were taken at 6 AM and 4 PM and were composited for each weekly period.

Forage samples were taken daily by hand-plucking to simulate grazing. Proximate analyses of forage and feces were made by conventional methods.

Milk yields were recorded at each milking and milk fat and total solids analyses were made weekly on a one-day (AM and PM) composite sample. Body weights were taken weekly on two successive days.

## RESULTS AND DISCUSSION

*Pasture evaluation.* The composition of the hand-plucked samples from the oat pasture during the various grazing periods is presented in Table 1. The dry matter and ether extract declined gradually from the second through the sixth week of grazing then increased slightly the last week. The other constituents varied slightly, but inconsistently, during the advancing season. The average values obtained corresponded well with those previously summarized (9).

Forage intake and digestibility data, as measured by three methods, are presented in Table 2. Dry matter digestibility averaged 77.5% and daily intake 32 lb for the chromogen and nitrogen formula methods. Hawkins and Autrey (4) reported a dry matter digestibility of 79.4% for harvested, immature (6-12 in.) oat forage; however, no previous reports on the digestibility or intake of pastured oats have been found.

Although small weekly fluctuation occurred in both intake and digestibility, there was no apparent quality deterioration during the successive weeks of this trial. Intake of forage dry matter averaged 3 lb/cwt. The mean consumption of green forage, based on the average of 17.8% dry matter, was 180 lb/cow daily.

The chromogen and nitrogen formulas used in evaluating digestibility and intake agreed closely; however, estimates obtained by the chromogen ratio method were about five percentage units lower. These results suggest that forage of higher chromogen content was obtained in manual sampling than was actually grazed by the animals. Therefore, it appears that the ratio method is not as satisfactory as the other two generally more acceptable meth-

TABLE 1

Composition of oat pasture

| Week of grazing | Dry matter | Crude protein | Ether extract | Crude fiber | N-free extract | Ash |
|---|---|---|---|---|---|---|
| | | *(% of dry matter)* | | | | |
| 2 | 20.7 | 23.8 | 4.3 | 18.4 | 44.6 | 8.9 |
| 3 | 18.6 | 20.1 | 3.2 | 17.7 | 48.9 | 10.1 |
| 4 | 17.9 | 20.2 | 2.6 | 21.8 | 45.7 | 9.7 |
| 5 | 16.7 | 22.9 | 1.9 | 21.4 | 44.0 | 9.8 |
| 6 | 15.6 | 25.2 | 1.5 | 21.1 | 42.0 | 10.2 |
| 7 | 17.6 | 24.5 | 1.8 | 19.0 | 44.9 | 9.8 |
| Avg | 17.8 | 22.8 | 2.6 | 19.9 | 45.0 | 9.8 |

TABLE 2

Consumption rate and digestibility of oat pasture by dairy cattle

| Week of grazing | Dry matter digestibility | | | Dry matter consumption | | |
|---|---|---|---|---|---|---|
| | Chromogen formula | Nitrogen formula | Chromogen ratio | Chromogen formula | Nitrogen formula | Chromogen ratio |
| | *(%)* | | | *(lb/day)* | | |
| 2 | 80.9 | 76.2 | 70.9 | 34.9 | 27.6 | 22.7 |
| 3 | 78.9 | 77.2 | 69.1 | 32.2 | 29.8 | 22.0 |
| 4 | 73.6 | 76.8 | 76.1 | 25.7 | 29.2 | 28.5 |
| 5 | 79.6 | 79.6 | 74.3 | 36.8 | 36.8 | 29.1 |
| 6 | 75.6 | 78.9 | 73.7 | 27.6 | 31.6 | 25.6 |
| 7 | 77.0 | 75.9 | 73.1 | 36.4 | 34.6 | 31.0 |
| Avg | 77.6 | 77.4 | 72.9 | 32.3 | 31.6 | 26.5 |

ods. Others (5, 14, 17) have also reported close agreement between the chromogen and nitrogen methods for evaluating forages of quality apparently similar to that used in this study.

An approximation of the TDN value of oat pasture was made, using digestibility values computed from Reid's formula and composition of the forage samples. Estimates of mean apparent digestibility were: crude protein, 77.9%; ether extract, 4.6%; crude fiber, 83.2%; NFE, 88.8%; and ash, 35.0%. The resulting TDN was 74.5%, on the dry matter basis. It is recognized that these values are biased to the extent that the forage sampled might have differed in composition from that consumed by the animals. Since the digestibilities of the major constituents did not greatly differ, it seems unlikely that the TDN value is seriously biased.

*Cow performance.* The lactation curve for cows in this study is shown in Figure 1. The significance of milk yields in this study is limited by the small numbers of cows and their low production levels. The two cows most advanced in lactation (seven months) dropped sharply during the last 2 wk on the experiment.

Figure 1 also shows the total milk solids and milk fat percentage curves. Although there was a slight increase in milk fat per cent during the first week of grazing, the values subsequent to the preliminary week averaged 3.3% and were nearly constant. In contrast, McClymont (8) reported a severe drop in milk fat test when cows grazed oat pasture; whereas, others (2, 3, 7, 10) observed no appreciable fat depression. Total milk solids averaged 11.9% and did not appear to vary appreciably during the course of the experiment.

Mean body weight of cows just prior to pasturing was 1,078 lb. During the first week of grazing, weight loss averaged 37 lb per cow. About one-half of this weight loss was regained during the following week and by the end of the experiment the average cow had gained 41 lb, compared to her body weight before pasturing.

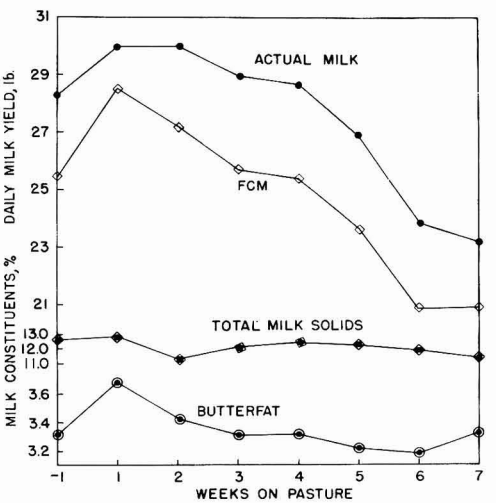


FIG. 1. Lactation performance of cows grazing oat pasture.

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# COLLECTION OF BLOOD FROM THE CAVERNOUS SINUS IN THE COW

Fluhman (7) first demonstrated pituitary gonadotropins in human blood when he injected 3.0–5.0 ml serum from ovariectomized women into infantile mice producing follicles and corpora lutea. Several authors (1, 3) have described methods of fractionation of plasma proteins with the isolation and detection of the gonadotropic fraction. Apostolakis (2) has described a method of concentration of plasma rendering it less toxic to bioassay animals. The method involved precipitation with acetone and dialyzing against normal saline.

Ganong and Hume (8), using a surgical approach, collected cavernous sinus blood in the dog and demonstrated the presence of ACTH. McFarland, Clegg, and Ganong (10) and Ellington, Contopoulos, and Clegg (6) were able to detect corticotropic, gonadotropic, and growth hormone activities in cavernous sinus plasma collected from gonadectomized, unanesthetized sheep. The blood was collected by passing a needle through the foramen ovale.

In sheep, Daniel and Prichard (5) have shown that much of the pituitary venous drainage is into the cavernous sinus, and a similar situation has been observed in the cow [Cummings (4)]. These circumstances led to the development of a technique in the cow to obtain cavernous sinus plasma by passing a needle through the foramen ovale.

## MATERIALS AND METHODS

A number of skulls were examined to locate and prove a passage through the foramen ovale into the cavernous sinus.

The cows were restrained in an operating stall with their heads tied to the side. The area below the ear was clipped, sterilized, and anesthetized with 3% procaine. A 12- or 14-gauge 6- or 9-in. needle was used. The starting point was half-way between the angle of the mandible and the zygomatic arch at the level of the caudal border of the mandible. The needle was directed at right angles to the mid-saggital plane, toward a point on the opposite side of the skull located by one of two different methods. In one method, the point was located ½ to 1 in. above the supraorbital foramen, which can be palpated in most cows. In the second method, the point aimed for was two-thirds the distance from the orbital rim of the frontal bone to the base of the horn, and one-third to one-half the distance from the lateral border of the frontal crest [Crista frontalis ext., Nickel, Schummer, and Seiferle (11)] and the midline (Surtura frontalis).

The foramen is 3.5–5 in. along the path of the needle in 600-800 lb heifers, and the foramina examined were about 1 cm in diameter. In some animals there was barely space for the entrance of the needle, because of the angle at which the needle approached the foramen. The direction of the foramen is dorso-ventral; the needle approaches from a postero-lateral direction.

Besides the mandibular nerve the foramen also contains a small inconsistent artery, a branch of the internal maxillary, called the proximal branch of the rete (proximaler Reteäst) by Nickel and Schwarz (12).

The penetration of the foramen is characterized by increased resistance, followed by release of pressure on the needle, after which a free flow of blood occurs. As the needle penetrates the foramen there is a characteristic general reaction from the animal. Care must be taken not to penetrate the diaphragma sellae and not to pass into the brain.

A ventral approach from the intermandibular space is prevented by the stylohyoid bone. Some variation in the point of aim occurs, mainly as a result of variation in the point of entry. Therefore, the muscular process of the temporal bone, the temporal condyle, the articular process of the mandible, the bulla tympanica are useful landmarks which can sometimes be recognized with the needle (Figure 1).

The blood may be drawn with a syringe, by a partially evacuated flask, or by a plastic cannula left in the sinus for periodic sampling.

## RESULTS AND DISCUSSION

Up to one liter of blood has been collected from each of the 40 heifers and calves. Twice, in early attempts, collection was unsuccessful and in another cow there was no passage through the foramen ovale. Occasionally, when

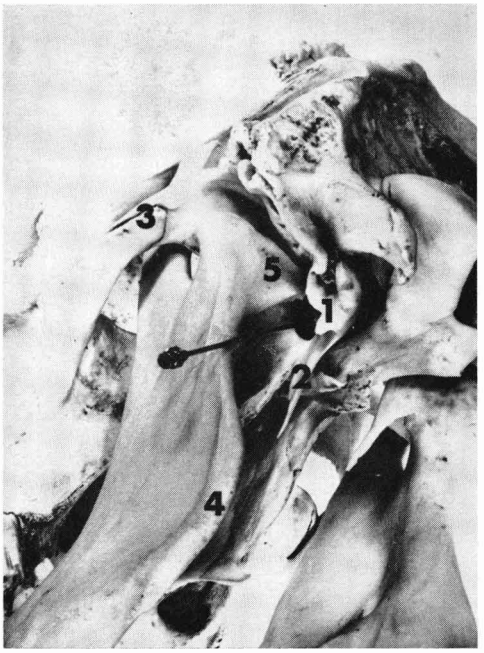


FIG. 1. The path of the needle is shown passing over the concave posterior border of the mandible, and through the foramen ovale on the skull of a Holstein heifer. 1. Bulla tympanica. 2. Muscular process of the temporal bone. 3. Zygomatic arch. 4. Angle of mandible. 5. Condyle of mandible.

the restraint has allowed head movement, the brain has been penetrated.

Blood plasma collected in this way from normal cows is being assayed for gonadotropins, TSH, and growth hormone.

Besides providing better access to pituitary venous drainage the approach offers the possibility of producing brain lesions in the hypothalamic area. The needle did not usually enter the pituitary but passed near its posterior dorsal border, and the possibility of introducing necrotizing substances to destroy pituitary tissue can be considered.

Care must be taken not to remain in the foramen ovale and draw blood from the proximal branch of the rete. This can happen when the angle of the needle is such that it will only pass into but not through the foramen. The nature of the blood collected could not be proved. It appeared predominantly venous, though on certain occasions some brightening of the color of the blood occurred, or a small pulse was evident. This blood was probably from the rete mirabile and the pulse could be stopped by slight repositioning of the needle.

Johnston and Callow (9) described a surgical approach for brain inoculation and biopsy which could well be done by passing the needle through the foramen ovale and into the brain.

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# OUR INDUSTRY TODAY

## UTILIZATION OF CENTRIFUGAL FORCE FOR REMOVAL OF MICROORGANISMS FROM MILK [1]

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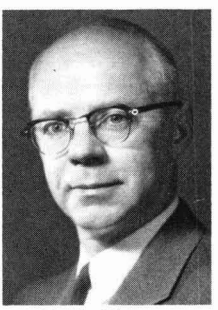
The process for removing bacteria from a liquid, e.g., milk, by means of centrifugal force has been termed commercially as bactofugation. It is similar to the force of that in a milk clarifier. This type of centrifuge is named bactofuge. The actual separation of bacteria occurs in the gravity field of the machine.

### BACTOFUGE DEVELOPMENT

About 10 yr ago, Professor Simonart at the University of Louvain, Belgium, believed that bacteria could be separated from milk by centrifugal force. He carried out the first experiments with a small Alfa-Laval clarifier. He soon found that this machine was able to remove bacteria. However, the efficiency of the removal lasted for only a few minutes. When the bowl of the clarifier was clean, efficiency was high. After about one-half hour the efficiency had dropped to zero, because the sediment space of the bowl was filled with bacteria and sediment. An equilibrium had then been reached, and the same amount of bacteria was reinfecting the milk. It appeared that Professor Simonart's progress would end, due to this equilibrium.



**G. A. Houran**

Professor Simonart found success with the bactofuge process by chance. During one of his test runs the rubber gasket of the clarifier bowl was damaged, resulting in a bowl leak. The analysis of the samples taken from this test showed that the efficiency of bacteria removal was maintained at a high level throughout the running period. Systematical work with the leaking bowl confirmed that continuous bacteria removal was important. A new centrifuge was built which had a small hole drilled through the vertical wall of the bowl.

Successful work with this machine led to the design of new machines with capacities up to 13,000 lb per hour. Professor Simonart discovered that optimum conditions were obtained at temperatures between 149 and 167 F, with a centrifugal force of 9,000 to 10,000 g.

### PRINCIPLE OF OPERATION

The bactofuge utilizes the difference in specific gravity between bacteria and the other constituents of milk. The specific gravity of bacteria varies between 1.07 and 1.13. Variation in their size is between 0.5 and 8 $\mu$. The specific gravity of skimmilk is about 1.035, with casein playing an important role. Specific gravity of the casein particles is 1.066 and their size varies between 500-800 $\mu\mu$.

The difference between the smallest bacteria and casein particles makes possible separation of bacteria from milk. However, for practical reasons the separation cannot be total. Difficulty in obtaining an ideal flow through the bowl of the centrifuge causes a decrease in separating effect. Present machines operate at an efficiency of about 90% of the bacteria removed from the milk.

The bactofuge is similar to a clarifier with the milk flowing through a hollow spindle into the bowl. The milk passes the distributor and flows into the disc stack through the holes in the discs. The bactofugated milk passes into the center of the bowl and is discharged on top. Sediment and bacteria-concentrated milk are thrown against the vertical wall of the bowl and evacuated through two 0.3-mm holes.

The bacterial concentrate, which amounts to 1.5% of treated milk, is gathered in the bowl casing. This is equipped with a special sludge groove for removal of the concentrate and cooling air. Air and bacterial concentrate are separated from each other in a cyclone located close to the bactofuge.

The frame hood and the bowl are double-jacketed. Cooling water is circulated in the jackets while the bactofuge is running. Cooling prevents the bacteria-concentrated milk from baking on to the inner surfaces of the hood and bowl casing.

### NEED FOR HEAT TREATMENT

The partial elimination of bacteria with the bactofuge makes it necessary to combine bactofugation with heat treatment. Heat is also essential to inactivate enzymes. It must be strongly emphasized that bactofugation cannot replace pasteurization, but will serve as an aid in attaining low total bacteria counts in processed milk.

[1] Presented in the Dairy Manufacturing Extension Subsection, 58th Annual Meeting of the American Dairy Science Association, June, 1963, Purdue University, Lafayette, Indiana.

### RESULTS IN BELGIUM

The first bactofuge was installed in Belgium by Cie Lacsoons of Rotselaar, in 1962. The plant is designed for two operating conditions. The bactofuges can be used either in connection with normal pasteurization or with sterilization by steam injection.

Milk enters the balance tank and is pumped to the plate heat exchanger, where it is preheated by regeneration and then heated to the pasteurizing temperature of 162 F with vacuum steam. Then the milk flows to the first-stage bactofuge and then to the second stage. After the bactofuge, the milk returns to the plate heat exchanger for regenerative cooling to the homogenizing temperature. More regenerative cooling follows and finally the milk is cooled with water and brine. Homogenization takes place after pasteurization, to reduce fat losses.

The bacterial concentrate from each bactofuge amounts to about 1.5% of the treated quantity. The concentrate leaving the first bactofuge has a very high bacterial count, but that collected from the second bactofuge has less than 10% of the bacteria found in the first-stage concentrate. To lessen losses from the double-stage process the concentrate from the second bactofuge is returned to the intake and recirculated. In this way, the total losses amount to only 1.5 instead of 3.0%.

In addition to significant reductions of bacteria, the keeping quality of the treated milk was tested at 59 and 65 F. The average keeping quality for 40 samples was 11 days at 59 F and seven days at 65 F.

### OTHER APPLICATIONS

Future prospects for application of the bactofuge are being studied. One application which has been discussed is in cheese-making. Trouble caused by spore-forming bacteria can be serious. If spore-formers are eliminated from the milk, better-quality cheese can be expected. Tests show that more than 99.0% of the spores are removed from milk with single-stage centrifugal treatment. Systematic investigations are planned in cooperation with the Dairy Research Station at Alnarp in Sweden for further studies on spore removal.

Spore-forming bacteria also cause trouble in the production of sterilized milk. If it is possible to lessen the heat treatment in sterilizers, an improvement in the quality of sterilized milk should result. Evaporated milk made from bactofugated raw milk has demonstrated the need for less heat treatment in sterilization. The quality of this evaporated milk was correspondingly better.

The bactofuge may also be useful in reducing the ultimate bacterial count in dry milk, which will improve quality.

### SUMMARY

Removing bacteria from milk centrifugally has passed the experimental stage and is ready for a wider application. A plant in Belgium has produced milk of better quality than common pasteurized milk. Through the removal of cells, bactofugated milk has better hygienic and commercial qualities.

Since the bactofuge is not a complicated machine, including it in various types of heat treatment systems is possible.

## CENTRAL LABORATORIES FOR DETERMINING THE HYGIENIC QUALITY OF MILK FOR MANUFACTURING [1]

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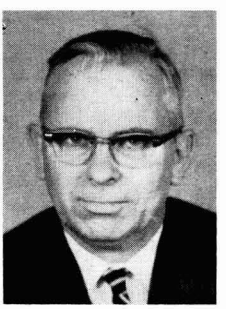
Kemptville Agricultural Schools, Kemptville, Ontario
Department of Dairy Science, Federated Colleges, Guelph, Ontario

In several large areas in the province of Ontario most producers sell their milk exclusively for manufacturing purposes. The price is considerably lower than that paid for milk for fluid consumption. As a result, the hygienic quality is sometimes poor. Comparatively few Ontario manufacturing milk shippers have bulk tanks. This has also delayed improvement of the manufacturing milk supply.

Since 1954 provincial regulations have required manufacturing plants purchasing milk to grade it on flavor, sediment, and bacterial activity. Competition among plants for increased patronage, however, has frequently resulted in grade standards being poorly enforced. The general level of milk quality suffered severely.



O. R. Irvine

Milk destined for fluid consumption is examined by laboratories maintained by dairies and also by 18 regional

[1] Presented in the Dairy Manufacturing Extension Subsection, American Dairy Science Association Annual Meeting, June 18, 1963, Purdue University, Lafayette, Indiana.

laboratories of the provincial Department of Health or by municipal authorities. In 1961 the Department of Health expanded this service and began testing milk for manufacturing. Marked improvement in quality has resulted.

The main test used by these laboratories on manufacturing milk is the 3-hr Resazurin Test. But the direct Microscopic Count or the Danish Mastitis Test is also used. The Danish Mastitis Test is essentially the same as the California Mastitis Test and henceforth will be referred to as the Gel Test.

In two large milk-producing districts not served by the Department of Health, the Ontario Department of Agriculture agreed to establish two laboratories. One of these was set up at the Department of Dairy Science, Federated Colleges, Guelph, and the second at the Dairy Division, Kemptville Agricultural School, Kemptville. The two laboratories are now operating smoothly, the former testing approximately 2,600-3,000 samples monthly, while the latter handles 3,600-4,000. Individual reports are mailed to the producer, plant, and fieldmen concerned.

FIG. 1. Unloading a case of 2-oz bottles as received from field staff.

## METHODS EMPLOYED

Each laboratory requires the full-time services of two women—a technician who performs the tests and a typist who prepares the reports. Two part-time junior fieldmen take many of the samples and transport them to the laboratories. Maintaining accurate mailing lists is important, particularly when producers switch from one market to another.

Except for bulk tank milk, sampling is done at the weigh tank either by one of the junior fieldmen of the Department of Agriculture or an employee of the plant. Approximately 1.5 fluid ounces of milk is collected into a square, screw-cap 2-oz bottle. The samples are placed in an ice bath immediately and later assembled in styrofoam-insulated cases which are kept cold with slugs. With reasonable care it has been possible to have samples reach the laboratory no higher than 45 F, even in July. The temperature of each case is checked on arrival at the laboratory.

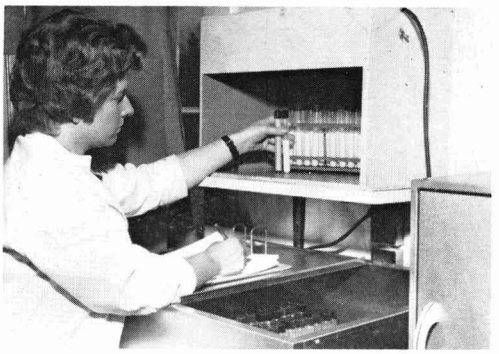


FIG. 2. A resazurin test is checked against color standard under uniform conditions of illumination.

Approximately one-half of the samples reach the laboratory early enough in the day for immediate resazurin testing. The remainder are tested the following morning. Small variations in methods are permitted between laboratories, but generally the 3-hr test as described in the A.P.H.A. Standard Methods is followed (1). Particular attention is given to proper illumination when reading results, and all borderline color change cases are compared to the 5P 7/4 color standard. Grades are recorded as No. 1, 2, 3, or 4. Samples which reduce in less than 30 min are specially marked.

Over the past few years the Department of Health Laboratories have gained considerable experience in applying the Gel Test to samples of herd milk. While the results of this test are undoubtedly of much more significance when applied to udder-quarter samples, good correlation has been found between Gel Test results and leucocyte counts on herd samples (2, 3).

The test is performed by pipetting 3 ml of milk into a flat-bottomed, white plastic dish, adding an equal quantity of test reagent and swirling the cup ten times in a period of 7 sec. Absence of any thickening indicates that the milk is Nonreactive (NR). On the other hand, a trace of coagulation or definite gelling is classified as Weakly Reactive (WR) or Reactive (R), respectively. If a sample shows a definite reaction it is a clear indication that mastitis exists in the herd. A weakly reactive test must be interpreted with considerable caution, because cows in early and especially in late stages of lactation often produce milk showing a slight reaction with this test.

The Gel Test is useful in several ways. The producer can be alerted of trouble in his herd. Cheese-factory operators are aided if they know when abnormal milk is received. Results of the test are sometimes helpful in explaining a Grade of No. 2 or 3 from the resazurin test.

FIG. 3. Gel Test reagent is added to a series of five samples.

Excessive leucocytes or other reducing agents may be present to such an extent that production of No. 1 milk is difficult.

In addition to the junior fieldmen who obtain the samples, other government-employed fieldmen work closely with the laboratories. Perhaps the major advantage of initiating this program was in allowing these men to spend more time calling on farms. They are furnished with complete information on test results and must make a personal visit to a farm before shipments can be stopped. The latter action is taken if three tests in succession are Grade 4. To be reinstated, a producer must produce milk of Grade 2 or higher. Since the government undertook this program, dairy plant laboratories concentrate on follow-up work. This has also been of real help in improving quality.

Another advantage of the system is in providing a large number of samples for research programs. Protein determinations by the Amido Black method are being made on approximately 100 samples per month. A program of testing for antibiotics and freezing point twice each year is also under way.

The cost of operating this service has not been estimated too precisely, but is undoubtedly high if calculated on a per-sample basis. In general, however, its reception has been very favorable. Both producers' and processors' organizations have gone on record as being willing to share the cost of the central laboratories.

SUMMARY

Some of the main features of two government-sponsored milk-testing laboratories have been described. Samples of raw milk sold for manufacturing purposes are collected and examined monthly. The Resazurin and Gel Test are being used to determine quality. The program has been found to have a number of advantages and has had a marked effect on quality improvement. Milk producers have confidence in test results and fieldmen have more time available for farm visits. Having a large number of samples available in the laboratory is also advantageous for research purposes.

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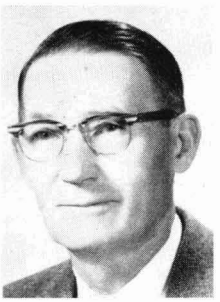
# ASSOCIATION AFFAIRS

## MEMORIALS

### D. V. KOPLAND

The Dairy Industry lost one of its most devoted servants in the death of D. V. KOPLAND on September 12, 1963, in Logan, Utah. He was born on January 26, 1899, in Wellington, Kansas, son of John K. and Daisy Irene Pickell Kopland. He was reared and schooled in South Dakota, graduating from South Dakota State College in 1921.



D. V. Kopland

After managing the South Dakota State herds, Professor Kopland was employed by the USDA at Ardmore, South Dakota, and was later assigned to the Huntley, Montana, Field Station, where he was in charge of dairy research for about 32 years. In May, 1961, the USDA moved the Dairy Research Center to Utah State University, Logan, Utah, and Dave Kopland moved with his cows which had become so much a part of his life.

Contributions to the Dairy Industry were many through the years. He helped pioneer the proved sire program for which the USDA has been so well known. His pasture research helped dairymen recognize the economic potential of this use of land. Amounts and types of grain to feed were studied and the rates of milk flow among many different cows were evaluated.

Dairymen relied on Dave's understanding of dairy cows and asked his advice personally, as well as listened when he spoke. Many of them attributed their success to the use of his methods and programs. Montana dairymen elected him as their delegate to the Holstein-Friesian Association of America several times, including the year he moved to Utah. He has been a long-time member of the American Dairy Science Association.

His passing was unexpected, following a brief illness and surgery. Friends and professional colleagues will miss him, as he was a most charitable, kind gentleman at all times. He was a baptized member of the Methodist Church and a member and leader in the Masonic Lodge. He routinely contributed to charities and to the Billy Graham crusades.

Surviving are his widow, Janet Buyers Kopland of Smithfield, Utah, whom he married December 10, 1962, and a sister, Mrs. Lucille Vagner of Rawlins, Wyoming.

### HENRY SOBORG

His many friends were saddened to learn of the death of HENRY SOBORG, Manager of the New England Selective Breeding Association, Woodbridge, Connecticut. Mr. Soborg had been with NESBA since November, 1960.

Henry Soborg

Mr. Soborg came to the United States in 1948 as a member of the staff of the Cooperative Artificial Breeding Association No. 1 of Clinton, New Jersey. He was named manager of that Association in 1950 and served in that capacity until he joined the Southern Illinois Breeding Association as Sire Analyst in 1953. He served as Manager of SIBA from July, 1959, until going to NESBA.

A graduate of the Royal Veterinary and Agricultural College of Copenhagen, Denmark, Soborg was farm manager of large dairy farms in Germany and Denmark, and for 14 years owned and operated a large dairy farm with over 300 head of dairy cattle. During this period, Soborg served as director of various cooperatives and noncooperative enterprises.

During the German occupation of Denmark, he served under Major General V. Bennike as a member of the Danish Resistance Movement. He also was an active member of the Jutland Staff, a sabotage group under the leadership of Captain Brandenburg of the United States Army.

He leaves a widow, Ingar, and two sons, Chris and Jack, aged 13 and 10. They will reside in Norway.

# THE UNIVERSITY OF ARIZONA

TUCSON

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To the Members of the American
Dairy Science Association:

The University of Arizona extends a cordial invitation to the American Dairy Science Association to hold its fifty-ninth annual meeting on the University campus in Tucson June 22-24, 1964.

You will receive a hearty welcome from the University's faculty and staff and from the Tucson Chamber of Commerce and Sunshine Climate Club, on behalf of all Tucson citizens. We will do all we can to make your visit an enjoyable and profitable one.

The facilities and personnel of the University of Arizona will be devoted to the success of your meeting. Comfortable and spacious rooms in several modern, air-conditioned buildings will be available for general and special meetings and committee sessions. The University of Arizona has experienced remarkable growth in the past decade and numerous new buildings have been constructed on our 136-acre campus. They provide a wide range of facilities admirably suited to the requirements of the American Dairy Science Association in planning its meeting.

Housing in air-conditioned residence halls will be available for about 300 men who wish to room on campus while attending the meeting. For others, Tucson has a wide range of hotels and motels in which accommodations are available and which will be especially suitable for those accompanied by their wives or families.

The resort facilities of the Tucson area are among the finest in the country and attract year-round visitors. Recreational opportunities include outdoor swimming pools, golf, tennis, horseback riding and excursions to old Mexico or the pine forests of adjacent mountains. Special programs being planned for the wives of members include sightseeing trips to major points of interest.

Assuring you of friendly Arizona hospitality and with best wishes for a successful meeting.

Sincerely,

Richard A. Harvill

Richard A. Harvill

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# ABSTRACTS OF PAPERS PRESENTED AT WESTERN DIVISION MEETING OF A.D.S.A. OREGON STATE UNIVERSITY, CORVALLIS, AUGUST, 1963

**Significance of California Mastitis Test trace and one reactions in quarter milk (I) yield of milk.** T. L. FORSTER,* U. S. ASHWORTH, AND L. O. LUEDECKE, Washington State University, Pullman.

One hundred and fifteen cows in University and privately owned herds were quarter-milked once, the CMT performed on the mixed-quarter milk, and the milk from each quarter weighed and sampled. Using opposite quarters of the same udders as controls, 47 negative-trace, 18 negative-one, and 40 trace-one comparisons were obtained. Average decreases in yield of milk per milking, attributable to the higher CMT reaction, for each of the comparisons mentioned above were 0.35, 0.92, and 0.84 lb, respectively. These differences were significant at values of P less than 0.05.

**Significance of California Mastitis Test trace and one reactions in quarter milk (II) leucocyte count.** L. O. LUEDECKE,* T. L. FORSTER, AND U. S. ASHWORTH, Washington State University, Pullman.

Leucocyte counts were made on quarter milk samples showing negative, trace, and one CMT reactions. Comparisons of leucocyte counts were made from opposite quarters showing negative-trace, trace-one, and negative-one reactions; 47, 38, and 18 comparisons were made, respectively. Statistical analysis showed that in each of the three comparisons the leucocyte count of the samples with the lower CMT reading was significantly lower (99% level) than the leucocyte count of the samples with the higher CMT reading. Similar results were obtained when the results of the catalase test were compared using the samples and comparisons mentioned above.

**Significance of California Mastitis Test trace and one reactions in quarter milk (III) composition.** U. S. ASHWORTH,* T. L. FORSTER, AND L. O. LUEDECKE, Washington State University, Pullman.

Small but significant differences in composition of 40 samples of milk produced from opposite quarters were found when comparing trace with one reactions of the CMT test. Solids-not-fat dropped on the average 0.08%, which proved to be significant at a P value of 0.01. Likewise, lactose showed an average drop of 0.10% between trace and one reactions, significant at P = .01.

The average increase in pH found for these same samples was .038 unit, measured on an expanded scale pH meter. This small difference had the same level of significance, P =

* Author who presented paper.

.01. It is concluded that subclinical mastitis does affect the composition of milk.

**Cost of upgrading dairy farms for Grade A milk-powder production.** R. V. WITHERS, Idaho State University, Moscow.

In 1960, a study was begun attempting to determine the effect of upgrading the production of milk for milk powder. A 2% random sample of farmers selling to milk powder plants was drawn in the Boise Valley and Twin Falls areas of Idaho. Each farm was visited and the dairy facilities inspected. Part of the farmers cooperated by keeping rather complete records of time spent and receipts and disbursements.

Cost of upgrading the dairy facilities, and alternatives to dairy production, including costs of converting existing buildings to other enterprises, were studied.

**Acid degree value and flavor of raw milk as influenced by certain selected factors.** R. C. HUNTER AND D. D. DEANE,* University of Wyoming, Laramie.

Milk samples were collected semimonthly for one year from 22 purebred Holstein cows. The cows were fed alfalfa hay, small grain silage, and concentrate, with no access to pasture until their dry period.

The ADV, determined by titrating an ether extract of the raw milk, varied considerably during the lactation of individual cows. The average ADV of fresh milk increased from 1.08 the first month of lactation to 1.38 the fourth and fifth months; then varied somewhat during the remainder of the lactation period, with a maximum ADV of 1.40 the tenth month.

The lowest ADV (0.96) was found in February, with an increase to 1.58, 1.44, and 1.49, respectively, in April, May, and June, followed by a drop to 1.10 in September and a second high of 1.38 in December. A rancid flavor was detected in fresh raw milk of only one cow, in December. Storing 48 hr at 35 ± 2 F slightly increased the ADV and occurrence of a rancid flavor. Agitation of raw milk in a Waring Blendor for 40 sec at 6,000 rpm, before cooling, caused a marked increase in ADV and definitely increased the occurrence and intensity of rancid flavor.

**Factors influencing methyl ketone formation in milk fat.** J. E. LANGLER AND E. A. DAY,* Department of Food Science and Technology, Oregon State University, Corvallis.

The ketone-producing ability of milk fat was studied quantitatively under various conditions. The characteristic series of odd-numbered methyl ketones ($C_3$ to $C_{15}$) were identified. Fat samples were heat-treated at temperatures ranging from 40 to 200 C for 30 min. Samples heat-treated in which the fat was sealed under vacuum to eliminate the presence of oxygen approached a plateau in ketone production near 140 C. Total ketones produced are dependent on the conditions of the treatment. Maximum amount of ketones formed in wet fat heated under vacuum was 1.364 mmoles per kilogram of fat and in anhydrous fat was 1.145 mmoles per kg. Addition of water to fat samples enhanced total ketone production. Ketone composition was nearly constant for all samples. Acetone was 24% of the total, 2-heptanone 22%, and 2-pentadecanone 18%. The other ketones were present in amounts less than 15% of the total. When fat was heated in air, 2-pentadecanone represented 21% of the total. The analytical procedures used were quantitatively evaluated. The hydrolytic ability of water as related to ketone formation from the fat will be discussed.

**Influence of milk citrate concentration on associative growth of lactic streptococci.** E. R. VEDAMUTHU,* W. E. SANDINE, AND P. R. ELLIKER, Oregon Agricultural Experiment Station, Corvallis.

In a study of the growth relationships in mixed cultures of lactic streptococci, separate mixtures of *Streptococcus diacetilactis* 8a with *Streptococcus cremoris* KH and *Streptococcus lactis* 8b were grown for 20 hr at 22 C in 10% nonfat milk fortified with different levels of citrate and plated on agar medium. Colonies were picked at random and identified as to species. The ratio of *S. cremoris* to *S. diacetilactis* was 1:3 in milk containing no added citrate and 1:99 in milk fortified with 0.2 to 1.0% citrate. In milk containing no added citrate, the ratio of *S. lactis* to *S. diacetilactis* was 3:2; this ratio changed to 1:1, 1:3, and 1:2.3 as the added citrate was 0.2, 0.6, and 1.0%, respectively. It was felt that the ability of *S. diacetilactis* to rapidly ferment citrate accounted for this strain dominance. Considering the wide seasonal fluctuations in milk citrate, this phenomenon may be another reason for starter culture variation.

**Factors affecting isolation and survival of bacteriophages for lactic streptococci.** D. R. HENNING,* DONA KIENLE, W. E. SANDINE, AND P. R. ELLIKER, Oregon Agricultural Experiment Station, Corvallis.

Survival of bacteriophages for *Streptococcus cremoris* 8, *Streptococcus lactis* C2, and *Streptococcus diacetilactis* 18-16 was studied to evaluate isolation procedures. Neutralization of phage-containing whey reduced survival for the *S. cremoris* phage; 84% survived storage for 1 wk in whey (pH 4.7) at 2 C, whereas 52% survived at pH 7.0. Storage at 2 C for 1 wk did not provide survival advantage over storage at 25 C for the *S. lactis* and *S. cremoris* phages; 50% of the *S. diacetilactis* phages were inactivated under these conditions. Only 48 to 67% viruses could be recovered from acid-coagulated milk. A tenfold increase in

phage titer was accomplished by concentrating whey preparations in dialysis tubing by packing them in polyethylene glycol. Repeated freezing and thawing (4 ×) of phages suspended in milk did not reduce the titer more than one log cycle. Glycerol afforded slight protection for phages stored up to 34 days at −20 C. Only 2 to 24% of phages could be recovered following Seitz filtration, whereas 92 to 99% were recovered following sterilization by membrane filtration.

**Enumeration of thermoduric bacteria in milk.** W. R. THOMAS,* University of Wyoming; G. W. REINBOLD, Iowa State University, Ames; AND F. E. NELSON, University of Arizona, Tucson.

Effect of the plate incubation temperature, incubation period, pH of the plating medium, and type of bacteriological peptone included in the plating medium upon the enumeration of pasteurization-resistant bacteria in milk was studied. Effect of the various plating procedures upon the recovery of pure cultures of some thermodurics before and after laboratory pasteurization was examined.

Of the plate incubation temperatures, 35, 32, 28, and 21 C, incubation at 28 C for four days was found, on the average, to be optimum for determining the maximum bacterial population of laboratory-pasteurized milk. The type of bacteriological peptone employed in the plating medium influenced the thermoduric colony count, particularly with milk containing appreciable numbers of thermoduric streptococci. Of the pH levels, 6.5, 7.0, 7.5, 8.6, and 9.1, the maximum mean thermoduric colony count was obtained with Plate Count Agar adjusted to pH 7.5.

Thermoduric cultures of *Arthrobacter* sp., *Micrococcus* sp., and *Streptococcus* sp. grew over a much wider temperature and pH range prior to than after being subjected to laboratory pasteurization. No appreciable differences in colony counts were observed when the unheated cultures were plated with media prepared with various peptones but, after laboratory pasteurization, colony counts differed substantially.

**Intracellular proteinases of some bacteria commonly associated with dairy products.** D. V. VADEHRA AND J. C. BOYD,* Montana State College, Bozeman.

Seven organisms *(Streptococcus lactis, Leuconostoc dextranicum, Lactobacillus casei, Proteus vulgaris, Bacillus subtilis, Pseudomonas fluorescens,* and *Staphylococcus aureus)* were selected and grown on liquid media. From these organisms the intracellular enzymes were separated and studied for their proteolytic activity on casein and whey protein substrates. The proteolysis was followed by the Folin-Ciocalteu method, with results being expressed in $\mu g$ of tyrosine and tryptophane.

Optimum pH for the various enzymes was found to be within the range of 6.0 to 7.0, although *S. lactis* showed a second peak at pH 5.5.

The age of the cells was found to be a factor and proteinases production increased as the age of the cell increased up to 96 hr. Cells 144 hr old showed a decrease in proteinase production.

The addition of gelatin and casein to the medium used to grow the organisms increased the proteolytic activity of the intracellular enzyme system, whereas addition of Casitone (Difco) had no effect. The absence of carbohydrates from the growth medium also increased the proteolytic activity of these enzymes.

**Observations on the volatile flavor fraction of blue-vein cheese.** D. F. ANDERSON* AND E. A. DAY, Department of Food Science and Technology, Oregon State University, Corvallis.

Fat was obtained from blue-vein cheese by centrifugation at 30,000 × $g$ for 20 min. Volatiles were removed from the fat by passing it through a Roto-film molecular still operating at 40 C and 4 $\mu$ pressure. The fat was redistilled a second time at 100 C.

The volatiles were trapped in a glass bead trap immersed in liquid nitrogen. Portions of the volatiles from the 40 C distillation were gas-chromatographed on polar and nonpolar columns at 70 and 100 C. The distillate from the 100 C distillation was taken up in ethyl ether, the ether removed by a stripping column, and the residue gas-chromatographed at 160 C on a phosphoric acid–treated polar column.

Analysis of the chromatographic data indicated there were at least 24 volatile components isolated from the cheese fat. Comparisons of relative retention times of authentic compounds and the unknowns were used for tentative identification of the chromatographic peaks. Members of the classes of compounds identified, which included aldehydes, ketones, fatty acids, and alcohols, will be discussed.

**Relationship between arylesterase of milk and mastitis.** R. R. MARQUARDT AND T. L. FORSTER,* Washington State University, Pullman.

Comparison of chloride (n = 120), catalase (n = 530), California Mastitis Test CMT (n = 530), and leucocytes (n = 382) with those of arylesterase (phenylacetate hydrolyzing enzyme present in high levels in mammalian blood and not inhibited by organophosphorus compounds such as parathion) for foremilk samples drawn from individual quarters, indicated that the level of arylesterase (A-esterase) activity is associated with severity of mastitis. A-esterase values were also compared with catalase, CMT, and leucocyte values

on the mixed milk obtained from individual quarters by complete quarter milking. The coefficients of correlation ($P < 0.01$) between the 425 milk samples were: A-esterase × catalase, 0.59; A-esterase × CMT, 0.53; and A-esterase × leucocytes, 0.66.

Another phase of this study involved a comparison of the per cent of herd quarters which had a CMT rating of three, with the A-esterase, catalase, and leucocyte values for milk taken from the bulk tank. The correlations between the per cent of herd quarters with a CMT rating of three and bulk tank A-esterase, catalase, and leucocyte values were 0.76 ($n = 43$), 0.79 ($n = 43$), and 0.74 ($n = 32$), respectively. These correlations were all significant at the 1% level of probability.

**Current research on aryl-esterase in bovine blood.** S. S. CHOI AND T. L. FORSTER,* Washington State University, Pullman.

Partial purification of the arylesterase of bovine blood plasm was accomplished by a combination of $(NH_4)_2SO_4$ fractionation, followed by column chromatography on DEAE cellulose. Methods used were as follows: Blood plasma was made 50-55% saturated with $(NH_4)_2SO_4$ and the filtrate dialyzed against a 0.005 M Tris-HCl buffer (pH 7.8) containing a 0.1 M NaCl and 0.004 M $CaCl_2$. Twenty milliliters of dialyzed filtrate were placed on a DEAE cellulose column 36 by 2.2 cm and eluted with the above-described buffer. The NaCl content of the eluting buffer was then increased stepwise. Three major protein peaks, designated I, II, and III, were observed. Peak I, the breakthrough peak, was obtained at 0.1 M NaCl concentration, and Peaks II and III at 0.2 M and 0.3 M NaCl, respectively. The highest specific activity was obtained at Peak III and purification was 12.1-fold. Peak III enzyme showed characteristics similar to the pre-albumin esterase which other workers obtained by electrophoresis. This enzyme was labile and EDTA-sensitive and appeared to be the principal phenyl acetate hydrolyzing arylesterase of bovine plasma.

**Fatty acid synthesis during fasting and bovine ketosis.** J. R. LUICK* AND L. M. SMITH, Departments of Animal Husbandry and Food Science and Technology, University of California, Davis.

It appears that one of the most characteristic biochemical aberrations attending fasting and bovine ketosis is the marked fall in the low molecular fatty acids. This fall amounts to 36 mol per cent for ketotic cows and 64 mol per cent for cows fasted five days. Further study reveals that the individual fatty acids are not uniformly depressed. On the contrary, the loss of each acid appears to be approximately proportional to its concentration in normal milk fat. This suggests that the short-chain fatty acids of milk fat are synthesized by more than one metabolic pathway. This, in turn, implies that the diminished lipogenesis from acetate noted during ketosis is probably not due to a simple enzymatic defect.

**Variations between breeds and among individual cows in levels of Cesium$^{137}$ secreted in milk.** GERALD M. WARD,* J. E. JOHNSON, AND H. F. STEWART, Colorado State University, Fort Collins.

While studying the metabolism of the fission product $Cs^{137}$ and potassium, the major element which most closely resembles cesium biochemically, it became of interest to investigate variations in the level of these two elements in milk which might be ascribed to breed or individual effects.

Determinations of both $Cs^{137}$ and $K^{40}$ were made with a 4- by 8-in. NaI (Ti) crystal and 400-channel pulse height analyzer.

Levels of $Cs^{137}$ and potassium in the milk of individual Holstein cows were found to be significantly different when a large volume of milk (5.4 liters) and a counting of 120 min were used. Statistically significant differences were also found between composite samples obtained from groups of cows containing either mixed Holsteins and Brown Swiss or Guernsey or Jersey cows.

To date, it has not been possible to establish clear-cut relationships between either nuclide and the level of milk production, the chemical composition of milk, or the stage of lactation.

**Effect of quality of hay and level of concentrate fed in conjunction with high-moisture grass silage on milk production and composition.** F. R. MURDOCK* AND A. S. HODGSON, Western Washington Experiment Station, Puyallup.

Two qualities of hay, a high-quality alfalfa and a poor-quality grass, were fed on a limited basis (½ lb/100 lb body weight) to milking Holstein cows in conjunction with grass silage ad lib. Also, two levels of concentrate were superimposed on each of the hay quality treatments in an extra-period Latin-square design.

Although cows produced slightly more milk on the high-quality alfalfa hay ration, the differences between qualities of hay were not significant ($P = > 0.05$). FCM production was significantly higher ($P = < 0.01$) for cows fed the higher level of concentrates. There was no significant interaction between qualities of hay and levels of concentrate.

Neither the percentages of fat, protein, nor SNF of the milk produced were significantly altered ($P = > 0.05$) by the ration treatments.

Results of this experiment suggest that the quality of hay is not of great importance when dairy cows are fed a ration of limited hay and good grass silage ad lib.

**Utilization of volatile fatty acids by goat liver.** R. M. COOK* AND R. H. ROSS, University of Idaho, Moscow.

Rumen vein, portal, carotid, and hepatic vein blood was sampled in anesthetized goats 3 hr after feeding. The molar per cent of acetate, propionate, and butyrate in rumen fluid of goats fed hay was 67, 25, and 8, respectively.

Concentrations ($\mu$mole per cent) in blood of propionate, acetate, formate, and B-OH-butyrate, respectively, were: arterial, 5, 116, 86, 28; rumen vein, 162, 592, 56, 47. The concentrations in portal and hepatic vein blood were the same and were 34, 160, 90, and 32. Butyrate was not detected in any of these blood samples. The molar per cent of acetate propionate and butyrate in rumen fluid of goats fed grain was 57, 25, and 18, respectively. Concentrations in blood of butyrate, propionate, acetate, formate, and B-OH-butyrate were: arterial, 0, 12, 116, 78, 30; rumen vein, 14, 125, 394, 30, 55; portal, 64, 302, 722, 34, 104; hepatic vein, 2, 13, 187, 38, 47.

For hay diets the data indicate little volatile acid absorption from the intestine. When the diet was grain, considerable quantities of volatile acids were absorbed from the intestine, since their concentrations were much higher in portal than in rumen vein blood. The liver removed considerable amounts of butyrate, propionate, acetate, and B-OH-butyrate.

**Effect of feeding different roughage levels and sodium acetate in high grain rations on milk production, milk constituents, and rumen volatile fatty acids.** R. W. STANLEY, K. MORITA, AND E. UEYAMA, University of Hawaii, Honolulu.

Two trials, ten animals in Trial 1 and 12 in Trial 2, were conducted to study the effect of feeding program on dairy cattle performance. Treatments in Trial 1 were: (1) 10 lb of pineapple bran plus concentrate, (2) Treatment 1 with 2.91% sodium acetate in concentrate, (3) 12 lb of pineapple bran, 50 lb of chopped Napier grass and concentrate. Treatments in Trial 2 were: (1) 10 lb of pineapple bran plus concentrate, (2) Treatment 1 with 2.83% sodium acetate in concentrate, (3) Treatment 1 with 5.66% sodium acetate in concentrate. Data from cows in Trial 1 on Treatments 1, 2, and 3, respectively, were: 4% FCM 35.35[a], 37.13[a], and 38.74[a] lb; milk fat 2.79[a], 2.94[a], and 3.79[b]%; total solids 11.05[a], 11.10[a], and 12.44[b]%; SNF 8.22[a], 8.16[a], and 8.45[b]%. In Trial 2, the values for Treatments 1, 2, and 3 were: 4% FCM 30.30[a], 32.85[b], and 34.17[c] lb; milk fat 3.42[a], 3.96[b], and 4.18[b]%; total solids 11.94[a], 12.49[b], and 12.63[b]%; SNF 8.50[a], 8.50[a], and 8.43[a]%. Values in the same category with different superscripts are significantly different by Duncan's multiple range test. Rumen fluid from cows on Treatment 3 in Trial 1 contained significantly more acetic and less propionic and valeric and longer-chain acids than from cows on Treatment 1.

**Comparative losses of carotene in alfalfa hay stored in the bale, or as wafers or pellets.** I. R. JONES,* L. A. HOGAN, AND P. H. WESWIG, Oregon State University, Corvallis.

Three lots of second-cutting irrigated alfalfa hay grown on three different Eastern Oregon farms in 1958, 1959, and 1960 were obtained in baled, wafered, and pelleted form, stored in the University dairy barn loft, and sampled at intervals for carotene determinations. The carotene values expressed as mg per lb of the 1958 baled alfalfa at 10, 24, 34, and 48 months were 40, 19, 15, and 9, respectively. At the same sampling periods, the wafered alfalfa had values of 23, 9, 7, and 6 and the pelleted alfalfa 19, 3, 2, and 1.

The 1959 baled, wafered, and pelleted alfalfa decreased from original carotene values of 40, 26, 18, respectively, to 27, 15, 10 at one year, to 10, 4, 3 at two years, and 7, 3, 2 at three years.

The carotene values for the 1960 baled, wafered, and pelleted alfalfa were 33, 31, 27 at two months, 27, 25, 25 at seven months, 19, 13, 12 at twelve months, and 14, 12, 11 at twenty-two months.

Results show the marked loss of carotene in hay processed into wafers and pellets and in baled and processed hay during storage.

**Relationships of various digestibility measurements and stage of maturity of orchardgrass and a timothy-Ladino clover mixture.** F. R. MURDOCK,* A. S. HODGSON, G. D. RAMAIAH, W. W. HEINEMANN, AND T. H. BLOSSER, Washington State University, Pullman.

Digestible dry matter (DDM) and digestible energy (DE) were determined on first-cutting orchardgrass (S-143) and a mixture of Climax timothy and Ladino clover at varying stages of maturity with yearling dairy heifers. The forages were cut daily and hand-fed. Samples of forage were dried, ground, and analyzed chemically. Portions of these dried samples were subjected to in vitro digestion as well as in vivo digestion by the nylon bag technique.

Highly significant correlations were obtained between DDM and DE as determined by digestion trials and percentages of lignin, crude fiber, crude protein, and cellulose in the forages.

In the in vitro studies the amount of cellulose in the residue was the independent variable most highly correlated with DDM and DE as determined in digestion trials. Of the variables studied by nylon bag digestion, digestible dry matter was the one most highly correlated with DDM and DE.

**Effects of feeding high molybdenum alfalfa hay to dairy cattle.** WALTER B. DYE, JOSEPH

F. STEIN, LEGRAND WALKER, Max C. Fleischmann College of Agriculture, University of Nevada, Reno, AND MARY RUTH SWOPE, Eastern Illinois University, Charleston.

Under the conditions of this preliminary reversal experiment with two lactating Holstein cows, it was found:

Blood. There was a direct correlation between the molybdenum content of the forage ingested (alfalfa hay) and molybdenum concentration of the blood serum; conversely, there was little if any correlation between molybdenum content of the forage and copper concentration in the serum; erythrocytes, hemoglobin, and hematocrit values were not influenced by the molybdenum content of ingested forage.

Milk. Xanthine oxidase concentrations in the milk were not correlated directly with the concentrations of molybdenum in the diet; xanthine oxidase increase in the milk did not follow or parallel the observed increase of molybdenum in the blood serum or milk; organoleptic results: 1. When Cow A was on low Mo hay there was no detectable oxidized flavor in the milk; with Cow B on the same hay, only a slightly oxidized flavor was detected. 2. When Cow A was on high Mo hay a mildly oxidized flavor was detected in 40% of the milk samples; with Cow B on the same hay, all three judges rated the samples as having a strongly oxidized flavor. Natural high Mo in hay appears to enhance oxidized flavor in milk.

**Dental changes as related to fluorine content of teeth and bones of cattle.** F. N. MORTENSON,* L. G. TRANSTRUM, W. P. PETERSON, AND W. S. WINTERS, Agricultural Department, Columbia-Geneva Division, United States Steel Corporation, Provo, Utah.

Incisor teeth, mandibles, and metacarpals of cattle from herds in fluorosis and nonfluorosis areas were studied. The teeth were classified into six degrees of severity, 0 through 5, for fluorine effects. Bones and teeth were analyzed for fluorine and amount of enamel in the teeth determined.

A close relationship was found between incisor score, fluorine content, and amount of enamel. As incisor scores increased in severity, fluorine levels increased, and the percentage of enamel decreased. Average fluorine content of enamel was less than one-half that contained in the dentin. These and other data obtained from unerupted teeth indicate that enamel is formed and acquires its fluorine before the incisors erupt from the gums.

Mandibles were found to contain the highest levels of fluorine, with metacarpals and whole incisors containing 96 and 84%, respectively. Some incisors were rated in Class 5 severity, even though fluorine content of bones and teeth from the same animal was decidedly below reported tolerable limits, further indicating that the appearance of severely affected teeth should not be used as the sole criterion upon which to make a diagnosis of definite fluorine toxicosis.

**Measuring the performance of 12,184 proved sire daughters in 75 large dairy herds in California.** HARLAN R. KOCH* AND ROBERT E. WALTON, American Breeders' Service, Palo Alto, California.

All available records started since 1951 were collected from 75 unselected Holstein herds and standardized to a 2×, 305-day, ME basis. A total of 12,184 daughters with 29,317 records average 14,803 lb milk, 543 lb milk fat at 3.6% test.

Records for the dams of 10,183 daughters were available for comparison: 10,183 dams (46,202 records)—13,417 m.—3.63%—487 f. 10,183 daughters (24,272 records)—14,827 m.—3.66%—543 f. Difference: + 1,410 m.; + .03%; + 56 f.

ABS daughter–herd-mate and ABS daughter–Non-ABS daughter contemporary comparisons were made. First- and second-generation contemporary daughters were also compared to show the effect of one generation of the bulls' inheritance.

The resulting 1,155 effective comparisons show a difference of +231 lb milk and +10 lb milk fat. Therefore, a genetic gain of +231 milk and +10 milk fat resulted in a daughter-dam difference of +1,410 lb milk and +56 lb milk fat in the herds studied.

**Detection and intensity of estrus in dairy cattle.** K. R. JOHNSON,* University of Idaho, Moscow.

Difficulty in detecting certain animals in estrus is one of the more common causes of delayed breeding or prolonged calving intervals in cattle. All females were observed for signs of estrus five times daily at 4-hr intervals. Estrus expectancy lists were used and animals were examined, per rectum, on day due in estrus or beginning 30 days postpartum, to aid in estrus detection. A scoring system of 1 to 4 was used. A score of 1 was given when no external signs of estrus were visible, but estrus was determined by rectal palpation; a score of 4 was given to animals that stood to be mounted. In 1961, of 215 estrous periods scored, 26% were given scores of 3 or less. The percentage dropped to 13% in 1962 and 9% in 1963. This would indicate that proper training and more careful observation will enable the detection of most animals in standing estrus sometime during the estrous period. It also indicates that the period of standing estrus is very short for some dairy cows and easily missed. The fertility of cows with lowered estrous intensities was not impaired.

**Corticosteroids in bovine jugular vein blood.** V. L. ESTERGREEN,* Washington State University, Pullman.

Approximately one-liter samples of jugular vein blood plasma from three mature, nonpregnant cows were extracted with organic solvents to determine the free and conjugated corticosteroids present. Cortisol was isolated and identified by chromatography of the free and acetylated compounds in three paper chromatography systems each and by its ultraviolet absorption spectrum in methanol. Levels of 5.45, 6.40, and 6.73 μg/100 ml plasma and 1.84 and 2.04 μg/ml packed cells were found by an isotope dilution technique using cortisol -4-$C^{14}$. A substance was isolated which absorbed ultraviolet light and chromatographed in two paper and two silica gel systems like corticosterone. However, the substance had an atypical ultraviolet absorption spectrum and did not form a chromogen typical of corticosterone. Two substances more polar than cortisol, one of which could be acetylated, were separated on paper chromatograms. Neither of these substances possessed ultraviolet absorbance typical of a $\Delta^4$, 3-ketone. No progesterone or conjugated 17-ketosteroids could be isolated from the blood samples.

**Biochemistry of fractionated ejaculates in the bovine.** L. FAULKNER,* J. F. MASKEN, AND M. L. HOPWOOD, Colorado State University, Fort Collins.

Lutwak-Mann and Rowson (J. Agr. Sci., 43: 131. 1953) used electric stimulation to obtain split ejaculates from the bovine. The sperm-rich fraction contained high levels of fructose, citric acid, and 5-nucleotidase. The presperm fraction, said to be of urethral origin, was clear, slightly viscous, and had no fructose or citric acid and little protein.

With relative ease we have been able to obtain both of these fractions using the artificial vagina. An additional post-sperm fraction has been obtained with greater difficulty. This fraction was essentially sperm-free and was intermediate between the presperm and sperm fractions with respect to nitrogen and fructose content.

Fractions collected with the artificial vagina were compared to similar fractions obtained by electric stimulation. By electroejaculation the presperm fraction was lowest in fructose and sperm concentrations, but was not completely free of the components. The post-ejaculate fluid was of variable nature, having from 0 to 843 mg fructose per 100 ml and between 10,000 and 89,000 sperm per $mm^3$.

**Freezability of spermatozoa obtained with the electro-ejaculator and with the artificial vagina.** C. W. COLLEARY AND M. H. EHLERS,* Department of Dairy Science, Washington State University, Pullman.

Experience in this laboratory has indicated that semen collected by the electro-ejaculator might have satisfactory initial spermatozoa motility, and yet not undergo successful freezing. These observations were based on bulls collected by electro-ejaculation because of failure to respond to the artificial vagina.

An experiment (I) was designed to compare freezability of electro-ejaculator and artificial vagina ejaculates collected on the same day from each of four Angus bulls. Collection procedures worked satisfactorily on a given week, near the beginning of the experiment, when the artificial vagina was used first and electro-ejaculator second. However, an increasing reluctance to serve the artificial vagina was noted. This experiment was abandoned after 8 wk because of an excessive number of failures.

A further experiment (II) was designed to collect two ejaculates, by the use of the artificial vagina, from each of two bulls and two ejaculates from each of two bulls with the electro-ejaculator. On the following week the collection method was alternated between the two pairs of bulls. Occasionally during the 8 wk of the experiment failure to secure two satisfactory ejaculates from a given bull was encountered.

Freezability comparisons indicated little difference between semen collected by the two methods, particularly for samples having similar original quality.

**Seminal fructose levels following induced hyperglycemia in bulls.** S. HARDWICK,* J. F. MASKEN, M. L. HOPWOOD, AND L. FAULKNER, Colorado State University, Fort Collins.

Alloxanized rabbits had elevated levels of seminal fructose diminished by administration of insulin (Mann and Parsons, Biochem. J., 46: 440. 1950). In the present study, the relationship between blood glucose and seminal fructose levels was examined. A Holstein bull was injected intravenously with 250 g of D-glucose. Blood and semen samples were obtained at intervals of 3 min to 24 hr following infusion and analyzed for glucose and fructose. Blood levels peaked at 633% above preinfusion levels in 5 min. Seminal fructose levels were maximal at 52 to 53% above preinfusion levels at 20 to 40 min after glucose administration. At 24 hr after injection the blood glucose was still 43% above the preinfusion level, whereas seminal fructose at this time was only elevated by 5%. No glucose was found in semen despite its elevated concentration in blood.

# BOOK REVIEW

THIN LAYER CHROMATOGRAPHY (TLC). J. M. Bobbitt. Reinhold Book Division, 430 Park Avenue, New York, New York. 208 pp. $8.50. 1963.

Dr. Bobbitt of the University of Connecticut has compiled a most useful book on the fundamental and applied aspects of TLC. This book will serve the beginner as well as the more mature researcher. It provides theory and working applications of experimental procedures in considerable detail, including reagents, binders, layer preparation, glass plates, separation equipment, development of chromatograms, and interpretations of Rf values. Analytical procedures include organic, inorganic, biochemical, and physical applications.

The historical aspects and personalities involved in the development of TLC are given concise, but adequate, treatment. This book will serve as a basic guide to the selection of techniques to solve specific problems. It sets forth clearly the applications and limitations of TLC.

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