Journal of
DAIRY SCIENCE

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CONTENTS

(Continued on inside front cover)

CONTENTS

(Continued from front cover)

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.[2] 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.

ORGANIZATION OF PAPERS

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

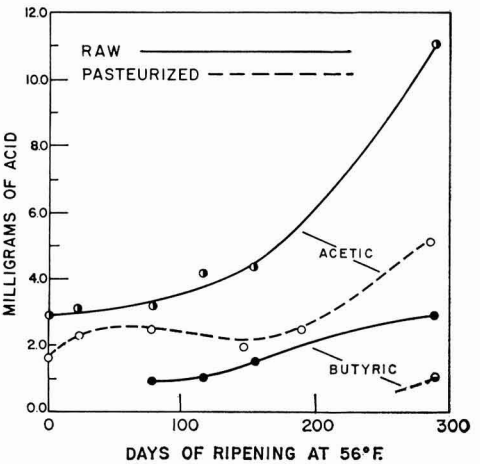

FIG. 1. Acetic and butyric acids in raw and pasteurized milk Cheddar cheese during ripening (milligrams in distillate obtained from 150 g of cheese oil).

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

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

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

Tables are costly. Use graphs whenever possible.

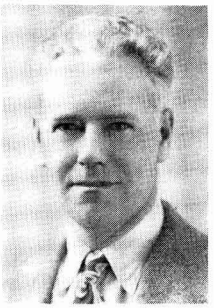
13. Colored illustrations can be reproduced. Authors should submit detailed specifications to the editor and costs will be supplied.
14. Abbreviations for titles of periodicals and for botanical, chemical, physical, mathematical, and statistical terms should conform to those in the Style Manual for Biological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

PEOPLE AND EVENTS

MEMORIALS

Samuel A. Lear

DR. S. A. LEAR, 53, professor of dairy industry at the College of Agriculture, Rutgers University, New Brunswick, New Jersey, died recently in Middlesex General Hospital after a long illness. He leaves his widow, Lucille; three children, Richard, 14; Janet, 12, and Bruce, 10; one brother and three sisters.



S. A. Lear

Dr. Lear won wide recognition in the milk industry for his research in milk enzymes and the bacteriology of milk. He taught courses in dairy administration, milk sanitation and milk testing, and assisted in a short course in ice cream making.

He was a native of Bucks County, Pennsylvania, and earned the Bachelor of Science and Doctor of Philosophy degrees from Pennsylvania State University and a Master of Science degree from the University of Minnesota. He was appointed to the Rutgers faculty in 1946.

Dr. Lear was recently president of the Metropolitan Dairy Society and was a member of several professional societies and fraternities.

James MacMillan Murphy

DR. J. M. MURPHY, research professor Veterinary Medicine at the University of Pennsylvania, died December 14, 1961, following major lung surgery. He was forty-nine years old.

Dr. Murphy was widely recognized as an outstanding authority on bovine mastitis. A native of Philadelphia, he obtained his V.M.D. degree from the University of Pennsylvania in 1935. The next 2 yr were spent at his Alma Mater, where he conducted research in periodic ophthalmia and bovine mastitis. He joined the faculty at Rutgers University as an associate professor of dairy husbandry in 1937 and remained at Rutgers until he accepted a position as professor of veterinary medicine at Cornell University in 1946. He left Cornell to rejoin the faculty at the University of Pennsylvania in 1959, where he was the director of the University's Basic Mastitis Research Project.

Dr. Murphy spent his entire professional life in the study of bovine mastitis. He was the author of over 40 papers devoted to his specialty. He also edited and published Mastitis Abstracts. In addition to his brilliant contributions in mastitis research, he served 4 yr as editor of The Cornell Veterinarian. He was a member of the American Dairy Science Association, the National Academy of Sciences, The New York Academy of Science, the National Institute of General Semantics, Phi Zeta honorary fraternity, the Northeastern Mastitis Council, The National Mastitis Workers' Conference, and The National Mastitis Council.

In addition to his sterling personal character, his colleagues credited Murphy with "one of the most brilliant research minds the scientific community has ever known. He had the rare ability to think logically and imaginatively and he was willing to settle for nothing less than perfection. His insistence upon factual data as the basis of hypothesis, the efforts of his work, the objectives that he set, and his systematic, analytical approach have made their marks on an untold number of other research workers."

As a token of the high esteem in which he was held by his Alma Mater, the laboratories he directed were dedicated as the James MacMillan Murphy Research Laboratories on June 1, 1962. The inscription on the dedicatory plaque is a fitting farewell to a beloved colleague, teacher and friend—"Dedicated to the memory of one who brought honor to his school as an alumnus and faculty member and to his profession as a research scientist and a gentleman."

He is survived by his widow, Helen, and their daughters, Betsy (Mrs. Edward Keough), Martha (Mrs. Alfred G. Tottey, Jr.), and Virginia.

Dr. George R. Greenbank, USDA Dairy Scientist, Retires

After 40 years of research on dairy products, DR. G. R. GREENBANK, a U. S. Department of Agriculture chemist, has retired. Dr. Greenbank, who has lived to see many of his original ideas on dairy processing adopted by the industry, worked in Washington, D. C., at the Dairy Products Laboratory of the Agricultural Research Service's Eastern Utilization Research and Development Division.

G. R. Greenbank

Dr. Greenbank's research established that dried milk for use in baking could be improved for this purpose and could be given greater storage stability by heat treatment,

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1932—July, September, November
1934—October
1938—November, December
1943—January, February, March, April, August, December
1946—January, March, April
1947—January, February, March, April, May, June, December

homogenization, and clarification. The veteran scientist also carried out extensive work on the keeping quality of milk fat. He pioneered the theory that milk acquires certain off-flavors as the result of a mild oxidation of one of its minor constituents. Other research of Dr. Greenbank's has contributed new knowledge about phosphatides and enzymes.

A native of Woodsfield, Ohio, Dr. Greenbank is a graduate of Ohio State University. After doing graduate work at Ohio State, Johns Hopkins University, and Massachusetts Institute of Technology, Dr. Greenbank received his Ph.D. degree from American University in 1930.

In 1949, Dr. Greenbank's research was recognized by the American Chemical Society, when it bestowed on him the coveted Borden Award in the Chemistry of Milk. The following year he received the U. S. Department of Agriculture's Superior Service Award. Dr. Greenbank is a member of the American Chemical Society and has served on a number of its important committees. He is also a member of the American Dairy Science Association and has been elected to the Washington Academy of Sciences. His name appears in Who's Who in Chemistry, Who's Who in Science and Industry, American Men of Science, Who's Who in the Southeast, and Who Knows What.

Dr. Greenbank is active in the Boy Scouts of America and in the Methodist Church. After a planned 6-month round-the-world tour, Dr. and Mrs. Greenbank plan to enjoy their six grandchildren and pursue their hobbies of floriculture and photography at their home in Arlington, Virginia. Dr. Greenbank may also devote some of his time to more research.

New Appointments at Missouri

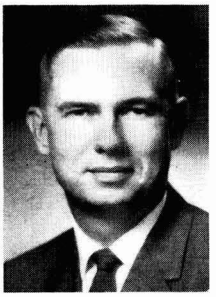
C. P. MERILAN, former chairman of the dairy husbandry department at the University of Missouri, has been named associate director of the University's Agricultural Experiment Station.

Merilan succeeds SAM B. SHIRKY, who retired September 1 at age 65, the University's mandatory retirement age for staff members in major administrative positions.

A native of Lesterville, Merilan received a B.S. degree from the University of Missouri in 1948; an A.M. in 1949; and his Ph.D. degree in 1952. He has specialized in teaching and research in dairy cattle breeding and nutrition, and in rumen bacteriology. He was named chairman of the dairy husbandry department a year ago.

Succeeding Merilan as chairman of the dairy husbandry department is J. E. EDMONDSON, who has been on the department staff since 1940.

Edmondson, a native of Springfield, received his B.S. and A.M. degrees from the University in 1939 and 1940, and his Ph.D. degree from Iowa State University in 1953. He has done research in mastitis and in product control and sanitation.



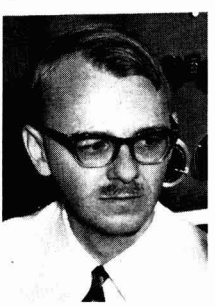
C. P. Merilan

J. E. Edmondson

Dr. Serge N. Timasheff, USDA Scientist, Named 1963 Winner of Borden Award in Milk Chemistry

A U. S. Department of Agriculture scientist, who solved a riddle about one of the proteins of milk that had baffled researchers for 20 yr, is to be honored for his work next year by the American Chemical Society.

DR. SERGE TIMASHEFF, of the Agricultural Research Service's Eastern Utilization Research and Development Division in Wyndmoor, Pennsylvania, will be the 1963 recipient of the Borden Award in the Chemistry of Milk. The announcement was made September 10 at the 142nd National Meeting of the American Chemical Society in Atlantic City, New Jersey. The Borden Award, consisting of $1,000 and a gold medal, will be presented to Dr. Timasheff at the Society's meeting next April in Los Angeles, where a symposium will be conducted in his honor.



S. N. Timasheff

Dr. Timasheff's research has been conducted on one of the principal proteins of milk known as beta-lactoglobulin. Because this protein exists in two almost identical forms, called beta-lactoglobulin A and beta-lactoglobulin B, it has yielded important clues on the relationship between a protein's chemical structure and its biological or physical behavior. By studying the A and B forms of beta-lactoglobulin, Dr. Timasheff has been able to cancel out their features that are exactly alike, and to explain their behavioral differences precisely on the basis of the very few amino acid residues that distinguish one form from the other.

Biologically, beta-lactoglobulin is a significant protein, since genetics determines whether the A form, the B form, or a combination of the two, will be found in the milk produced by a given cow. Dr. Timasheff discovered that the molecules of beta-lactoglobulin A, at low temperatures and within a certain acidity range, clump together in loose aggregates with four times the normal molecular weight. No such aggregation of the B molecules occurs.

The curious fact about beta-lactoglobulin (both A and B) is that when its solution is increased in acidity beyond a certain point, the molecules of the protein split in half. Then when the acidity is lowered again, the halves recombine. Through ingenious research, involving many precise analytical techniques, Dr. Timasheff was able to prove that in a mixture of beta-lactoglobulin A and B, the halves of the A molecules always rejoin halves of the other A molecules, never of B. Likewise, only B half-molecules rejoin each other. This indicates that the half-molecules of the two forms are joined in distinctive ways.

Born in Paris in 1926, Dr. Timasheff became an American citizen in 1944 and was graduated magna cum laude from Fordham University in 1946. He received his M.S. and Ph.D. degrees from the same university, where he also taught until 1950. He became a postdoctoral fellow at the California Institute of Technology in 1951, and at Yale University from 1951 to 1955. Since that time he has been a member of the staff at the USDA laboratories in Wyndmoor. In 1958, Dr. Timasheff won an American Chemical Society travel grant to the International Congress of Biochemistry in Vienna. In 1959 he was awarded a senior postdoctoral fellowship by the National Science Foundation for studies at the University of Strasbourg, in France.

Dr. Timasheff's wife, Dr. Marina Timasheff, is also a scientist at the Eastern Utilization Research and Development Division. The Timasheffs live at 705 E. Hartwell Lane, Wyndmoor, Pennsylvania.

New Appointments at North Carolina State College

Two recent staff additions to the Department of Food Science, North Carolina State College, are J. F. WILES and V. A. JONES. Mr. Wiles has assumed the position of Extension Industrial Engineer in Food Science and Mr. Jones is on the Food Science staff in the area of Food Engineering.

Mr. Wiles has a Bachelor of Industrial Engineering degree from North Carolina State College and has been working in industry for the past 5 yr. Mr. Jones is now in the process of completing his Doctor of Philosophy degree from Michigan State University.

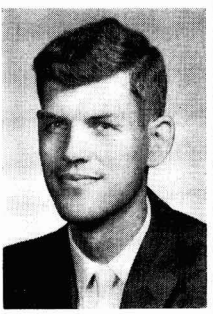
Mr. Wiles will work with the Food processing plants in North Carolina; Mr. Jones will have research and teaching responsibilities.

J. F. Wiles

V. A. Jones

Dr. Waldern Appointed at Washington State

A Canadian dairy nutritionist, DR. D. E. WALDERN, has just joined the staff of WSU's Department of Dairy Science. He will hold a joint research-teaching appointment with the rank of assistant professor and his principal assignment will involve teaching and research in nutrition.



D. E. Waldern

He has been a staff member of the Canadian Department of Agriculture's experimental farm at Prince George, British Columbia, for the past several years.

Dr. Waldern was awarded a Ph.D. degree in Dairy Science by Washington State University this past June. Subject of his doctoral research and thesis was the effect of diet on volatile fatty acid production and absorption in the bovine.

The new WSU dairy scientist was born at Lacombe, Alberta, Canada. He received both his B.S. and M.S. degrees from the University of British Columbia, Vancouver. He also attended high school in Vancouver, B. C.

Professor King Lectures at Iowa State

PROFESSOR N. KING, Division of Dairy Research, C.S.I.R.O., Highett, Victoria, Australia, was a guest of the Department of Dairy and Food Industry, Iowa State University, on August 2, 3, and 4. Professor King presented two seminars concerning his concept of the nature of the orientation of surface-active materials at the milk fat globule interface and his hypothesis of the mechanism of churning. In addition, he conferred with the members of the Department concerning topics of mutual interest.

Professor King has been a member of the Dairy Technology staff at the University of Illinois since February 1, 1962. He returned to Australia on August 15 by way of Copenhagen, Denmark, where he participated in the program of the International Dairy Congress which was held September 3 to 7.

David Wieckert Appointed to Southern Illinois University

DAVID WIECKERT, Appleton, Wisconsin, is a new assistant professor of animal industries at Southern Illinois University, effective September 15, 1962. He is a doctoral candidate (1963) at the University of Wisconsin. He received his Bachelor's and Master's degrees from Wisconsin in 1952 and 1956, respectively.

His doctoral problem has been a genetic study of the well-known Pabst Farm Herd of Holstein cattle in Wisconsin. Wieckert's specialization is dairy breeding. He held a Fulbright research grant in 1957-58 for ten months of study in New Zealand in the field of dairy cattle physiology. For 1 yr, 1956-57, he served as a 4-H extension agent in Wisconsin. He is a member of the American Dairy Science Association and the American Society of Animal Production.

Professor E. O. Anderson Assumes New Position in Connecticut

E. O. ANDERSON, Emeritus Professor of Dairy Manufacture, University of Connecticut, was recently appointed to the newly created post of Food Quality Control Specialist.

Professor Anderson, who retired after 30 yr of service at the University of Connecticut, will work with the dairy industry in the state to help maintain and protect the natural flavor of milk from the cow to the consumer. The United States Department of Agriculture and the University of Connecticut will cooperate with the department in this endeavor. Professor Anderson will also assist dairy organizations in the setting up of milk-testing panels, and will organize milk flavor clinics for plant employees and farmers.

Dairy Fieldmen's and Dairy Plant Operators' Conferences at Purdue

F. N. ANDREWS, Head of the Animal Sciences Department at Purdue University, and F. J. BABEL, professor in charge of the Dairy Manufacturing Section, have announced two, one-day meetings to be held in November, 1962, in the Memorial Center at Purdue University. The Dairy Fieldmen's Conference will be held on November 13 and the Dairy Plant Operators' Conference on November 14. The conferences are an annual affair sponsored in cooperation with the Indiana Dairy Products Association.

The Dairy Fieldmen's Conference will include papers on High Corn Silage Rations, Recent Developments in Milking Systems, Comparisons of Daily Fresh Milk Tests and Stratified Random Fresh Milk Tests, The Milk Flavor Problem, and Solids Testing from the Producer's Point. The program will also include a discussion of The Cooperative Extension Service and a report on the World's Dairy Congress and European Dairying.

The Dairy Plant Operators' Conference is to include discussion on Quality Programs for Manufacturing Grade Milk, The Dairy Industry and Governmental Policies, Effect of the European Common Market on Midwest Dairying, Processing Sterile Products, Variations in Total Solids and Protein Content of Bulk Tank Producer Milk, and a discussion on Recent Developments in Dairy Product Packaging.

Short Course at Wisconsin

A special 3-wk winter dairy course in cheese-making will be offered by the University of Wisconsin department of dairy and food industries November 26 to December 14.

The lecture portion of the course will feature principles of cheese-making, milk composition and testing, dairy arithmetic, dairy mechanics, dairy bacteriology, starter cultures, and dairy sanitation. Using the University's facilities in Babcock Hall, the students will gain experience in the actual manufacture of many varieties of domestic and foreign type cheese, as well as Cottage and other soft types of cheese.

An examination for a Wisconsin cheesemaker's license will be given by the Wisconsin State Department of Agriculture for qualified students at the end of the course.

The course is open to both residents and nonresidents of Wisconsin who have completed high school or its equivalent. For further details write H. E. Calbert, chairman, Department of Dairy and Food Industries, University of Wisconsin, Madison, Wisconsin.

Completed Theses

M.S. Degree:

ROBERT RAY WILSON. Weight and volume relationships in fluid dairy products. A. and M. College of Texas.

ASTON S. WOOD. Nitrofurans in infant dairy calf diets. University of Minnesota.

Ph.D. Degree:

WILLIAM A. OLSON. Factors influencing utilization of lipid material by the dairy calf. University of Minnesota.

ABDUL WAHAB QURESHI. Genetic trends in milk and butterfat production of Texas Dairy Herd Improvement Association cows. A. and M. College of Texas.

American Association for the Advancement of Science to Sponsor Symposium

A symposium on food quality as affected by production practices and processing will be sponsored by the AAAS in Philadelphia, Pennsylvania, on December 27, 1962. DR. R. E. HODGSON, Director, Animal Husbandry Research Service, USDA, Beltsville, Maryland, will preside over the section concerned with Dairy Products. The program is as follows:

The Evaluation and Measurement of Quality of Dairy Products. V. H. NIELSEN, Professor and Head of Department of Dairy and Food Industry, Iowa State University, Ames, Iowa.

Genetic and Environmental Factors in Development and Performance. NED D. BAYLEY, Assistant Director, Animal Husbandry Research Division, Agricultural Research Service, USDA, Beltsville, Maryland.

Protection of Milk Quality Through Management Practices. W. M. ROBERTS, Professor and Head of Food Science Department, North Carolina State College, Raleigh, North Carolina.

Effects of Processing Factors on Quality of Dairy Products. STUART PATTON, Professor of Dairy Science, Pennsylvania State University, University Park, Pennsylvania.

Similar sections will be devoted to cereal grains, poultry and eggs, meats, and fruits and vegetables.

Forage Symposium Program

A symposium program on The Economics and Role of Forages in Dairy and Beef Production has been scheduled by the Pastures and Forages Program Committee of the American Society of Animal Production. This symposium is part of the annual meeting program of the American Society of Animal Production and will be held on the afternoon of November 24, 1962, at the Sherman Hotel in Chicago, Illinois. The symposium will consist of six papers by agricultural economists, animal nutritionists, and one agronomist, covering various phases of forage production and utilization. These are as follows:

Economic Evaluation of Forages for Dairy Cattle. C. R. Hoglund, Michigan State University; Forage and Nutritional Factors Influencing Potential and Economic Use of Forage for Dairy Animals. M. E. McCullough and J. R. Russell, Georgia Experiment Station; Irrigated Pastures, an Energy Source of Supplemental Feed. J. H. Mayer and J. L. Hull, University of California; Roughage Testing, Its Implications and Values. J. R. Baylor and co-authors, Pennsylvania State University; Measuring Economic Impacts for Modern Forage Technology. N. C. Hadley, Purdue University; Economics of Forage Mechanization. R. N. Van Arsdall, University of Illinois.

STUDENT NEWS SECTION

W. W. SNYDER, Editor

A Section Devoted to News of Student Members

A.D.S.A. Invests in the Future

The American Dairy Science Association is very much interested in dairy students—the future leaders of our Association. An example of this interest can be shown by outlining the purposes and activities of one of the committees which functions as a unit of A.D.S.A. The Student Affiliate Committee, composed of nine members of the American Dairy Science Association, is appointed by the President of the Association. The prime function of this committee is to create student interest in the activities of the Association. Its objectives may be stated as follows:

1. To develop student appreciation of the magnitude of the Dairy Industry, the opportunity it offers, and the training required for success in the field.
2. To acquaint students with the American Dairy Science Association, its scope and purposes and the role of student affiliate members in this over-all program.
3. To encourage closer relationships between students, faculty, industry, and other educational institutions.
4. To work with the National Student Branch, encourage strong local affiliate chapters, and coordinate the student activities with those of the parent association.

The Student Affiliate Committee sponsors many activities in an effort to accomplish these objectives. One important activity is the promotion of the Student Chapter News Section in the JOURNAL, which serves to keep dairy students informed and bind them closer together. Other activities include:

1. To promote student affiliate memberships.
2. To conduct the student chapter activity contest during the annual meeting.
3. To conduct the Graduate Student Scientific Paper Presentation Contests.
4. To advise the National Student Branch officers and aid in planning and conduct of the annual convention.

A series of articles giving additional information on the chapter activity contests, the National Student Branch, and the Graduate Student contests will appear in future issues

of the JOURNAL. The officers of each affiliate club are encouraged to read these articles and discuss them at a club meeting.

L. J. BOYD, Chairman
Student Affiliate Committee

SPOTLIGHT

[This is the first in a series of feature articles to appear under this heading. The purpose of these articles is to help all clubs improve their activities. The officers and advisors should be willing to study these reports in order to achieve this purpose.

The Dairy Science Club of the University of Georgia was selected as the first affiliate club to be honored, because of its outstanding record in the club achievement award program. The spotlight club won the A.D.S.A. trophy at the annual meeting for two successive years, 1961 and 1962. We salute the Dairy Science Club of the University of Georgia for a job well done.—W .W. Snyder]

The University of Georgia's Dairy Science Club

The Dairy Science Club at the University of Georgia had its beginning in 1946 when enrollment began the post-war surge. Since the Dairy Department had been officially organized just 5 yr earlier, almost at the beginning of the war, 1946 really was the beginning of its student program. Olin C. Rhodes, currently district sales manager for Nopco Chemical Company, was the first president of the club. He was followed by Richard Sendelbach, who today is a district sales manager for Foremost Dairies. Thirty-five students were charter members.

The purpose of the Club from its beginning has been threefold:

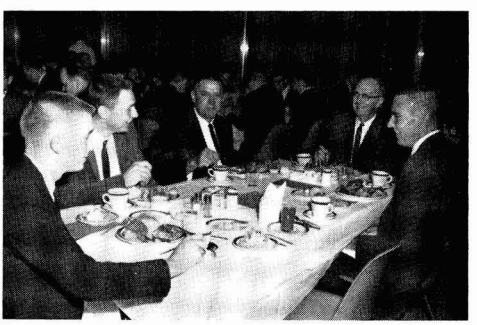
1. To promote understanding among dairy students and other students interested in any phase of dairying.
2. To promote fellowship among dairy students and between the dairy department staff and the students.
3. To be of service to the Dairy Department and to the dairy industry.

To say that the Club has been successful in the above aims would be an understatement. The Dairy Club is an essential part of the Dairy Department, and probably no other department in the University has such a cosmopolitan group—geographically as well as field of interest—and at the same time enjoys such a close working relationship among its students. Likewise, the interest and support of industry in the activities of the department are commendable, most of which can be attributed to the Dairy Science Club.

In the current organization several staff members serve as advisors, thus dividing the areas of responsibility. Prof. H. B. Henderson serves as general advisor and works with all of the committees. Dr. Olin T. Fosgate works with the program committee and assists in scheduling events during the year. Mr. Robert E. Roberts works with the staff of the Georgia Dairyman, the scrapbook committee, and coordinates the social activities of the Club. Dr. Carl Clifton works closely with the Dairy Day committee.

The major activities of the Club are handled through committees. These committees work with the advisors in developing the project for which each committee is responsible. At the semimonthly meeting of the Club, the president calls for a report from each committee. The vice-president is an ex-officio member of all committees.

The social program of the Club is varied enough to keep the students interested, but not so extensive as to be too time-consuming or expensive to the average student. The year is started each fall with a chicken barbecue sponsored by a local dairy. This is the time when students can get acquainted with each other, the faculty, University administrators, etc. This is followed by the fall banquet just before the conclusion of the fall quarter. At this time, scholarship awards are made and a large number of industry people are present. The spring banquet concludes the year's activity. At this time the Georgia Dairy Princess and the American Dairy Princess are always present. It is the time, too, for presentation of the Honorary Member of the Year, and the dedication of the annual.



Parents and students enjoy the fellowship of the Dairy Science Club banquets.

Miss Louise Knolle, American Dairy Princess, and Miss Marlyn Smith, Georgia Dairy Princess, with the President of the Dairy Science Club, Mr. David Hoyer.

The Annual published by the Club has been of great value. Named "The Georgia Dairyman" by the members of the Club in 1946, it has been published annually since 1947. The editor of the first edition was Jim Stone, who today is owner of a hardware and building supply firm. From the very beginning, the aim was to publish a very high-class annual, which has been accomplished only because of the support of the people who advertised in it. Thus far, the publication has never lost money although, most of the time, very little is left, after all expenses are paid.

"Let's get this job done," seems to be the central theme as these Dairy Science Club leaders get together to plan the annual publication, "The Georgia Dairyman."

An important aspect of the student-industry relationship has been the Honorary Member program. One member from the industry is elected to honorary membership each year. Generally, this is a person who has shown some special interest in the Club and the activities of the Dairy Department. The 1961-62 honorary member, for example, was Mr. George Farrar, an outstanding livestock auctioneer, who serves as auctioneer for the annual Dairy Day heifer sale. It should be noted that Mr. Farrar has never charged the Club for this service.

Another aspect of the student department–industry relationship is exemplified by the presentation at the fall banquet of Certificates of Appreciation to industry people who have assisted the department with some aspect of its program during the past year. These certificates go to firms and individuals who have made contributions to the Dairy Scholarship Fund, made grants to the department for research, or in some other way assisted the program of the department.

Officers of the Club are elected for terms of one-half the school year. Such an arrangement provides opportunity for more students to obtain leadership experience. Likewise, it creates more student interest. A new president may have ideas which he has been accumulating for a long time. He has four and a half months in which to accomplish his program. Thus, he works hard, and gets his classmates to work hard.

One of the early projects of the Club was the establishment of a scholarship fund. This was proposed by Mr. George Griffin, for several years now in public health work in Douglas, Arizona. From a meager beginning with Mr. Griffin's $3 contribution, the fund has grown to more than $1,700. It was the intent of Mr. Griffin and the other members of the Club at that time, that this fund be used only for emergencies, such as when some student was going to drop out because of financial difficulties but who could be kept in school by a contribution of a small sum of money. It has been used only twice, but for two very deserving young men.

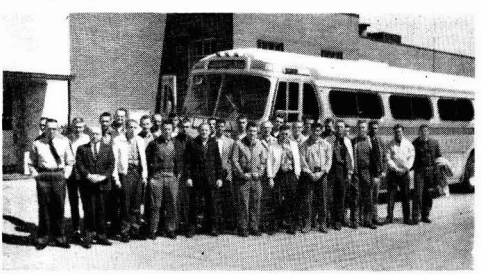
The Club has several money-making projects. The major one is the Dairy Day program held annually the last Saturday in April. As a part of this program 36 dairy heifers are auctioned to certified 4-H and FFA members. The animals are consigned by breeders in the Southeast and represent the three major breeds in this area. The Dairy Science Club receives a commission of 10% of the gross. The net profit from the sale has been approximately $500 each year. The publication of The Georgia Dairyman also nets a small amount of profit. A concession stand at Dairy Day generally nets a nice profit. Recently, the Club took on a project of selling subscriptions to dairy magazines, which proved rather profitable. Club dues are $1.50 per year. The total receipts for last year were approximately $12,000; expenditures amounted to more than $11,000.

The Dairy Science Club meets twice each month during the school year. Variety in the type of program is intended, to create interest. Programs range from talks by dairy industry

Mr. Emory Cocke, President of Suni Citrus Products Company, Haynes City, Florida, a benefactor of the Dairy Department and the Dairy Science Club, receives a Certificate of Appreciation from Dean C. C. Murray, Dean and Coordinator College of Agriculture, at the Dairy Science Club Banquet.

leaders, University officials, movies (even including cartoons), to tours of other departments in the University. An important feature of all programs is that the students run the show, and that no longer than the allotted 1 hr is used for a meeting. Refreshments are always served, which provides opportunity for fellowship following the program.

Perhaps the high light of the over-all program of the Club is the biennial trip for Juniors and Seniors. The major portion of the expense is borne by the Club from the profits of the dairy heifer sale. With a profit of about $500 annually, this provides about $1,000 for each trip. This year the tour was held during the break between the winter and spring quarters and included visits to some 15 dairy establishments in Georgia, Tennessee, and North Carolina. More than 30 students made the 1,200-mile trip by chartered bus during the five-day period. Seniors who made the trip acclaimed it the high light of their college program.



Ready to board the bus for the biennial tour of dairy plants and farms in the Southeast. More than 30 Juniors and Seniors made the trip.

FROM THE SECRETARY'S DESK

Classification of New 1962 Affiliate Membership

It is of interest that of 255 new 1962 affiliate members, 60% are graduate students and 40% undergraduate. This is different from previous years. From 1956 through 1960 graduate students varied from 34.8 to 54%. The 1962 figures result from a recent survey made by your Secretary, whereby it was found there were more graduate students in dairy technology getting advanced degrees in 1961 than there were undergraduate students getting B.S. degrees.

Of the 1962 new undergraduate student affiliates, there were seniors, 18%; juniors 16%; sophomores, 4%; freshmen, 2%.

Of the 275 new 1962 members as of August 21, 1962, 66, or 24%, were student affiliates in 1961.

VOLUMES OF THE JOURNAL OF DAIRY SCIENCE FOR SALE

1. Complete set starting with 1928, Volumes 11 through Volume 34 (1951). These are unbound with the exception of 1931 through 1935, which are bound. Contact Dr. R. V. Hussong, Research and Development Division, National Dairy Products Corporation, 801 Waukegan Road, Glenview, Ill.

ADVERTISERS' INDEX

SOME CHANGES IN CATION COMPOSITION OF MILK CAUSED BY ION EXCHANGE TREATMENT [1]

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SUMMARY

Solutions containing various concentrations of calcium, magnesium, sodium, and potassium have been used for the conditioning of ion exchange resins, the resins then being used for the removal of radiostrontium and/or radiocesium from milk. Using the batch technique at a milk:resin ratio of 20, resins in the calcium form caused an increase in calcium concentration in the milk of about 22 meq/liter balanced by losses in sodium, magnesium, and potassium. Likewise, sodium resin treatment increased the sodium concentration about 59 meq/liter of milk, balanced by losses in calcium, magnesium, and potassium. Resins conditioned with solutions containing mixtures of these four elements caused changes in the composition of milk which reflected largely the concentration of that element in the regenerant. In general, the changes were minimized when the resin regenerant composition, on the basis of meq-fraction, approached that of milk. The concentration of regenerant, between 1,200 and 1,700 meq/liter had but little influence. Approximately 50% of the radiostrontium and 60% of the radiocesium were removed by this method. By the use of a column technique the strontium removal was shown to be more constant than the cesium removal. The concentration of Ca, Mg, Na, and K in the milk changed as the milk continued to flow through the column.

The exposure of milk to ion exchange resins in the calcium form has been shown to be effective in the removal of radiostrontium from milk (3, 4, 11). Resins in the sodium form have been used to decrease the calcium content of milk for stabilization of evaporated milk (6). Nervik et al. (11) used a sodium resin for removal of radiostrontium from milk and found an accompanying decrease in the calcium concentration. Easterly et al. (3) found an increase of about 22% in the calcium concentration of milk by treatment of milk with a calcium-resin at a milk:resin ratio of 20. Migicovsky (9) found no change in the concentration of calcium, sodium, or potassium in milk after ion exchange treatment when the resin was regenerated with a solution containing these elements in the same ratio as present in milk. Landgrebe et al. (8), however, state that because the cations in milk exist in the form of several complexes, one cannot use the concentration in the milk as the most effective concentration to use on the resin. By lowering the pH of milk to 5.2, followed by treatment of the milk with a resin conditioned with a mixture of Ca, Mg, Na, and K ions, Murthy et al. (10) were able to remove larger amounts of radiostrontium and radiocesium than when the milk, at its normal pH, was treated with a resin. The present study deals with some of the effects upon milk of calcium-resin treatment, sodium-resin treatment, and treatment to a resin conditioned with solutions containing various concentrations of calcium, magnesium, sodium, and potassium ions. No attempt was made to alter the pH of the milk.

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[1] This manuscript is published with the permission of the Director of the University of Tennessee Agricultural Experiment Station, Knoxville, Tennessee. The radioactive materials used in the work were obtained from the United States Atomic Energy Commission. The work was completed under Contract No. AT-40-1-GEN-242 between the University of Tennessee College of Agriculture and the Atomic Energy Commission.

CALCIUM RESIN TREATMENT

Procedure. Five grams of Dowex 50 W-X12, in the hydrogen form as received, was washed several times with 100-ml portions of 10% $CaCl_2$, then with water until the wash water was negative to the chloride test using $AgNO_3$. One hundred milliliters of pasteurized, homogenized, vitamin D-fortified milk was agitated

with this resin for 15 min at approximately 15 C. After allowing the resin to settle, the milk was filtered and analyzed on a Beckman DU flame spectrophotometer for sodium and potassium, using wave lengths of 589 and 768 mμ, respectively. Samples prepared by the method of Fiske and Subbarow (5) were analyzed for phosphorus by the use of a colorimeter. Calcium and magnesium were determined by the method described by Kamal (7). Samples of the untreated milk were analyzed also.

Results. Data in Table 1 show that the ion exchange treatment as used in these trials increased the calcium concentration 21.6 ± 3.3 meq/liter of milk, or about 37%. This increase was balanced by decreases in magnesium, sodium, and potassium. The phosphorus content of the control milk was essentially the same as that of the treated: 956 ± 34 and 944 ± 27 ppm, respectively.

SODIUM RESIN TREATMENT

Procedure. Four lactating Jersey cows were each given a single oral dose of 1 mc of Cs^{131} and six mc of K^{42} at approximately 2 PM. Seven consecutive milkings, beginning with the milk obtained the morning following dosing, were used for these trials. Milk from the four cows was mixed together and a composite taken. Trial I was conducted in October and Trial II, using four other animals, was conducted in January. Assuming a 35% counting efficiency for Cs^{131} and 50% for K^{42}, and with a background count of 550 per minute, the level of Cs^{131} in the milk ranged from about 0.24 to 6.4 μc/liter of milk and K^{42} ranged from 0.16 to 0.62 μc/liter. These minimums are the approximate lower limits of reliable detection by the instruments used under the experimental conditions.

The batch procedure used was as described above, except 10% NaCl was used instead of $CaCl_2$. Duplicate 3-ml samples of milk obtained before and after treatment were analyzed for gamma activity by use of a scintillation counter. The samples were then stored at approximately 40 F and counted 1 wk later. The original count was due to both Cs^{131} and K^{42}. The second count was due to Cs^{131} only; the K^{42} having decayed to a stable state (half-life of K^{42} is 12.5 hr; that of Cs^{131} is 2.3 yr). The K^{42} count was then corrected to compensate for decay during the counting of the samples. Fresh resin was used for each sample of milk. The effects of such resin treatment upon the Na, K, Ca, and Mg content of the milk were determined, using another sample of milk. For radiological safety reasons no sample containing added radioactivity was analyzed on the flame photometer.

The ion exchange columns used were 2.0 cm in diameter and each contained 10 g of resin in the hydrogen form as received in shipment. The resin was supported by a layer of Ottawa sand approximately one-half inch in thickness. Five hundred milliliters of a 10% solution of NaCl were passed through each column. The pH of the influent and the final effluent were the same. Seven hundred milliliters of distilled water was then passed through the column. This quantity was sufficient to give a negative chloride test in the effluent.

Two hundred milliliters of milk were passed through each column. The first 10-ml portion of effluent was discarded due to its dilution with water in the column. Duplicate 3-ml samples from the 2nd, 6th, 10th, 15th, and 20th 10-ml portions of effluent were analyzed for the concentration of Cs^{131} and K^{42} and compared to the composition of milk not passed through the column.

TABLE 1

Changes in composition of milk treated to a calcium resin [a]

	Before treatment	After treatment	Change
	(meq/liter)		
Na	24.4 ± 2.0 [b]	21.0 ± 1.9	− 3.4 ± 0.4
K	36.2 ± 0.6	23.0 ± 0.5	−13.2 ± 0.5
Ca	58.6 ± 3.1	80.2 ± 3.2	+21.6 ± 3.3
Mg	10.6 ± 1.8	7.4 ± 2.5	− 3.2 ± 1.9
Net	129.8	131.6	+ 1.8

[a] Seven trials on Na, K, and Ca; six on Mg.

[b] Standard deviation $= \sqrt{\frac{\Sigma x^2 - \frac{(\Sigma X)^2}{N}}{N-1}}$.

Regeneration was accomplished by rinsing the column with distilled water until all traces of milk were removed, followed by the NaCl solution and water as described above. The same columns were used for seven consecutive milkings from the dosed cows.

Results. The data in Table 2 show that about 74% of the Cs^{131} and about 70% of the K^{42} present in milk is removed by the batch ion exchange techniques used in these experiments. The percentage removals were rounded to the nearest whole number, except where that would have resulted in a figure of 100% removal.

Analysis of the eight samples of milk showed an increase in Na content of 59.2 ± 4.6 meq/liter and losses in Ca, Mg, and K of 31.1 ± 1.2, 4.8 ± 1.4, and 22.9 ± 1.3, respectively. The concentration of an element in the untreated milk and the loss of the element due to the treatment were shown to have correlation coefficients of 0.87 for Ca, 0.98 for Mg, and 0.68 for K. The pH of the milk was increased from 6.8 to about 7.3, due to the treatment.

Comparison of the two methods shows the column to be more efficient in isotope removal than the batch technique (Table 2). The removals of K^{42} in the second trial were slightly less than those in the first trial, which might be attributable to differences in the milk due to slightly different feeding conditions or differences between the animals.

MIXED ION RESIN REGENERANTS

Due to the alterations in milk composition by treatment with resins either in the Ca form, as shown previously (3), or the Na form, as shown above, experiments were conducted using regenerant solutions made up of mixtures of the four most abundant cations in milk.

Procedure. Four series of solutions for resin regeneration were formulated using the experimental design described by Box (1). Twenty-five different solutions comprised each series. The first series contained HCl, added to keep the total ionic strength of all solutions constant at 1,400 meq/liter, since the work of Strelow showed that the amount of a specific ion taken up by a resin is partially dependent upon the ionic strength of the regenerant solution (12). The pH of the solutions in Series I was approximately 1.0, except in the one solution which required no HCl to maintain constant ionic strength.

Milk used throughout the remainder of the study was reconstituted nonfat dry milk solids made up to 9% total solids approximately 20 hr before resin exposure. The use of the same milk source eliminated variations in the ratio of the elements in the milk before resin treatment. The batch procedure as described above was used. When radionuclides were used they were added at the time the milk was reconstituted. Sr^{89} was added at the rate of about 0.6 μc/liter, Sr^{85} at the rate of 12.4 μc/liter, and Cs^{134} at the rate of 1.8 μc/liter. Sr^{89} was determined by the oxalate method (2); Cs^{134} and Sr^{85} by counting 3-ml liquid samples in a scintillation well counter.

TABLE 2

Per cent Cs^{131} and K^{42} removed from milk by use of a resin in the sodium form

Milking after dosing	Batch method		10-ml portions through column									
			2nd		6th		10th		15th		20th	
	Cs	K	Cs	K	Cs	K	Cs	K	Cs	K	Cs	K
						Trial I (4 cows)						
2nd	75	68	99	99	99	99	99	99	99	98	99	95
3rd	75	77	99	99	99	99	99	97	99	97	98	99
4th	74	72	99	99	99	98	99	99	99	97	98	95
5th	74	83	99	98	99	99	99	92	99	93	97	95
6th	75	70	99	98	99	99	99	96	98	99	98	95
7th	75		99		99		99		99		99	
8th	74		99		99		99		99		96	
						Trial II (4 cows)						
2nd	74	64	99	99	99	99	99	97	98	96	96	91
3rd	73	69	99	99	99	96	99	94	99	93	97	93
4th	73	74	99	99	99	98	99	98	97	99	94	92
5th	73	59	99	99	99	96	99	98	98	99	96	99
6th	74	72	99	99	99	99	99	88	97	85	95	80
7th	73		99		99		98		98		94	
8th	73		99		99		99		99		94	

Results. The low pH of the first series of regenerant solutions caused the pH of the treated milk to be reduced from the original 6.7 to as low as 5.85 and increased the titratable acidity from 0.16 to as high as 0.23%. Thus, the use of acid to maintain constant ionic strength was not considered advisable, and the ionic strength of the other series of solutions was not constant. The range of each of the elements in each series is shown in Table 3.

The removal of Sr^{85} from milk by resins conditioned with solutions in Series I ranged from 55 to 70% and Cs^{134} removal was 54 to 69%.

The pH of regenerant solutions in Series 2 ranged from 6.0 to 7.8 and the milk treated with resins conditioned with these solutions was lowered not more than 0.1 pH unit. All solutions in this series, however, caused an increase of 2.0 to 7.5 meq/liter in the calcium content of the milk. Removal of Sr^{85} was 49-56%, and removal of Cs^{134} was 56-60%.

Milk treated with resins conditioned with solutions in Series 3 had virtually the same pH as before treatment, but most samples lost Na and K and gained Ca and Mg. The removal of Sr^{89} ranged from 34-51%, and the removal of Cs^{134} ranged from 59-67%. The standard deviation for within treatment variation for strontium removal was 3.89 and for cesium removal was 1.71.

Solutions in Series 4 were formulated to cover a wider range of each of the four elements. The pH of milk treated with resins conditioned with any solution in this series was not lowered more than 0.1 pH unit. Sr^{85} removal ranged from 48-54%, and Cs^{134} removal was 59-63%. The standard deviation for within treatment variation for strontium removal was 1.14 and for cesium removal was 0.66.

A least-squares fit was made for the data from Series 4, using a model containing all linear, quadratic, and interaction terms for the four variables in the regenerant solution. Using only those terms with which statistically significant contributions are associated, the relationships are:

$$Y_1 = 2.05 + 1.46x_1 - 0.58x_2 - 0.51x_3 - 1.23x_4$$
$$Y_2 = 0.014 + 0.114x_1 + 0.035x_2 - 0.045x_3 - 0.134x_4 - 0.007x_1^2$$
$$Y_3 = -2.02 - 0.42x_1 + 0.53x_3$$
$$Y_4 = -1.29 - 0.72x_1 + 1.30x_4$$

where Y_1, Y_2, Y_3, and Y_4 are the changes noted in the composition of milk in meq from pre-resin to post-resin treatment for Ca, Mg, Na, and K, respectively, and x_1, x_2, x_3, and x_4 are the coded values for levels of Ca, Mg, Na, and K, respectively (Table 4) in the regenerant solution. The standard errors for the estimation of the partial regression coefficients in these experiments for Y_1, Y_2, Y_3, and Y_4 were 0.078, 0.006, 0.135, and 0.172, respectively, for the linear terms and 0.379, 0.029, 0.662, and 0.842, respectively, for the intercept. The quadratic coefficient for Y_2 had a standard error of 0.009.

The use of these equations might be illustrated by calculating the expected change in K concentration in milk by assuming a resin to be conditioned with a solution containing 420 meq Ca (−2 level of Table 4), 610 meq K

TABLE 3
Range in composition of mixed regenerants

Series	Ca	Mg	Na	K	HCl	Total
	(meq/liter)					
1	420–580	0–160	180–340	320–480	0–320	1,400
2	600–760	45–125	185–265	320–480	0	1,150–1,630
3	500–740	45–125	190–310	320–480	0	1,055–1,655
4	420–820	5–165	200–400	290–610	0	915–1,985

TABLE 4
Concentration of ions used in resin regeneration solutions in Series 4

Ion	Interval between levels	Coded value				
		−2	−1	0	1	2
	(meq/liter)					
Ca	100	420	520	620	720	820
Mg	40	5	45	85	125	165
Na	50	200	250	300	350	400
K	80	290	370	450	530	610

(+2 level), 5 to 165 meq Mg, and 200 to 400 meq Na. The concentration between these ranges of Mg and Na in the regenerant solution did not influence the amount of K gained or lost from the milk; therefore, it does not enter into the calculation.

$$Y_4 = -1.29 - 0.72x_1 + 1.30x_4$$
$$= -1.29 - 0.72\,(-2) + 1.30\,(2)$$
$$Y_4 = 2.75 \text{ meq K change}$$

The standard deviations based on the milk-treatment interaction for Ca, Mg, Na, or K change (meq/liter) in milk were ± 0.64, ± 0.08, ± 0.86, and ± 0.67, respectively.

Interactions of ions were not statistically significant in these experiments. Most of the changes noted in milk composition had a linear relationship with the concentration of that ion in the regenerant solution. The correlation coefficients between the meq fraction of the regenerant made up by a particular element and the change in concentration of that element in milk due to resin treatment was: for calcium, 0.86; for Mg, 0.11; for Na, 0.83; and for K, 0.78.

The concentration of total ions in the regenerant between the limits of 1,200 and 1,700 meq/liter did not influence to any great extent the change in milk composition or removal of radiostrontium or radiocesium. The correlation coefficients between the total ionic strength of the regenerant on the one hand and changes in milk composition on the other hand were: Ca −0.006; Mg −0.037; Na −0.31; K +0.07; total ionic change −0.17; % Sr^{85} removal, + 0.018 and % Cs^{134} removal, −0.394. Although slightly different concentrations of Ca, Mg, Na, and K in different milks exist, the general relationships expressed in these equations would be expected to apply.

A column technique was employed to determine changes in milk composition as the quantity of effluent increased. Twenty grams of Dowex 50 W X-12 in the hydrogen form was conditioned with two liters of regenerant solution passed downward through the column at a rate of 2 ml per min, followed by two liters of distilled water. The milk effluent was collected in 100-ml portions and analyzed for changes in composition and removal of Sr^{85} and Cs^{134}. Two different regenerants were tested in this manner; Regenerant A, the same as + 1 Level in Table 4, contained 720 meq Ca, 125

TABLE 5

Effect of ion exchange resin column treatment upon composition of milk and the removal of Sr^{85} and Cs^{134}

Effluent from column	Sr^{85}	Cs^{134}	Ca	Mg	Na	K
			Regenerant A			
			No. of trials			
	4	4	12	11	3	3
100-ml	% Removed		meq/liter change			
2nd	49.2	99.9	+1.08	−0.16	+1.52	+0.21
3rd	49.3	99.6	+1.11	−0.15	0	+0.40
4th	48.4	98.8	+0.75	−0.08	+0.30	+0.36
5th	47.9	95.1	+0.71	−0.02	+0.50	+0.30
6th	48.0	86.0	+0.54	+0.07	+0.10	−0.13
7th	47.3	69.5	+0.66	+0.16	0	−0.13
8th	47.4	44.6	+0.96	+0.02	−0.15	−0.55
9th	47.1	26.8	+0.96	+0.16	−0.15	−0.08
10th	47.2	9.7	+0.71	+0.13	+0.19	0
			Regenerant B			
			No. of trials			
	2	1	7	7	4	4
	% Removed		meq/liter change			
2nd	50.9	99.9	+2.79	−0.04	+1.30	0
3rd	50.7	99.9	+2.71	0	−0.20	+0.30
4th	51.1	98.9	+2.71	+0.04	−0.20	0
5th	50.7	94.3	+2.21	+0.10	−0.10	−0.15
6th	50.2	84.6	+1.93	+0.16	−0.10	−0.15
7th	49.5	62.4	+1.86	+0.18	0	−0.15
8th	49.8	37.7	+1.71	+0.17	−0.20	−0.30
9th	48.7	19.7	+1.29	+0.12	+0.01	−0.15
10th	49.2	9.9	+0.86	+0.08	+0.01	−0.10

meq Mg, 350 meq Na, and 530 meq K/liter, and Regenerant B, less concentrated, contained 644 meq Ca, 88 meq Mg, 300 meq Na, and 435 meq K/liter. After passage of the milk, the columns were flushed with distilled water, followed by 500 ml of 0.1 N HCl, again flushed with water, and reconditioned with the regenerant solution.

Results listed in Table 5 show the strontium removal to be lowered only slightly as the milk passes through the column. The cesium removal, however, is much less efficient as the quantity of milk passed through a column increases.

Regenerant B contained less calcium than Regenerant A, yet the calcium addition to the milk is greater with resin regenerated with Solution B than with Solution A. The suggestion is made that because Regenerant B contained a larger percentage of the total meq in the form of calcium (43.8) than did Regenerant A (41.7), more calcium was taken up by the resin from Solution B than from Solution A.

ACKNOWLEDGMENT

The authors express their appreciation to D. G. Easterly for a portion of the laboratory analysis reported in this paper.

REFERENCES

(1) Box, G. E. P. The Exploration and Exploitation of Responses Surfaces: Some General Considerations and Examples. Biometrics, 10: 16. 1954.

(2) Comar, C. L. Radioisotopes in Biology and Agriculture. McGraw-Hill Book Company, Inc., New York. 1955.

(3) Easterly, D. G., Demott, B. J., and Cragle, R. G. Strontium in Milk. I. Removal by Means of Reverse-Flow Ion Exchange Columns. J. Dairy Sci., 43: 137. 1960.

(4) Easterly, D. G., Demott, B. J., and Cragle, R. G. Strontium in Milk. II. Removal by Batch Ion Exchange Methods. J. Dairy Sci., 43: 146. 1960.

(5) Fiske, C. H., and Subbarow, Y. The Colorimetric Determination of Phosphorus. J. Biol. Chem., 66: 375. 1925.

(6) Josephson, D. V., and Reeves, C. B. The Utilization of the Mineral-Ion Exchange Principle in Stabilizing Evaporated Milk. J. Dairy Sci., 30: 737. 1947.

(7) Kamal, T. H. Complexometric Titration of Calcium and Magnesium in the Presence of Phosphate in Milk and Blood Plasma. Agr. and Food Chem., 8: 156. 1960.

(8) Landgrebe, A. R., Edmondson, L. F., and Douglas, F. W., Jr. Optimum Ratio of Cations on Charged Resin for Removal of Radio-strontium from Milk. J. Dairy Sci., 44: 1167. 1961.

(9) Migicovsky, B. B. Radioisotopes and Environmental Circumstances; Reduction of the Hazard in the Food Chain. Radioisotopes in the Biosphere. (A Symposium.) p. 293. University of Minnesota. 1960.

(10) Murthy, G. K., Masurovsky, E. B., Campbell, J. E., and Edmondson, L. F. Method for Removing Cationic Radionuclides from Milk. J. Dairy Sci., 44: 2158. 1961.

(11) Nervik, W. E., Kalkstein, M. I., and Libby, W. F. Purification of Milk for Calcium and Strontium with Dowex 50W Resin. University of California Radiation Laboratory, UCRL-2674. 1954.

(12) Strelow, F. W. E. An Ion Exchange Selectivity Scale of Cations Based on Equilibrium Distribution Coefficients. Anal. Chem., 32(9): 1185. 1960.

OXIDATION OF MILK FAT GLOBULE MEMBRANE MATERIAL. I. THIOBARBITURIC ACID REACTION AS A MEASURE OF OXIDIZED FLAVOR IN MILK AND MODEL SYSTEMS [1]

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SUMMARY

The thiobarbituric acid (TBA) reaction was used for investigating oxidized flavor in model systems containing fat globule membrane material and ascorbic acid. Trichloroacetic acid was used to flocculate the proteins and the TBA reaction was carried out and determined in the filtrate. The method is highly satisfactory in reproducing and measuring rapid oxidation rates in the model system.

When applied to milk, lactose was found to contribute considerable interference in the TBA reaction. This was shown by chromatographic separation and spectrophotometric analyses of TBA pigments. A satisfactory application for milk uses trichloroacetic acid to remove fat and protein and ethanolic-TBA to increase the rate of color formation at 60 C, a temperature at which lactose degradation is minimized. Results are presented showing quantitative recovery of oxidized milk from mixtures of oxidized and nonoxidized milk. Effect of exposing homogenized milk to direct sunlight for 20-min intervals is readily detected by the method. Relation between organoleptic and TBA analyses is indicated.

Detection of lipid oxidation in foods organoleptically is considered the most sensitive and reliable method, though it does not lend itself well to quantitative measurements. The natural complexity of milk and the relatively small amounts of material or changes therein responsible for oxidized flavor have limited most studies to organoleptic evaluations. The use of a model system will overcome some of the inherent problems associated with the complexity of milk, but may introduce others such as the comparison of organoleptic sensations between milk and simpler systems.

Olson and Brown (4) reported that small quantities of ascorbic acid added to copper-contaminated washed cream resulted in intense oxidized flavor. Fat globule membrane material obtained by churning washed cream reacts in a similar manner, even without the addition of copper (3). The reaction proceeds very rapidly and the intense flavors are difficult to evaluate organoleptically. The 2-thiobarbituric acid (TBA) reaction has been widely applied for the detection of lipid oxidation in foods including dairy products (1, 7, 9, 10, 13). Ottolenghi (5) used the TBA reaction to measure the reaction between mitochondrial lipids and ascorbic acid.

This study was undertaken to evaluate the TBA reaction as a measure of lipid oxidation in simplified systems containing milk fat globule membrane material (FGMM) that simulate development of oxidized flavor. Various factors influencing the reaction and methods for application to milk or model systems are presented.

EXPERIMENTAL PROCEDURE

Model systems. Fat globule membrane material was prepared by churning washed cream. The washed cream was prepared from fresh, uncooled milk separated at 40 C, using a De Laval Model 518 machine. The cream was washed four times with four volumes of 40 C tap water. Experiments using P^{32} indicated that at least 80% of the organic phosphorus is retained by the washed fat globules by this procedure. The membrane material was sedimented from the buttermilk by centrifugation at about 1,000 × G in 250-ml glass bottles, after adjusting the pH to 4.9 with 1 N HCl. The sedimentation is facilitated if the buttermilk is reseparated to remove unchurned fat globules and

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free fat. The clear supernatant was discarded and the sediment redispersed in phosphate buffer, pH 6.6, containing 4.4 g Na H_2PO_4 and 2.6 g Na_2HPO_4 per liter. The solids content of the redispersed membrane preparation was about 6%. The basic model system contained various amounts of the 6% membrane preparation, L-ascorbic acid, and additional buffer. These membrane preparations can be stored under refrigeration for several weeks without oxidative deterioration.

TBA TEST FOR MILK

Pipet 17.6 ml milk into a flask fitted with glass stopper, warm to 30 C, add 1.0 ml trichloroacetic acid solution containing 1 gram per milliliter, followed by 2.0 ml 95% ethanol, stopper, and shake vigorously for 10 sec. After 5 min, filter the contents through no. 42 Whatman paper. To 4.0 ml of the clear filtrate add 1.0 ml of TBA solution made by dissolving 1.4 g 2-thiobarbituric acid in 95% ethanol to 100 ml. Stopper container, mix contents, and place in 60 C water bath for 60 min. Cool, determine optical density of 532 mμ with a Beckman DU spectrophotometer, using distilled water as a reference.

Preparation of the TBA reagent is facilitated by heating in a 60 C water bath. The reagent undergoes deterioration (12) and should not be stored longer than about three days.

The TBA test for the model system is carried out in a similar manner, except that the addition of ethanol to the sample is not required. The quantity of trichloroacetic acid will depend on the composition of the model system, but should not be more than that needed to lower pH to 1.4 (1). An alternative procedure requires 2.0 ml aqueous TBA (0.05 M) to 4.0 ml filtrate and the reaction is carried out at 100 C for 15 min. Interference caused by high heat treatment is not a problem in the model system described.

Chromatographic separation of TBA pigments. The trichloroacetic acid filtrate-TBA mixture is poured on to a cellulose column prepared from an aqueous slurry of powdered cellulose packed under 5 lb of nitrogen to a height of 10 cm in a 2-cm diameter column. From acid solution the pigments are strongly absorbed at the top of the packing. The column is then rinsed with 0.1 N HCl until the effluent is clear and eluted with 5 ml 0.1 N Na_2HPO_4, followed by 0.1 N NaOH. This results in two distinctly separated bands, both of which move rapidly down the column. The first fraction emerging from the column consists of a brown pigment and the second the red pigment.

RESULTS

In evaluating the possible usefulness of the TBA reaction for the study of oxidizing milk fat globule membrane systems, the following criteria were considered: relation to organoleptic sensations; reproducibility; definite end point of the reaction; simplicity and rapidity. Other applications of the TBA reaction to milk or milk fat (1, 6) do not satisfy one or more of these criteria and suggest that the TBA reaction should not be carried out in the presence of the oxidized or oxidizing lipids, since further oxidation may occur during the reaction with TBA.

The method initially adopted for use in the present study is similar to that proposed by Keeney and Bassette for detection of the browning reaction (2). Some results obtained using this method are shown in Figure 1. Rates of oxidation for model systems of two different compositions are shown. Each curve contains two sets of data representing separate experiments, thus illustrating the reproducibility of the model system and the TBA reaction. The rapid rate at which the oxidative reaction proceeds is apparent when compared to a badly oxidized milk which will yield an optical density in the range of 0.06-0.10, a value reached in these model systems in about 10 min. This also indicates the futility of organoleptic methods for measuring these changes accurately.

Trichloroacetic acid serves several functions in this method. It effectively flocculates the

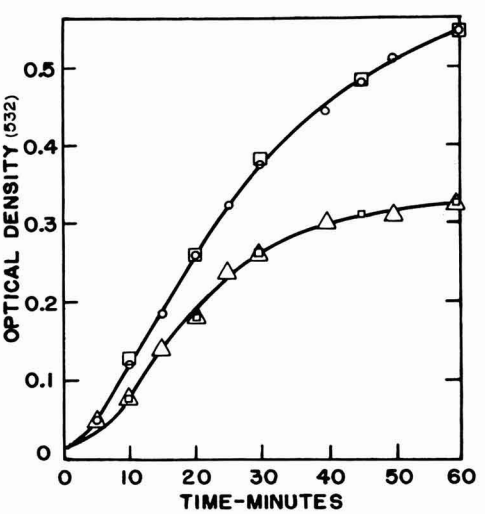

FIG. 1. Rate of oxidation of FGMM at 25 C as measured by the TBA reaction. Two compositions of the model system and two sets of rate data for each are represented.

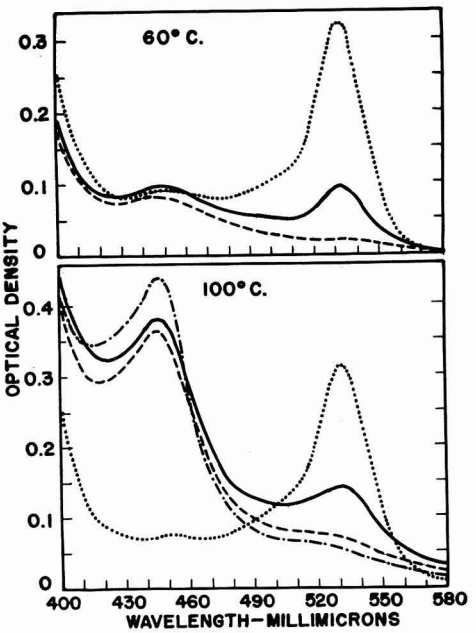

FIG. 2. Comparison of TBA pigments formed at 100 and 60 C for ..... oxidized FGMM, —·— 5% lactose, ---- fresh milk, and ———— oxidized milk.

membrane material, thus facilitating its removal by simple filtration. At the same time, it provides the acidity necessary to carry out the subsequent reaction with TBA. The addition of trichloroacetic acid effectively stops the lipid oxidation which is particularly important with such a rapid reaction. The filtrates are suitably clear after reaction with TBA to allow direct determination of absorption, thus eliminating the need for extraction and simplifying the entire procedure. Sampling frequencies of 2-min intervals have been successfully used in measuring reaction rates.

The method developed for the model systems could not be applied directly to milk. When trichloroacetic acid filtrates from milk are heated with TBA at 100 C a reddish-brown color develops, the intensity of which is related to the degree of heat treatment rather than the flavor of the original milk. The red pigment associated with oxidized lipids will form at temperatures less than 100 C, but at a slower rate. At temperatures below 60 C formation of the brown pigment is markedly reduced and interference with red pigment absorption is essentially eliminated. A 5% lactose solution in phosphate buffer, when treated in a similar manner to milk, also produces a brownish-red color when heated with TBA at 100 C. The absorption characteristics of the TBA pigments formed in trichloroacetic acid filtrates of fresh milk, oxidized milk, oxidized FGMM, and 5% lactose solution, as affected by the temperature at which the TBA reaction is carried out, are shown in Figure 2. Fresh milk and lactose yield very similar curves at 100 C and both show considerable absorption in the region of maximum absorption of the red pigment. The curve representing FGMM is relatively unaffected by the different heat treatments reflecting the absence of lactose. The difference in absorption at 532 mμ between fresh milk and oxidized milk (same milk after 36 hr of storage) is similar for both temperatures; however, the relative difference is much greater at 60 C.

The 100 C reaction mixtures (represented in Figure 2) were passed through cellulose columns and the adsorbed pigments from 100 ml were eluted with 0.1 N NaOH into a final volume of 15 ml adjusted to pH 1.5. The absorption spectra for these unresolved TBA pigments are shown in Figure 3. The original reaction mixtures show considerable absorption at 443 mμ (Figure 2) which passes through the cellulose column. The pigments retained by the column show two major peaks at 450 and 532 mμ. The curves representing fresh milk and lactose are quite similar. The oxidized milk shows the same two peaks but at much greater

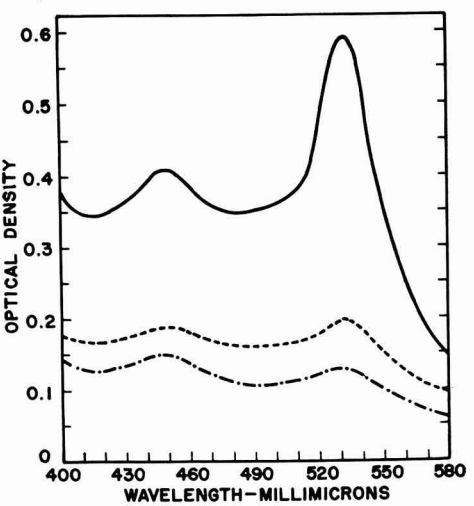

FIG. 3. TBA pigments from 100-ml reaction mixture carried out at 100 C, 15 min, adsorbed on cellulose, and eluted into final volume of 15 ml, pH 1.5. —·— 5% lactose solution; ---- fresh milk; ———— oxidized milk.

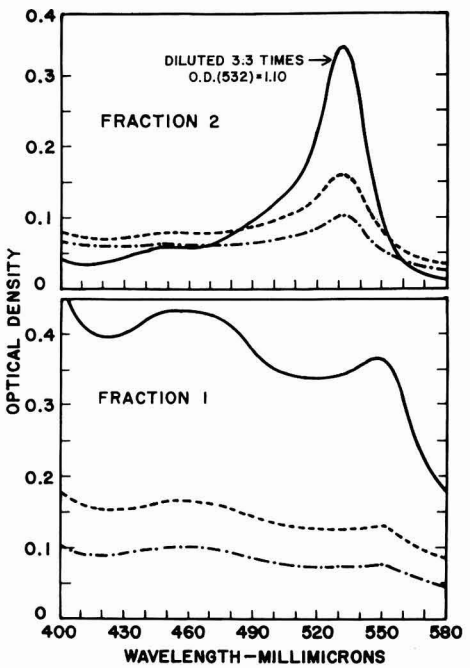

FIG. 4. Separation on cellulose of crude TBA pigments (Figure 3) into brown (Fraction 1) and red (Fraction 2) components. Final volume 6 ml, pH 1.5. —·— lactose; – – – – fresh milk; ———— oxidized milk.

concentration; however, the red pigment is predominant.

The pigments shown in Figure 3 were resolved into two components as described previously. These results are shown in Figure 4. The lower curves represent Fraction 1 (brown) and the upper, Fraction 2 (red). The brown pigment shows two peaks at 450 and 550 under acid conditions. In basic solution the peak at 450 flattens out and the one at 550 shifts to 540. This fraction is much less soluble in acid than base. Fraction 2, the red pigment, has a minor peak at 450 which probably represents a slight contamination with Fraction 1. The major peak absorbs at 532 mμ in acid conditions and at 545 mμ in basic solution. The absorption measured at 532 mμ of the original reaction mixture is contributed to by both of these fractions.

A source of the interference caused by copper contamination is demonstrated in Figure 5. Fresh milk and 5% lactose solutions were prepared containing increasing amounts of Cu^{++}, after which the TBA reaction was carried out at 100 C for 15 min. The TBA pigments from 50 ml of the reaction mixtures were adsorbed onto cellulose and eluted with 0.1 N NaOH into a final volume of 15 ml, pH 1.5. Both lactose and milk produced similar pigments; however, the quantity was greater in milk. The pigments formed as a result of copper contamination are similar if not identical to those represented by Fraction 1 in Figure 4.

A portion of the reaction mixture representing a 5% lactose solution containing 1.0 ppm Cu^{++} was extracted with an equal volume of pyridine-isoamyl alcohol and the absorption compared with the original mixture. These results are shown in Figure 6 and indicate that interference caused by copper contamination as reported by Dunkley and Jennings (1) is partly if not wholly due to lactose degradation.

To obtain additional information concerning the origin of substances reacting with TBA, the following experiments were conducted: Samples of fresh milk containing 0.1 ppm added copper were stored in quart containers into which were suspended dialysis tubing containing 20 ml water. After intervals of storage at 3 C, with infrequent mixing, the milk and

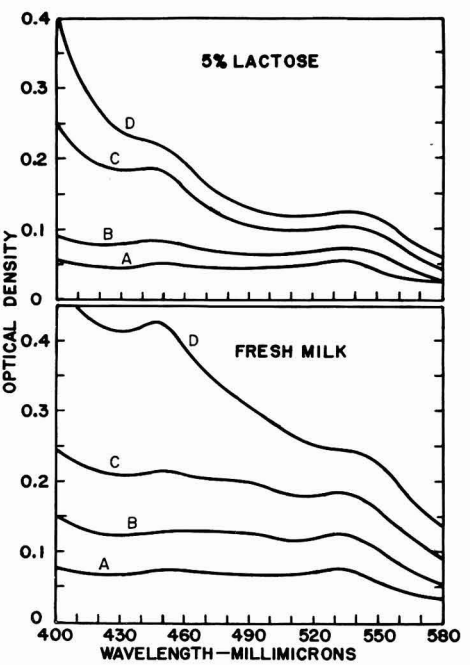

FIG. 5. Effect of copper on TBA reaction at 100 C for 15 min in fresh milk and lactose solutions. TBA pigments adsorbed from 50-ml reaction mixture in final volume 15 ml, pH 1.5. A—control, B—0.5 ppm Cu^{++}, C—1.0 ppm Cu^{++}, D—1.5 ppm Cu^{++}.

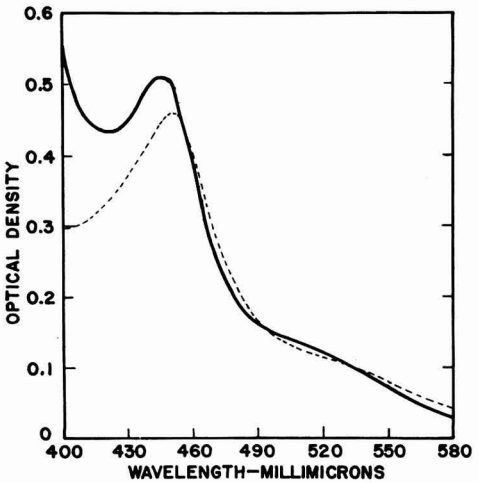

FIG. 6. TBA pigments from lactose containing 1 ppm Cu^{++} after heating at 100 C for 15 min. ——— reaction mixture; - - - - pyridine-isoamyl alcohol extract of reaction mixture.

the contents of the bag were subjected to the TBA reaction as herein described. Similar experiments were carried out on the model system. Some of these results are shown in Table 1 and indicate that the amount of TBA pigment formed is not related to the addition of trichloroacetic acid to the milk.

TABLE 1

Comparison of TBA values for intact samples and their dialysates

Description	Optical density (532 mμ)	
	Sample	Dialysate
Milk 1	0.072	0.052
Milk 2	0.111	0.103
Milk 3	0.217	0.203
FGMM	0.305	0.275

To eliminate the interference due to lactose degradation other reaction conditions were investigated. It was found that ethanol as a solvent for TBA allows an increase in the concentration of the reagent and also has an apparent catalytic effect on the color reaction. The quantity of trichloroacetic acid needed to yield a pH of 1.4 does not consistently yield complete protein precipitation, as evidenced by a slight turbidity when the ethanolic TBA is added to the filtrate. To overcome this, a small amount of ethanol is added to the milk before filtration. A temperature of 60 C was found to cause minimal lactose degradation and the color reaction proceeds at a reasonable rate. The rate of formation of the red pigment for several samples is shown in Figure 7. The upper curve represents a FGMM filtrate produced by the original procedure at 100 C. The reaction is essentially complete in about 15 min. The other curves represent pigment developed using ethanolic-TBA at 60 C and the reaction is complete in about 45 min. In all cases a definite end point is indicated which is contrary to results reported using different procedures (1, 7). The relative flatness of the curves at 60 min indicates that plus or minus a few minutes will have little effect on the result.

Samples of oxidized milk were diluted with various amounts of nonoxidized milk and the samples were evaluated organoleptically and by the TBA method. Results of two separate experiments using different milks are shown in Figure 8. Both of these curves indicate a linear relation between optical density and relative concentration of oxidized milk, thus indicating good recovery and sensitivity. Ranking by a flavor panel was good for samples yielding optical density values above 0.04; however, correlation fell off among the samples having low flavor intensity.

The observed relation between organoleptic evaluation and optical density is shown in Table 2. Multiple determinations have been

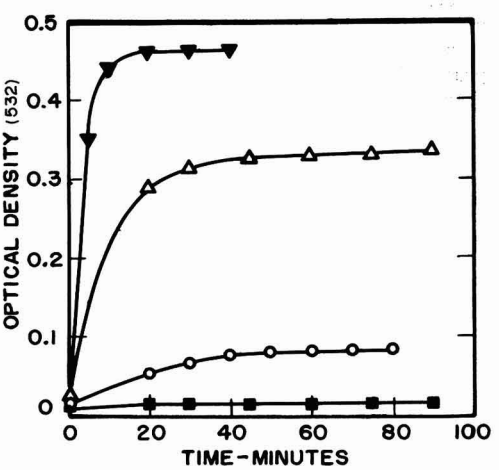

FIG. 7. Rate of formation of TBA pigment in trichloroacetic acid filtrates. The curves representing FGMM are not related.

▼———▼ oxidized FGMM, 100 C
▽———▽ oxidized FGMM, 60 C
○———○ oxidized milk, 60 C
■———■ 5% lactose solution, 60 C

TABLE 2

Relation between organoleptic and TBA values

Flavor score	Description	Range of optical density (532 mμ)
0	No oxidized flavor	0.010-0.023
1	Questionable to very slight	0.024-0.029
2	Slight but consistently detectable	0.030-0.040
3	Distinct or strong	0.041-0.055
4	Very strong	>0.056

found to agree within ± 0.003 optical density units. Contamination of the milk with copper up to about 0.5 ppm does not alter the result.

A further test of sensitivity of the method was demonstrated by measuring changes in homogenized milk during exposure to direct sunlight. These results are shown in Figure 9. The lower curve represents milk exposed in a quart glass milk bottle on a cold day. The point outside the figure represents the value obtained for this 140-min-exposed milk after 24 hr of cold storage, indicating the reaction did not proceed much after the exposure was discontinued. This was consistent with the organoleptic evaluation. The upper curve in Figure 9 represents 17.6-ml samples exposed in 50-ml Erlemeyer flasks on a mild day. When compared with the lower curve, the influence of degree of exposure and, probably, heat are indicated. These data are presented as a test of sensitivity; however, the method would appear to have some value in the study of flavor changes induced by light exposure.

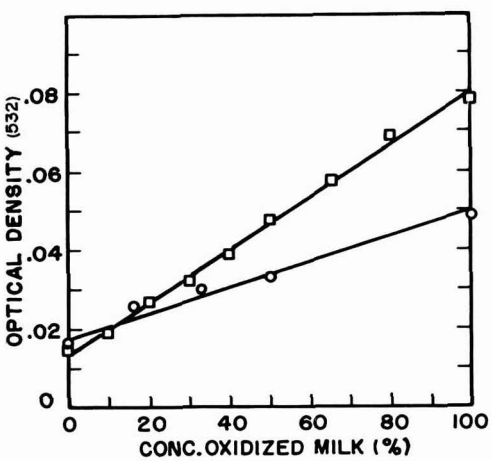

FIG. 8. Recovery of known relative amounts of oxidized milk as measured by the TBA reaction.

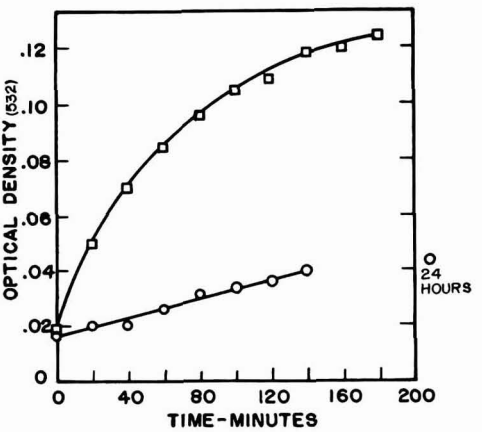

FIG. 9. Exposure of homogenized milk to direct sunlight as measured by TBA reaction. Point outside figure represents milk in lower curve after 24 hr of refrigerated storage.

DISCUSSION

The substance(s) reacting with TBA to produce the red pigment as well as the composition of the pigment have been rather thoroughly investigated (6, 8, 11). The origin of the total TBA-reactive material is not so well understood. Results reported in this paper show that this material can be derived from lactose as well as oxidized lipids. When the reaction is carried out in whole milk or in the presence of milk fat, the pigments derived probably do not entirely reflect the organoleptic property of the intact sample, but must include material produced by the reaction conditions. This is evident from the lack of a definite endpoint. Such procedures may indicate the susceptibility of the lipids to undergo oxidation under accelerated conditions. Other sources of interference in this reaction are reported by Tarladgis et al. (12).

In the present study, it was found that the addition of trichloroacetic acid to the sample did not affect the amount of TBA pigment formed. Dialysates from oxidized milk or FGMM yielded approximately the same amount of TBA pigment as did the intact samples. The material reacting with TBA in this procedure is not bound to the fat globules or the FGMM, but is freely dialyzable.

It is concluded that the procedure as presented produces results that closely reflect the organoleptic condition of the intact sample and embodies the sensitivity, reproducibility, and simplicity to be useful in studying the kinetics of the reaction in model systems as well as flavor changes in milk.

REFERENCES

(1) DUNKLEY, W. L., AND JENNINGS, W. G. A Procedure for the Application of the Thiobarbituric Acid Test to Milk. J. Dairy Sci., 34: 1064. 1951.

(2) KEENEY, M., AND BASSETTE, R. Detection of Intermediate Compounds in the Early Stages of Browning Reaction in Milk Products. J. Dairy Sci., 42: 945. 1959.

(3) KING, R. L. Variation and Distribution of Copper in Milk in Relation to Oxidized Flavor. Ph.D. thesis, University of California Library, Davis, California. 1958.

(4) OLSON, F. C., AND BROWN, W. C. Oxidized Flavor in Milk. XI. Ascorbic Acid, Glutathione, and Hydrogen Peroxide as Mechanisms for the Production of Oxidized Flavor. J. Dairy Sci., 25: 1027. 1942.

(5) OTTOLENGHI, A. Interaction of Ascorbic Acid and Mitochondrial Lipides. Arch. Biochem. Biophys., 79: 355. 1959.

(6) PATTON, S. Response of Epihydrin Aldehyde and Glyceraldehyde in the Thiobarbituric Acid Test for Fat Oxidation. Food Research, 25: 554. 1960.

(7) PATTON, S., AND KURTZ, G. W. 2-Thiobarbituric Acid as a Reagent for Detecting Milk Fat Oxidation. J. Dairy Sci., 34: 669. 1951.

(8) PATTON, S., AND KURTZ, G. W. A Note on the Thiobarbituric Acid Test for Milk Lipid Oxidation. J. Dairy Sci., 38: 901. 1955.

(9) RAMOS-CARDOVA, M., AND CONTRERAS, L. F. Chemical and Tasting Threshold Limits of Identification of Oxidative Rancidity in Butter for Freezing by 2-Thiobarbituric Acid Method. Proc. XVth Intern. Dairy Congr., 3: 1790. 1959.

(10) SIDWELL, C. G., SALWIN, H., AND MITCHELL, J. M., JR. Measurement of Oxidation in Dried Milk Products with Thiobarbituric Acid. J. Am. Oil Chem. Soc., 32: 13. 1955.

(11) SINNHUBER, R. O., YU, T. C., AND YU, TE CHANG. Characterization of the Red Pigment Formed in the 2-Thiobarbituric Determination of Oxidative Rancidity. Food Research, 23: 626. 1958.

(12) TARLADGIS, B. G., PEARSON, A. M., AND DUGAN, L. R., JR. The Chemistry of the 2-Thiobarbituric Acid Test for the Determination of Oxidative Rancidity in Foods. I. Some Important Side Reactions. J. Am. Oil Chem. Soc., 39: 32. 1962.

(13) YU, T. C., AND SINNHUBER, R. O. 2-Thiobarbituric Acid Method for the Measurement of Rancidity in Fishery Products. Food Technol., 11: 104. 1957.

HEAT-INDUCED CHANGES IN MILK FAT [1, 2]

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SUMMARY

A study on the effect of heat on milk fat shows that acetone, pentanone-2, heptanone-2, nonanone-2, and undecanone-2 are formed in the absence of oxygen and moisture. The use of gas chromatography with capillary columns and flame ionization detection also reveals the presence of many other components. Among these, and present in relatively small quantities, are the even-numbered carbon ketones butanone-2, hexanone-2, and octanone-2, as well as the n-alkanals C_1 to C_9.

Temperatures above 100 C appear to be critical for the development of an off-flavor. Samples of milk fat so heated and homogenized into fresh skimmilk had a marked resemblance to reconstituted milk powder when subjected to a taste panel.

The effects of heat in the absence of oxygen on the color of anhydrous milk fat are shown.

Although a considrable amount of research has been carried out on the oxidation of milk fat (4-7, 12), a clear distinction has not always been made between the effect of heating and the effect of oxidation. The only comprehensive study on thermal degradation of milk fat is that of Patton and Tharp (10), in which they stripped the fat with steam for a period of 3 hr, holding it at a temperature of 200 C and a pressure of 0.1-0.25 mm Hg. They identified a homologous series of methyl ketones containing the n-alkyl members with odd numbers of carbons from C_3 to C_{15}. These workers have also identified δ-decalactone (8), and δ-dodecalactone (13) in the steam distillate of milk fat.

The purpose of the present study was to investigate in some detail the effect of heat on milk fat in the absence of oxygen and moisture. Particular emphasis was placed on those compounds which might be produced in trace quantities but could contribute significantly to the flavor of heated dairy products.

EXPERIMENTAL PROCEDURE

Preparation of milk fat. Fresh milk from the University herd was separated at 30 C and the cream promptly cooled to 5 C and churned. The butter was washed three itmes with cold water, warmed, and centrifuged. Only the clear oil was collected, placed in low actinic ℉ Erlenmeyer flasks (350 g fat in a 500-ml flask), and bubbled with a stream of nitrogen for 3 min. The flasks were then sealed by insertion of the appropriate size rubber serum caps and securing the sleeves of the caps with several tight turns of copper wire.[4] The temperature of the milk fat never exceeded 45 C during preparation. The tightly stoppered flasks were stored at −15 C for use as needed.

Heat treatment. While still cold, the pressure in the sealed flasks was reduced to approximately 100 μ by means of insertion via the serum cap, of a hypodermic needle connected to a vacuum pump and a McLeod gauge. To reduce the possibility of breakage, the flasks were warmed in a 70 C water bath for 2 min before being placed in a constant oil bath set at the desired heating temperature. As the temperature of the fat increased, the evolution of entrapped nitrogen became apparent.[5] Pre-

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[4] The effectiveness of the seal was determined on empty flasks which had been evacuated to a pressure of 100 μ. The rate of leakage was of the order of 1 cc of air per hour as determined by measuring the volume of the entrapped bubble resulting from replacement of the residual flask volume by water.

[5] Gas chromatographic analysis of oxygen and nitrogen in the headspace of the flasks was carried out by the method of Brenner and Cieplinski (1) utilizing an Aerograph model A-90-P instrument. Oxygen concentration prior to the heat treatment was less than 0.3% with no detectable increase during heating. The evolution of nitrogen from the fat during heating reduces the pressure differential and thereby assists in maintaining the low oxygen concentration.

liminary studies showed an increase in the production of volatiles with increasing temperatures of heating in the range 100–170 C. Below 100 C, the production of volatiles is relatively low. Unless otherwise noted, heat treatment of samples was carried out at 130 C for 3 hr.

Gas chromatographic analyses. Volatiles were analyzed by both the direct and the enrichment techniques reported previously (9). In the direct technique, a portion of the atmosphere is simply transferred by means of a syringe into the gas chromatographic instrument. The enrichment technique consists of a continuous cycling system involving gas-liquid extraction. The volatiles are collected in a special cold trap which can then be capped, warmed, and its enriched headspace analyzed as in the direct technique. The heated fat as well as an unheated control were held at 40 C during the collection period. A Perkin Elmer Vapor Fractometer Model 154C with a hydrogen flame ionization detector was used with both capillary and packed columns.

Since it is likely that, under some conditions, two or more compounds could have the same retention time, and since it is difficult to achieve optimal operating parameters for the satisfactory resolution of all of the variety of components present, attention was focused separately on each group of carbonyl compounds having similar boiling points or carbon chain lengths. Preliminary runs were made with authentic compounds to determine conditions which would permit good resolution of the different members of each group. These previously determined conditions were used for comparing the retention times of unknowns with those of known chemicals. As an example, authentic butanone-2, n-butanal, isobutanal, and biacetyl did not separate well on a diisodecylphthalate packed column at most of the temperatures used. Efficient separation, however, was easily achieved by means of a capillary polypropyleneglycol (PPG) column at 35, 40, and 50 C. At higher column temperatures, these low-boiling compounds emerge so close to each other that retention data are of little value. Our triethanolamine (TEA) column was particularly interesting, since aldehydes were apparently retained on the column while ketones were eluted normally.

Reaction with DNPhydrazine[6] *and chromatography of the hydrazones.* The volatiles collected in the cold trap by means of the cycling system mentioned above were allowed to react with 4 N HCl saturated with DNPhydrazine for 2 hr at room temperature. The hydrazones were extracted repeatedly with n-heptane, concentrated, and chromatographed on paper using a modification of the method described by Clements and Deathrage (2), which employs a system of N, N-dimethyl formamide-pyridine (5:1) as the stationary phase and cylohexane as the mobile phase. The DNPhydrazones were also separated by column chromatography according to the method of Day, Bassette, and Keeney (3).

Spectrophotometric analyses. Absorption characteristics in the ultraviolet region of the hydrazones eluted from paper or column chromatography (in 95% ethanol) were obtained by means of a Cary 14 recording spectrophotometer. A Spectronic 20 spectrophotometer was used for transmittance and reflectance measurements on the heated milk fat samples.

Flavor evaluation. The effect of heating of the fat phase alone on the flavor of milk was studied by homogenizing the heated fat into fresh skimmilk to the level of 4% fat. A series of dilutions with an unheated control was prepared and presented at random to a panel of eight judges. The threshold concentration of the off-flavor was determined as described by Whitney and Tracy (14).

RESULTS

Figure 1 shows a composite gas chromatogram of volatiles from milk fat heated at 130 C for 3 hr. The components shown are mainly carbonyl compounds with the methyl ketones of odd carbon numbers present in relatively high concentrations, heptanone-2 being the highest. The figure also shows that also present but in much smaller amounts were the even-

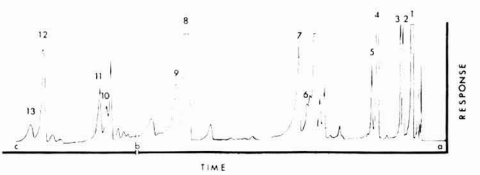

FIG. 1. Gas chromatographic analyses of volatiles from milk fat heated at 130 C for 3 hr, 150 ft UCON LB-550-X capillary columns. Flame ionization detector. Column temp. a-b 70 C, b-c 100 C. 1. Methanal, propanal, and acetone. 2. n-butanal. 3. butanone-2. 4. pentanone-2. 5. n-pentanal. 6. hexanone-2. 7. n-hexanal. 8. heptanone-2. 9. n-heptanal. 10. octanone-2. 11. n-octanal. 12. nonanone-2. 13. n-nonanal.

[6] The abbreviation DNP is used throughout for 2,4-dinitrophenyl-.

numbered carbon ketones C_4, C_6, and C_8, as well as the n-alkanals C_4, C_5, C_6, C_7, C_8, and C_9. Agreement of retention times of the unknowns with those of authentic compounds was obtained under a wide variety of gas chromatographic conditions, as shown in Table 1. Since resolution on capillary columns is far superior to that of packed columns, retention times obtained from the use of the former under varied conditions of temperature are more reliable. In addition to the above-mentioned compounds, the presence of methanal, ethanal, propanal, and possibly undecanal is revealed. Unfortunately, the use of temperatures higher than 100 C for packed columns and 120 C for capillary columns was limited by excessive bleeding of column substrate. This made difficult the analyses of carbonyl compounds with longer chain lengths than C_9 and also limited the detection of lactones reported to be present in heated milk fat by Patton and his co-workers (8, 13). However, a peak which appeared to correspond to butyrolactone could be observed only at a column temperature of 120 C. Further work is needed to confirm the presence of this compound. Chromatograms of the control showed the presence of a number of volatile compounds in the unheated fat. However, these were present in exceedingly low concentrations with acetone being predominant.

Additional evidence for the presence of aldehydes and ketones was secured by the following:

1. Gas chromatographic patterns of the headspace in the cold trap (of the cycling system) before and after reaction with DNPhydrazine were compared. The major peaks shown in Figure 1 were markedly reduced, whereas most of the minor peaks disappeared altogether.

2. Bands of DNPhydrazones separated by paper chromatography were treated with α-ketoglutaric acid (11), the released carbonyls analyzed by gas chromatography under different conditions and their retention times compared with known compounds. R_f values were also determined. The data reported in Table 2 show that each band contains a number of minor components in addition to the major compound. Trace compounds of different R_f values could possibly be carried along with neighboring bands.

3. Hydrazones separated by column chromatography were likewise treated. The gas chromatographic analyses of the released carbonyls are shown in Table 3. The presence of small amounts of minor components, along with the major component in each band, is again demonstrated. It is possible that in the purification often necessary for melting-point determinations of the major components several minor components are lost.

4. Ultraviolet spectra of the hydrazones separated by column chromatography (Table 3), as well as those obtained from paper chromatographic bands, confirmed that the carbonyl compounds present were mainly aliphatic saturated aldehydes and ketones.

TABLE 1

Summary of gas chromatographic analyses of volatiles from heated milk fat on various columns at various temperatures

	UCON LB-550-X capillary					Diisodecyl-phthalate		Triethanolamine	
Compound	35°	50°	70°	100°	120°	70°	100°	60°	90°
Ethanal	R	R	R	NR	NR	R	R		
Methanal	R	NR	NR	NR	NR	R	R		
Propanal	R	NR	NR	NR	NR	NR	NR		
Acetone	R	R	NR	NR	NR	NR	NR	R	R
n-Butanal	R	R	R	NR	NR	R	NR		
Butanone-2	R	R	R	NR	NR	R	NR	R	R
Pentanone-2	R	R	R	NR	NR	R	R	R	R
n-Pentanal	R	R	R	NR	NR	R	R		
Hexanone-2	R	R	R	NR	NR	R	R	R	R
n-Hexanal	R	R	R	NR	NR	R	R		
Heptanone-2	ND	R	R	R	NR	R	R	R	R
n-Heptanal	ND	R	R	R	NR	ND	R		
Octanone-2	ND	ND	ND	R	R	ND	R	ND	ND
n-Octanal	ND	ND	ND	R	R	ND	R		
Nonanone-2	ND	ND	ND	R	R	ND	ND	ND	ND
n-Nonanal	ND	ND	ND	R	R	ND	ND		
Undecanone-2	ND	ND	ND	ND	R	ND	ND	ND	ND

R—Resolved from adjacent peaks.
NR—Not well resolved, brackets indicate groups of unresolved peaks.
ND—Not discernible at this temperature.

TABLE 2

Gas chromatographic retention times of carbonyl compounds released from DNPhydrazones partially separated by paper chromatography

Band no. on paper	R_f	Tentatively identified compounds	GC analyses on diisodecyl-phthalate at 100 C: RT of vapors released from eluted band	GC analyses on diisodecyl-phthalate at 100 C: RT of authentic compounds	GC analyses on triethanolamine at 60 C: RT of vapors released from eluted band	GC analyses on triethanolamine at 60 C: RT of authentic compounds	R_f of authentic compounds
			(min)				
		Heptanone-2*	38.7	39	20.4	20.4	0.74
2***	0.65	n-Heptanal	41.6	42	.....	.....	0.73
		Hexanone-2	19.1	19.1	11.2	11.4	0.66
		n-Hexanal	20.5	20.1	.....	.....	0.68
		Pentanone-2*	9.5	9.5	6.8	6.8	0.56
3	0.61	n-Pentanal	10.0	10.0	.....	.....	0.56
		Hexanone-2	19.2	19.1	11.4	11.4	0.66**
		n-Hexanal	20.0	20.1	.....	.....	0.68**
4	0.46	Butanone-2* or n-Butanal	5.4	5.4	4.1	4.2	0.48
		Pentanone-2	9.5	9.5	6.8	6.8	0.56**
5	0.40	Acetone or* Propanal	3.0	3.0	2.8	2.8	0.43

*—Major component.
**—Trace carry-over from other bands.
***—A band with an R_f value of 0.85 preceded Band 2. However, GC analyses were not adequate, possibly due to low concentration of higher-boiling compounds.

TABLE 3

Gas chromatographic retention times of carbonyl compounds released from DNPhydrazones partially separated by column chromatography

Band no. (from column)	Absorption maximum of band	RT* of vapors released from band	RT* of authentic carbonyl compounds	Tentatively identified compounds	Absorption maxima of authentic compounds
	(mμ)		*(min)*		*(mμ)*
1	360	38.4	38.2	Nonanone-2	360
		40.4	40.0	n-Nononal**	
2	362	20.8	20.8	Octanone-2	360
		40.2	40.0	n-Nonanal	358
		38.3	38.2	Nonanone-2**	360
3	359	11.1	11.1	Heptanone-2	361
		21.8	21.8	n-Octanal	358
		12.5	12.5	n-Heptanal**	358
4	362	6.6	6.6	Hexanone-2	360
		12.5	12.5	n-Heptanal	358
		7.1	7.0	n-Hexanal**	358
5	363	4.3	4.3	Pentanone-2	360
		7.0	7.0	n-Hexanal	358
		6.6	6.6	Hexanone-2**	360
6	362	4.3	4.3	Pentanone-2	360
		4.5	4.5	n-Pentanal**	360

*—On a 150-ft UCON LB-550-X capillary column at 100 C.
**—Possibly carry-over from other bands.

Effect of heating milk fat on flavor. The off-flavor found in milk containing milk fat heated at 100 C or above was described by the panel as having a marked resemblance to reconstituted milk powder. There did not appear to be any qualitative difference in flavor, with temperatures of heating between 100 and 170 C. There did appear to be an increase in intensity of off-flavor with an increase in temperature in this range. While the flavor threshold for the 100 C heated fat was nearly 100%, it was below 20% for samples containing the 170 C treated fat.

Effect of heating on the color of milk fat. The effect of heat in the absence of oxygen on the color of milk fat is shown in Table 4. The control was held at 40 C for 3 hr. A progressive increase in light transmittance, with higher temperatures of treatment, is evident. A similar pattern was shown by reflectance measurements.

DISCUSSION

The results of this investigation confirm the presence of an homologous series of methyl ketones with odd numbers of carbon atoms in heated milk fat as reported earlier by Patton and Tharp (10). However, this study indicates that the presence of moisture is not necessary for the formation of these compounds. In addition, the formation of smaller amounts of several other carbonyl compounds as a result of this heat treatment is demonstrated. The latter components may also contribute significantly to the flavor of heated dairy products, since there is evidence to show that in some cases compounds present even at subthreshold levels exhibit a synergistic effect (9).

The origin and mechanism of formation of carbonyl compounds arising in milk fat upon heating are not well understood. Wong et al. (15) proposed that beta-keto-acids in the milk fat glycerides might represent possible precursors for the methyl ketones. These authors further point out that it would be unlikely that the formation of these compounds in such products as evaporated milk and heated cream occurs via atmospheric oxygen attack on the lipids, in view of the abundance of reducing substances present.

The possibility that some form of oxidation might be involved is suggested by the accompanying bleaching of color observed in milk fat upon heating. However, the loss of color by heat seems to proceed via a different mechanism than the bleaching normally produced by intensive oxidation of milk fat. No oxidized flavor was detected when the fat was heated to the point of discoloration in the absence of oxygen and moisture. Gas chromatographic patterns of the heated fat were markedly different from those of milk fat oxidized at low temperatures. Results reported by several investigators (4-6) indicate that aldehydes predominate as products of oxidative mechanisms, whereas this study shows that ketones may be the major components formed by heat. In actual practice, both mechansims may be operating simultaneously, giving rise to an extremely complex system. Attempts to obtain a correlation of flavor change with chemical composition are further complicated by the quantitative relationships involved. Further work is needed to clarify the mechanism by which heat-generated products are formed, as well as the role they play in contributing to flavor.

TABLE 4

Optical transmittance of milk fat heated for 3 hr at different temperatures

Temperature of heating (C)	Per cent transmittance at 490 mμ
40	11.5
100	16.0
130	23.0
150	27.0
170	42.0

REFERENCES

(1) BRENNER, N., AND CIEPLINSKI, E. Gas Chromatographic Analysis of Mixtures Containing Oxygen, Nitrogen, and Carbon Dioxide. Annals of the N. Y. Academy of Sci., 72: 705. 1959.

(2) CLEMENTS, R. L., AND DEATHRAGE, F. E. A Chromatographic Study of Some of the Compounds in Roasted Coffee. Food Research, 22: 22. 1957.

(3) DAY, E. A., BASSETTE, R., AND KEENEY, M. Identification of Volatile Carbonyl Compounds from Cheddar Cheese. J. Dairy Sci., 43: 463. 1960.

(4) DAY, E. A., AND LILLARD, D. A. Autoxidation of Milk Lipids. I. Identification of Volatile Monocarbonyl Compounds from Autoxidized Milk Fat. J. Dairy Sci., 43: 585. 1960.

(5) EL-NEGOUMY, A. M., DE PUCHAL, M. S., AND HAMMOND, E. G. Relation of Linoleate and Linolenate to the Flavors of Autoxidized Milk Fat. J. Dairy Sci., 45: 311. 1962.

(6) FORSS, D. A., DUNSTONE, E. A., AND STARK, W. The Volatile Compounds Associated with Tallowy and Painty Flavors in Butterfat. J. Dairy Research, 27: 381. 1960.

(7) KEENEY, M., AND DOAN, F. J. Studies on Oxidized Milk Fat. J. Dairy Sci., 34: 713. 1951.

(8) KEENEY, P. G., AND PATTON, S. The Coconut-like Flavor Defect of Milk Fat. I. Iso-

lation of the Flavor Compound from Butteroil and Its Identification as δ-Decalactone. J. Dairy Sci., 39: 1104. 1956.

(9) Nawar, W. W., and Fagerson, I. S. Direct Gas Chromatographic Analyses as an Objective Method for Flavor Measurement. Food Technology. (In press.)

(10) Patton, S., and Tharp, B. W. Formation of Methyl Ketones from Milk Fat During Steam Distillation or Saponification. J. Dairy Sci., 42: 49. 1959.

(11) Ralls, J. W. Rapid Method for Semiquantitative Determination of Volatile Aldehydes, Ketones and Acids. Anal. Chem., 32: 332. 1960.

(12) Tamsma, A. F. A Study of the Volatile Fraction Isolated from Oxidized Milk Fat. II. Further Characterization of Compounds Responsible for the Oxidized Flavor. J. Dairy Sci., 38: 487. 1955.

(13) Tharp, B. W., and Patton, S. Coconut-like Flavor Defect of Milk Fat. IV. Demonstration of δ-Dodecalactone in the Steam Distillate from Milk Fat. J. Dairy Sci., 43: 475. 1960.

(14) Whitney, R. McL., and Tracy, P. H. Stale-Flavor Components in Dried Whole Milk. I. The Distribution of Stale Flavor Between Fractions of Reconstituted Stale Whole Milk Powder. J. Dairy Sci., 32: 383. 1949.

(15) Wong, N. P., Patton, S., and Forss, D. A. Methyl Ketones in Evaporated Milk. J. Dairy Sci., 41: 1699. 1958.

SENSITIVITY OF MILK LIPASE TO ANTIBIOTICS[1]

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SUMMARY

A study was made to determine the effect of various concentrations (0.5–50.0 ppm) of antibiotics upon milk lipase. Aureomycin, penicillin, streptomycin, and terramycin inhibited the lipase activity by 9.5 to 42.4, 7.9 to 49.8, 9.2 to 39.6, and 7.6 to 44.2%, respectively. Up to 10.0 ppm, there was a direct relationship between the concentrations of the antibiotics and the per cent lipase inhibition, but with higher concentration the increase in lipase inhibition became disproportional to the antibiotic concentration. Fractional determinations of the fatty acids released showed that antibiotics did not possess any selectivity in inhibiting the release of short-, medium- or long-chain fatty acids by the enzyme. An increase in the fat content of milk lipase systems containing antibiotics nullified the inhibitory effect of antibiotics. Evidence is presented which indicates that the antibiotics apparently work as competitive inhibitors of milk lipase.

The lipase system of milk has been shown to be sensitive to several physical and chemical treatments. Several workers have reported the heat-inactivation of lipase(s) in milk by pasteurization temperatures (15). The destructive effect of light has been studied in detail by Stadhouders and Mulder (21), who observed a considerable inactivation of the enzyme by even a short exposure of milk to diffused daylight. Several chemical treatments affecting lipase activity have been used to elucidate the nature of the enzyme system. Using formaldehyde as an inhibitor, Schwartz et al. (17, 18) demonstrated that milk contains a multiple lipase system which is active throughout a pH range of 5.2 to 9.8, and that formaldehyde behaves as a competitive inhibitor. Tarassuk and Yaguchi (22) observed that N-ethyl maleimide inhibited milk lipase and concluded that the enzyme probably contained sulfhydryl groups which are essential for its hydrolytic action. Also, sodium chloride has been shown to inhibit the lipase activity in milk and cheese (7, 23).

As a consequence of the extensive use of antibiotics in mastitis therapy and their incidence in milk, several studies have been made concerning the effect of antibiotics upon starter cultures and upon the preservation of dairy products (1, 6). Shahani et al. (19) observed that pimaricin and mycostatin inhibited the yeast and mold growth and rancidity development in Cottage cheese and thereby extended its shelf life. Kooy and Pette (13) noted that the presence of the antibiotic-producing strain of *Streptococcus lactis* inhibited the butyric acid fermentation in milk inoculated with *Clostridium tyrobutyricum*.

Goldberg (6) and Eagle and Saz (5) have reviewed several studies relative to the mode of action of antibiotics on several enzyme systems. Tetracyclines have been reported to inhibit the action of urease (6). Kraskin and Stern (14) showed the competitive nature of the inhibition of coenzyme diphosphopyridine nucleotide (DPN) by oxytetracycline using the Lineweaver-Burk plot. Working with *Mycobacterium butyricum* and *Mycobacterium tuberculosis*, Cohen et al. (3) found that low concentrations of polymyxin B inhibited the esterase activity of the organisms. Scholz et al. (16) reported that under in vivo conditions nystatin, an antifungal antibiotic, inhibited markedly the activities of the enzyme aldolase, glyceraldehyde phosphate dehydrogenase, alcohol dehydrogenase, and phosphoglycerate kinase of yeast. Until recently little attention has been given to the effects of antibiotics upon the microbial and milk lipase systems which bear significance to the dairy industry. In a previous paper (2), the inhibitory effect of several antibiotics on microbial lipase was presented. The studies were continued and the effects of several antibiotics upon milk lipase systems were investigated. The results are presented in this paper.

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EXPERIMENTAL PROCEDURE

The effect of various concentrations of antibiotics was determined upon the naturally present lipase system in raw milk. Raw milk was obtained from the University herd, cooled immediately, and used within 30 min after milking. Prior to the addition of antibiotics, the enzyme system in the raw milk was subjected to the activation treatment, in order for lipolysis to proceed at a rapid rate (7). The activation treatment consisted of mixing five parts of homogenized, pasteurized milk with 95 parts of raw milk and subsequent cooling to 4 C, warming to 30 C, and cooling again to 4 C. This permitted the easy detection of even minute inhibitory effects of antibiotics on milk lipase(s). Appropriate quantities of 0.1-0.5 ml of aqueous solutions of aureomycin,[2] penicillin,[3] streptomycin,[4] or terramycin[5] were added to 24.5 ml of different samples of the raw milk system to yield antibiotic concentrations of 0.5, 1.0, 5.0, 10.0, and 50.0 ppm in milk. These antibiotics were used primarily because they are more commonly employed in mastitis therapy and may be present in residual quantities in market milk. The final volume of the reaction mixture was 25 ml and the fat content approximately 3.5% in all the samples. The control samples were prepared the same way, except that sterile distilled water was used in place of the antibiotic solution. Each of the control and experimental samples was divided into three lots. One lot was assayed immediately for the free fatty acid content, and the other two lots were stored in corked 100-ml Erlenmeyer flasks in a refrigerator at 4-6 C and assayed for the free fatty acid development after 24 and 48 hr storage, respectively. The free fatty acid content, measuring the extent of lipolysis, was determined by the silica gel method of Harper et al. (10). This method was selected because of its accuracy; in the preliminary trials the recovery of added free fatty acids from milk was of the order of 91 to 99%. For each assay, 10-ml aliquot of the incubated milk system was used, and the free fatty acids were eluted with 150 ml of chloroform-butanol mixture. The titration was performed with 0.01 N alcoholic potassium hydroxide, using phenol red as the indicator. The titer value representing the free fatty acids content (FFA) is used to express the lipase activity.

For the fractionation of the total free fatty acids into long-, mdium-, and short-chain fatty acids, the general assay procedure as outlined by Harper and Armstrong (9) was used. Long chain implied C_{12} to C_{18} fatty acids, medium chain C_6 to C_{10}, and short chain less than six carbon fatty acids. Penicillin and aureomycin in concentrations of 10.0 and 50.0 ppm were added to the raw milk system, and the samples were stored at 4-6 C for 24 and 48 hr. Employing different threshold volumes of the eluant (9), each fraction containing different chain length fatty acids was collected and titrated with 0.01 N alcoholic potassium hydroxide.

To determine the effect of varying the concentration of substrate (milk fat) on the antibiotic inhibition, and to determine whether the inhibition upon the lipase(s) was of a competitive or noncompetitive type, reaction mixtures containing various concentrations of the substrate were prepared. The procedure reported for studies with microbial lipases (2) was modified slightly to adapt it to milk lipase. The experiments were conducted at the natural pH of milk to simulate the condition encountered in milk. In brief, the following technique was employed: The raw milk system containing activated lipase activity was prepared as just described. Varying amounts of freshly separated pasteurized whipping cream (40% fat) were added to the activated enzyme system to obtain a fat content ranging between 3.5 and 25%, as determined by the Babcock method. One-half milliliter of an aqueous solution of appropriate concentration of aureomycin or penicillin was added to the system to obtain antibiotic concentrations of 5.0 and 50.0 ppm. The final volume of each mixture was 50 ml. Concurrently, control samples containing the substrate and the enzyme but no antibiotic were also prepared and treated the same way as the experimental samples. Each sample was divided into two lots and stored in a refrigerator at 4 C. After 24 hr of storage, one lot was removed and mixed thoroughly, and 10-ml aliquot was used for the determination of free fatty acids liberated. Similarly, the second lot was analyzed after 48-hr storage.

RESULTS AND DISCUSSION

Effect of various concentrations of antibiotics on milk lipase. The data relative to the effect

[2] Aureomycin (R), Chlorotetracycline Hydrochloride, Oral grade, American Cyanamid Co., New York 20, N. Y.

[3] Procaine Penicillin G, Commercial Solvents Corporation, New York 17, N. Y.

[4] Streptomycin Sulfate, Abbott Laboratories, North Chicago, Ill.

[5] Terramycin Hydrochloride, Chas. Pfizer and Co., Brooklyn 6, N. Y.

TABLE 1

Inhibition of milk lipase by various concentrations of antibiotics

Time of storage at 4 C	Control (no antibiotic) FFA [a]	Antibiotic concentration (ppm)									
		0.5		1.0		5.0		10.0		50.0	
		FFA [a]	Inhibition	FFA [a]	Inhibition	FFA [a]	Inhibition	FFA [a]	Inhibition	FFA [a]	Inhibition
(hr)			*(%)*		*(%)*		*(%)*		*(%)*		*(%)*
					Aureomycin						
0	0.96	0.96	.....	0.96	.....	0.96	.....	0.96	.....	0.96	.....
24	2.54	2.39	9.5	2.25	18.3	2.16	24.1	2.07	29.7	1.93	38.6
48	3.41	3.15	10.6	2.92	20.0	2.77	26.1	2.57	34.2	2.37	42.4
					Penicillin						
0	0.84	0.84	.....	0.84	.....	0.84	.....	0.84	.....	0.84	.....
24	2.86	2.70	7.9	2.40	22.8	2.22	31.7	2.03	41.1	1.89	48.0
48	3.13	2.93	8.7	2.58	24.0	2.36	33.6	2.17	41.9	1.99	49.8
					Streptomycin						
0	0.89	0.89	.....	0.89	.....	0.89	.....	0.89	.....	0.89	.....
24	3.38	3.15	9.2	3.03	14.0	2.84	21.7	2.70	27.3	2.52	34.5
48	4.10	3.73	11.5	3.51	18.4	3.22	27.4	3.01	34.0	2.83	39.6
					Terramycin						
0	0.82	0.82	.....	0.82	.....	0.82	.....	0.82	.....	0.82	.....
24	2.53	2.40	7.6	2.28	14.6	2.15	22.2	2.02	29.8	1.87	38.6
48	2.88	2.70	8.7	2.52	17.5	2.37	24.8	2.15	35.4	1.97	44.2

[a] FFA—Milliliters of 0.01 N alcoholic KOH used to titrate free fatty acids extracted from 10 ml milk.

of various concentrations of aureomycin, penicillin, streptomycin, and terramycin upon the liberation of free fatty acids by milk lipase are presented in Table 1. The data represent average values of four to five trials conducted with each antibiotic and each concentration. It may be observed that the control sample containing no antibiotic exhibited a maximum liberation of the FFA. In the samples containing 0.5 to 50.0 ppm of antibiotic, a decrease in FFA was observed in the milk samples, indicating that all the antibiotics suppressed the lipolytic activity.

At lower concentrations (0.5 to 1.0 ppm), there was a corresponding increase in lipase inhibition with increase in the concentration of the antibiotic. However, at higher concentrations (5.0 to 50.0 ppm) of the antibiotics, the increase in the enzyme inhibition was not in direct proportion to antibiotic concentration. One-half ppm of the antibiotics inhibited the lipase activity by 7.6 to 11.5%. On increasing the antibiotic concentration to 50.0 ppm, the corresponding inhibitions were found to vary between 34.5 and 49.8%.

In the control samples, of the total free fatty acids liberated in 48-hr storage, 64.5 to 88.2% were liberated in the first 24 hr of storage; whereas, 65.3 to 91.3% were liberated in the case of samples containing antibiotics.

During the first 24-hr period of storage, 0.5 to 50.0 ppm of aureomycin inhibited the lipase activity by 9.5 to 38.6%. The corresponding inhibitions found in the case of penicillin, streptomycin, and terramycin were 7.9 to 48.0%, 9.2 to 34.5%, and 7.6 to 38.6%, respectively. Further storage for 24 hr effected an additional inhibition of 0.8 to 6.7% only, indicating that most of the inhibitory effect of the antibiotic occurred in the first 24 hr of storage.

In these studies, at the lowest concentration of 0.5 ppm, streptomycin was the most inhibitory antibiotic; whereas, at the highest concentration of 50.0 ppm, penicillin was the most inhibitory antibiotic.

In an earlier study (2), it was observed that 0.5 to 50.0 ppm of penicillin inhibited 3.0 to 100% of the lipase activity produced by *Achromobacter lipolyticum*, *Geotrichum candidum*, *Aspergillus niger*, and *Penicillium roqueforti*. A comparison of the antibiotic-sensitivity of milk lipase to that of the microbial lipases indicated that the milk lipase system was less sensitive to antibiotics than the microbial lipases.

Inhibitory effect of antibiotics upon the release of various fatty acids by lipase. Milk lipase appears to be specific in splitting off various fatty acids from milk fat. Harper (8) reported that freeze-dried raw milk split off 14.7% butyric acid from milk fat. More recently, Harwalker (11) studied the release of relative amounts of lauric-and-higher, capric, caprylic, caproic, and butyric acids from milk fat by the action of milk lipase. He observed that the lauric-and-higher fraction was the major component released during lipolysis. Jensen et al. (12) also noticed that milk lipase released long-chain fatty acids from milk fat. This study was undertaken to determine the effect of antibiotics upon the liberations of long-, medium-, and short-chain fatty acids by the enzyme. The data pertaining to this investigation are presented in Table 2.

Both aureomycin and penicillin inhibited the liberation of all the three groups—long-, medium-, and short-chain fatty acids. In general, the inhibition of the release of the individual groups of fatty acids was of the same magnitude for different concentrations of the antibiotics and for different times of storage. In 24 hr, 10.0 ppm of aureomycin inhibited 37.5% of the release of the long-chain fatty acids, 37.5% of the medium, and 35.0% of the short-chain fatty acids. Storage for an additional 24 hr resulted in the inhibitions of the three groups of fatty acids up to 38.9 to 40.7%. At 50.0-ppm level of aureomycin in milk, the release of three groups of fatty acids was arrested by 43.8 to 50.0% in 24 and 48 hr of storage. Essentially similar results were obtained in the studies with penicillin. Since the inhibition of the liberation of all the three groups of fatty acids by antibiotics followed the same pattern, it was felt that the antibiotics did not exhibit any selectivity in regard to their inhibitory effects upon the release of various fatty acids by milk lipase. The results reported in Table 2 are in harmony with the observation of other workers (11, 12), that long-chain fatty acids comprise the major component of total fatty acids released during lipolysis of milk fat.

Relationship between antibiotic inhibition and substrate concentration. The results relative to the effect of increasing substrate concentration on the inhibition of milk lipase by antibiotics are presented in Figure 1. The figure presents the average values of three to four trials conducted with each antibiotic and at each concentration level of the antibiotics and the substrate. Curves marked I are the control curves representing the lipolysis at various levels of milk fat in the absence of antibiotics. Curves II and III show the effect of 5.0 and 50.0 ppm of antibiotics on the liberation of free fatty acids.

TABLE 2

Effect of antibiotics on the release of long-, medium-, and short-chain fatty acids during lipolysis

Time of storage	Antibiotic concentration	Fraction I Long-chain		Fraction II Medium-chain		Fraction III Short-chain	
		FFA [a]	Inhibition	FFA [a]	Inhibition	FFA [a]	Inhibition
(hr)	*(ppm)*		*(%)*		*(%)*		*(%)*
				Aureomycin			
24	0.0	1.60	.....	0.80	.....	1.00	.....
	10.0	1.00	37.5	0.50	37.5	0.65	35.0
	50.0	0.86	46.3	0.45	43.8	0.50	50.0
48	0.0	2.25	.....	1.18	.....	0.90	.....
	10.0	1.36	39.6	0.70	40.7	0.55	38.9
	50.0	1.25	44.0	0.60	49.1	0.45	50.0
				Penicillin			
24	0.0	1.37	.....	0.58	.....	0.48	.....
	10.0	0.65	52.6	0.25	56.9	0.25	48.0
	50.0	0.60	56.2	0.25	56.9	0.20	58.4
48	0.0	1.70	.....	0.71	.....	0.67	.....
	10.0	0.85	50.0	0.37	47.9	0.35	47.8
	50.0	0.77	54.7	0.33	53.6	0.30	55.2

[a] FFA—Milliliters of 0.01 N alcoholic potassium hydroxide used to titrate free fatty acids extracted from 10 ml milk sample.

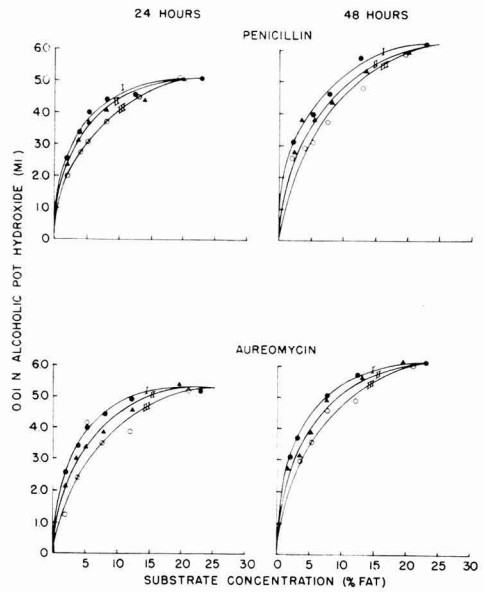

FIG. 1. Influence of substrate concentration upon the reaction velocity of milk lipase in the absence and in the presence of antibiotics. I. Control samples (no antibiotics); II. Samples containing 5 ppm of the antibiotic; and III. Samples containing 50 ppm of the antibiotic.

In general, an increase in the fat content of the reaction mixture counteracted the inhibitory effect of the antibiotic against the enzymic action.

As the fat content increased in the reaction mixture containing 5.0 and 50.0 ppm of antibiotic (Curves II and III), the inhibitory effect of the antibiotic against the enzyme was nullified and Curves II and III tended to merge with Curves I. This phenomenon was observed in the study of both the antibiotics. The maximum reaction velocities of both the control and the antibiotic became the same as the fat content reached the 25% level. Also, in the reaction mixtures containing more than 25% fat, the antibiotics exhibited no inhibitory effect upon the lipase activity of the system. The presence of fat seemed to protect the lipase against antibiotic inhibition. Nilsson and Willart (15) found a similar protection of lipase activity by milk fat against heat inactivation.

The reaction velocities in the absence and in the presence of two different concentrations of the antibiotics as plotted in Figure 1 were used to determine the type of inhibition. Employing the procedure outlined by Dixon and Webb (4), it was observed that the lipase of milk was apparently inhibited by the antibiotics in a competitive manner. A similar inhibition observed with microbial lipases was reported in an earlier paper (2).

REFERENCES

(1) ALBRIGHT, J. L., TUCKEY, S. L., AND WOODS, G. T. Antibiotics in Milk: A Review. J. Dairy Sci., 44: 779. 1961.

(2) CHANDAN, R. C., CARRANCEDO, M. G., AND SHAHANI, K. M. Sensitivity of Microbial Lipases to Antibiotics. J. Dairy Sci. In press. 1962.

(3) COHEN, S., PURDY, C. V., AND KUSHNICK, J. B. Inhibition of Mycobacterial Esterases by Polymyxin B. Antibiotics and Chemotherapy, 4: 18. 1954.
(4) DIXON, M., AND WEBB, E. C. Enzymes. p. 180. Academic Press, Inc., New York. 1958.
(5) EAGLE, H., AND SAZ, A. K. Antibiotics. Ann. Rev. Microbiol., 9: 173. 1955.
(6) GOLDBERG, H. S. (Ed.). Antibiotics, Their Chemistry and Non-Medical Uses. D. Van Nostrand Co., Princeton, N. J. 1959.
(7) GOULD, I. A. Effect of Certain Factors upon Lipolysis in Homogenized Raw Milk and Cream. J. Dairy Sci., 24: 779. 1941.
(8) HARPER, W. J. Apparent Selective Liberation of Butyric Acid from Milk Fat by the Action of Various Lipase Systems. J. Dairy Sci., 38: 1391. 1955.
(9) HARPER, W. J., AND ARMSTRONG, T. V. Measurement of Butyric Acid in Fat with Reference to the Detection of Substitute Fats in Dairy Products. J. Dairy Sci., 37: 481. 1954.
(10) HARPER, W. J., SCHWARTZ, D. P., AND EL-HAGARAWY, I. S. A Rapid Silica Gel Method for Measuring Total Free Fatty Acids in Milk. J. Dairy Sci., 39: 46. 1956.
(11) HARWALKAR, V. R. Specificity of Milk Lipases. Ph.D. thesis, University of Wisconsin. 1961.
(12) JENSEN, R. G., GANDER, G. W., SAMPUGNA, J., AND FORSTER, T. L. Lipolysis by a B-Esterase Preparation from Milk. J. Dairy Sci., 44: 943. 1961.
(13) KOOY, J. S., AND PETTE, J. W. The Inhibition of Butyric Acid Fermentation in Cheese by Using Antibiotic Producing Streptococci as Starter. Netherlands Milk Dairy J., 6: 4. 1952.
(14) KRASKIN, K. S., AND STERN, A. M. Terramycin Inhibition of Gluconate Oxidation by *Escherichia coli*. J. Bacteriol., 73: 608. 1957.
(15) NILSSON, R., AND WILLART, S. Lipolytic Activity in Milk. II. The Heat Inactivation of the Fat Splitting in Milk. Rept. No. 64. Milk and Dairy Research, Alnarp, Sweden. 1961.
(16) SCHOLZ, R., SCHMITZ, H., BÜCHER, T., AND LAMPEN, J. O. Über die Wirkung von Nystatin auf Bäckerhafe. Biochem. Z., 331: 71. 1959.
(17) SCHWARTZ, D. P., GOULD, I. A., AND HARPER, W. J. The Milk Lipase System. I. Effect of Time, pH, and Concentration of Substrate on Activity. J. Dairy Sci., 39: 1364. 1956.
(18) SCHWARTZ, D. P., GOULD, I. A., AND HARPER, W. J. The Milk Lipase System. II. Effect of Formaldehyde. J. Dairy Sci., 39: 1375. 1956.
(19) SHAHANI, K. M., NILSON, K. M., AND DOWNS, P. A. Effects of Antifungal Antibiotics upon the Keeping Quality of Cottage Cheese. Proc. 15th Intern. Dairy Congr., 2: 926. 1959.
(20) SJOSTROM, G. Lipase Problems in Milk and Dairy Products. A Review. Rept. No. 58. Milk and Dairy Research, Alnarp, Sweden. 1959.
(21) STADHOUDERS, J., AND MULDER, H. Some Observations on Milk Lipase. II. The Destructive Effect of Light on Milk Lipase Activity. Netherlands Milk Dairy J., 13: 122. 1959.
(22) TARASSUK, N. P., AND YAGUCHI, M. Inhibition of Lipase and Lipolysis in Milk with N-ethylmaleimide. J. Dairy Sci., 42: 864. 1959.
(23) WILLART, S., AND SJOSTROM, G. The Influence of Sodium Chloride upon the Hydrolysis of Milk-Fat in Milk and Cheese. Proc. 15th Intern. Dairy Congr., 3: 1482. 1959.

EFFECT OF HIGH-LEVEL GRAIN FEEDING ON MILK PRODUCTION RESPONSE OF LACTATING DAIRY COWS[1]

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SUMMARY

Eighteen lactating dairy cows were divided into three comparable groups approximately 36 days postpartum to study the effect of feeding high levels of grain for a complete lactation. Grain was fed at three levels: Group 1, 1.0 lb grain per 3.5 lb milk; Group 2, 1.0 lb grain per 2.5 lb milk, and Group 3, grain ad libitum. The cows fed the two higher levels of grain produced considerably more milk than expected from the first part of lactation, whereas cows fed the low level of grain produced slightly less milk than expected. The averages of milk produced during the 260-day experimental period were 9,861, 10,650, and 12,543 lb per cow for Groups 1, 2, and 3, respectively. In the same order, the average percentages of milk fat, protein, and SNF were 3.7, 3.8, 8.9; 3.6, 3.8, 8.9; and 3.7, 3.8, 9.0, respectively. The average gains in body weight were 0.64, 0.91, and 0.94 lb per cow per day for Groups 1, 2, and 3, respectively.

For many years roughages have played the predominant role in dairy cattle rations, primarily because they were recognized as the most economical sources of energy. However, in recent years much has been done to increase the yield of corn which, at least in the Corn Belt, is rapidly becoming the Number One economy feed. In many cases, the cost of 100 lb of TDN from ear corn is less than that from hay-crop silage or hay. Therefore, from an economic standpoint, maximum use should be made of the cheapest source of energy within the physiological limits of the cow.

High-level grain feeding of dairy cows has recently been reviewed by Huffman (4). Castle and Watson (2) reported increased milk yields of cows fed high levels of concentrates over those fed low levels of concentrates. The increased milk yield amounted to approximately 1.0 lb of milk per 1.0 lb additional starch equivalent. In a 10-wk field trial in New York State, Charron (3) observed that 95% of the cows responded in milk production to increased grain feeding, with approximately 40% showing a profitable response.

This experiment was conducted to determine the milk production response of cows fed high levels of grain for the major portion of the lactation period.

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EXPERIMENTAL PROCEDURE

Eighteen Holstein cows were divided into three comparable groups on the basis of milk production, age, and body weight, and assigned to one of three experimental treatments approximately 36 days postpartum. During the subsequent 260-day experimental period all cows were fed 40 lb corn silage per day. In addition, cows in Group 1 received alfalfa hay ad libitum and grain at the rate of 1.0 lb grain for each 3.5 lb of milk. Cows in Group 2 were limited to 15.0 lb of alfalfa hay per day and the grain feeding increased to 1.0 lb of grain for each 2.5 lb of milk. Group 3 cows were limited to only 5.0 lb of alfalfa hay per day and grain was fed ad libitum. The hay and corn silage were considered to be of excellent quality.

The grain mix was composed of 1,500 lb of corn and cob meal, 500 lb of soybean oil meal (44% C.P.), 20 lb of trace mineralized salt, 20 lb of dicalcium phosphate, and 100 lb of feed-grade molasses.

Milk samples were collected bi-weekly for milk fat analyses and monthly for solids-not-fat (SNF) and protein analyses. Milk fat was determined by the standard Babcock procedure; protein content was determined by formol titration (7); and SNF measurements were made by the lactometric method (8).

TABLE 1

Estimated and actual milk production of cows fed different levels of hay and grain

Group	Rate of grain feeding	260-Day production		Difference	
		Estimated	Actual	Act.-Est.	Std. error
1	1:3.5	10,505	9,861	− 644	444.1
2	1:2.5	9,542	10,650	+1,108	627.8
3	ad lib.	9,926	12,543	+2,617	926.5

RESULTS AND DISCUSSION

Milk production was used as the principal criterion in evaluating treatment effects. Since the amount of milk produced in a lactation is closely correlated with milk produced during the early stages of lactation, the amount of milk expected during the 260-day experimental period was estimated from the amount of milk produced during the preliminary period as follows: $P_{260} = P_p\left(\frac{f_p}{f_{p+260}} - 1\right)$, where P_{260} is the expected cumulative production of milk for the 260-day experimental period, P_p is the cumulative milk production during the preliminary period, f_p is the ratio factor (chosen for appropriate age, season of calving, and days in the preliminary period) for estimating 305-day production from the preliminary production and f_{p+260} is the ratio factor (chosen for appropriate age, season of calving, and number of days to the end of the 260-day experimental period) for estimating 305-day production from preliminary and 260-day experimental production. The response of milk production to treatment was measured by comparing the expected production with the actual production for the 260-day experimental period (Table 1).

The estimated potential production of all three groups was relatively close, ranging from 9,542 to 10,505 lb per cow. However, when the actual production of each cow was compared with the expected production, the milk response definitely increased as the level of grain feeding increased. Cows in Group 1 produced 644 lb less milk than expected, whereas cows in Groups 2 and 3 produced 1,108 and 2,617 lb, respectively, more milk than expected. This difference was significantly higher ($P < 0.01$) for cows in Group 3 than for cows in Group 1. Differences between Groups 1 and 2 or 2 and 3 approached significance ($P < 0.05$). If the accuracy of the production equation was constant for all three groups, the average net increase in milk production from feeding higher levels of grain would be 1,752 and 3,261 for Groups 2 and 3, respectively.

All cows did not respond to high-level grain feeding. Four of the six cows in Group 3 produced from 2,550 to 4,970 lb more milk than expected, whereas the two other cows produced at their respective calculated level. The reason these two cows did not respond is not known, but may have been due to the lack of genetic potential for high-level production. These data indicate that increasing the nutritional level of cows that do not have the genetic potential for high-level milk production will not result in increased milk production for a major portion of the lactation period.

The average daily milk production, feed consumption, and changes in body weight by groups are given in Table 2. The differences in hay and grain consumption among groups were primarily due to experimental treatment. The highest daily grain consumption for any one cow was 46.0 lb per day, with an average of 43.2 lb per day for the 260-day period. In no case was difficulty encountered with digestive disturbance or cows going off feed. Corn silage consumption decreased as the level of grain in the ration increased. There were no significant differences in corn silage consumption between Groups 1 and 2; however, both groups consumed significantly more corn silage than Group 3 ($P < 0.05$). Cows consuming the two higher levels of grain gained slightly more in body weight than the low-level grain group. How-

TABLE 2

Average daily milk production, feed consumption, and body weight change for the 260-day experimental period

	Group		
	1	2	3
	(lb/day)		
Milk production			
Actual	37.9	41.0	48.2
FCM	36.6	39.9	46.0
Feed consumption			
Grain	11.9	17.0	36.0
Hay	24.4	13.9	4.9
Corn silage	38.2	37.7	34.2
Body weight change	+0.64	+0.91	+0.94

ever, due to considerable within-group variation the differences among treatment groups in body weight gain were not statistically significant. Cows in Group 3 produced significantly ($P < 0.01$) more milk and FCM than cows in Groups 1 or 2. In general, the response to added grain was through a slight initial increase in production and increased persistency throughout the 260-day period (Figure 1). The average group

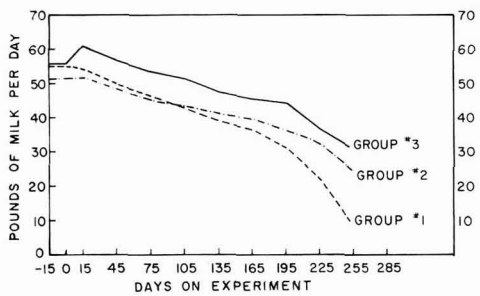

FIG. 1. Actual milk production by 30-day periods for the three experimental groups.

persistency values were 85.0, 92.7, and 94.0% for Groups 1, 2, and 3, respectively. The increased persistency of cows in Groups 2 and 3 was somewhat more evident in late lactation than in early lactation. The average initial milk production values were slightly higher for Groups 1 and 3 than for Group 2. However, due to the lower persistency of Group 1 cows, at the end of 90 days the average daily milk production was approximately the same as Group 2 and declined rather rapidly throughout the remainder of the 260-day period. The relationship between increased grain feeding and total milk production is evident.

This experiment was not designed to study the efficiency of milk production in terms of pounds of TDN required per pound of milk. However, when net efficiency was calculated, the cows in Group 2 required 0.39 lb of TDN per pound of FCM as compared to 0.49 and 0.52 lb, respectively, for Groups 1 and 3 ($P < 0.05$). In this calculation the following assumptions regarding TDN values of feeds were made: grain, 72% TDN; hay, 52% TDN; and corn silage, 19% TDN. The TDN requirements for maintenance were taken from Morrison (6) and the requirements for body weight gain from Brody (1). A correlation of -0.79 was observed between level of milk production for the 260-day period and pounds of TDN required per pound of milk. The increased efficiency of the higher-producing cows over the lower-producing cows may have been partially due to over-feeding the cows of inherent low production. The specific reason for the high correlation between level of milk production and efficiency of production cannot be ascertained from these data. The pounds of TDN required per pound of FCM for cows producing over 10,000 lb of milk averaged 0.44 lb, as compared to 0.32 lb recorded in various feeding standards (5, 6). In this comparison, however, the limitation of the calculations employed, as well as the fact that the cows receiving ad libitum grain were overfed in late lactation, should be considered.

The average milk fat, protein, and solids-not-fat percentages are shown in Table 3. Each period represents 30 days, with the exception of Period 9, which represents only 20 days. In general, the protein, fat, and solids-not-fat content of milk increased with stage of lactation. The small differences observed among

TABLE 3

Effect of high-level grain feeding on milk composition

Periods	Group 1			Group 2			Group 3		
	Protein	Fat	SNF	Protein	Fat	SNF	Protein	Fat	SNF
	(%)								
1	3.14	3.4	8.8	3.34	3.8	8.8	3.25	3.4	8.9
2	3.31	3.5	8.9	3.38	3.6	8.6	3.30	3.5	8.9
3	3.40	3.6	8.8	3.20	3.7	8.9	3.60	3.4	8.9
4	3.60	3.9	8.8	3.42	3.9	8.9	3.60	3.6	9.0
5	3.80	3.8	8.8	3.74	3.7	8.8	3.90	3.9	9.3
6	3.80	3.8	8.9	4.00	4.0	9.0	4.00	4.0	9.0
7	4.00	4.0	8.9	4.00	3.8	9.0	3.90	3.9	8.9
8	3.90	4.3	9.2	4.14	4.0	9.0	3.80	4.1	8.0
9	4.10	4.3	9.2	3.64	4.1	9.2	3.81	4.0	9.1
Average	3.7	3.8	8.9	3.6	3.8	8.9	3.7	3.8	9.0

treatment groups were not significant and did not appear to be associated with the feeding regime.

From the economic standpoint, cows in Groups 2 and 3 returned approximately $35 more over feed cost than cows in Group 1. When only the four highest producers from each group were considered, the return over feed cost was approximately $60 per cow higher for Group 3 than for Groups 1 and 2. In these calculations the following prices were assumed: grain, $40 per ton; hay, $20 per ton; corn silage, $7 per ton; and 4% FCM, $4.60 per 100 lb. Without question, the return over feed cost could have been increased considerably for cows in Group 3 by reducing grain to the lowest possible level without affecting milk production after cows reached their peak of production. Based on this work, it appears that feeding grain to appetite will increase milk production markedly and thus will aid in determining the genetic potential of the cow for high milk production.

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REFERENCES

(1) BRODY, S. Bioenergetics and Growth. Reinhold Publishing Corporation, New York. 1945.

(2) CASTLE, M. E., AND WATSON, J. N. The Effect of Level of Concentrate Feeding Before and After Calving on the Production of Dairy Cows. J. Dairy Research, 28: 221. 1961.

(3) CHARRON, E. C. Higher TDN Feeding of Dairy Cows. Applied Research Department, G.L.F. Exchange, Inc., Ithaca, New York. July 12, 1960.

(4) HUFFMAN, C. F. High Level Grain Feeding for Dairy Cows. J. Dairy Sci., 44: 2114. 1961.

(5) LOOSLI, J. K., BECKER, R. B., HUFFMAN, C. F., PHILLIPS, P. H., AND SHAW, J. C. Nutrient Requirements of Dairy Cattle. Natl. Acad. Sci., Natl. Research Counc. Publ. 464. 1958.

(6) MORRISON, F. B. Feeds and Feeding. 22nd ed. Morrison Publ. Company, Ithaca, New York. 1956.

(7) RICHARDSON, G. A., YOUNG, J. O., DALAL, S. H., PEARCE, S. J., AND NARULA, P. N. The Use of Protein Test (Formol Titration) to Estimate the Solids-not-fat Content of Milk. Proc. 34th Ann. Meet., Western Div., Am. Dairy Sci. Assoc. 1953.

(8) WATSON, P. D. Determination of the Solids in Milk by a Lactometric Method at 102° F. J. Dairy Sci., 40: 394. 1957.

PERFORMANCE OF DAIRY COWS FED PELLETED AND BALED COASTAL BERMUDAGRASS AND ALFALFA HAY[1]

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SUMMARY

Twenty-one cows were fed: (a) baled Coastal bermudagrass, (b) ground and pelleted Coastal bermudagrass plus 2.0 lb of baled Coastal hay per day, and (c) baled alfalfa hay. Following a standardization period, in which both concentrates and baled Coastal hay were fed ad libitum, the treatment rations were fed continuously for the 8th-37th wk of the lactation in one replicate, and the 8th-27th wk in a second replicate. Initially, concentrates were fed with the treatment forages at the rate of 1 to 5 lb of FCM, with the amount reduced 6% each 4 wk. Cows fed the pelleted Coastal produced more milk and FCM, ate more forage, gained more weight, and produced milk higher in milk fat, solids-not-fat, and protein than those fed the baled Coastal. They also produced milk higher in solids-not-fat than those fed alfalfa hay. Consumption of alfalfa hay was higher than that of baled Coastal but lower than that of pelleted Coastal. Cows receiving alfalfa hay produced more FCM than those fed baled Coastal. All of these differences were significant statistically.

Because of its high yields, dependability, and other desirable agronomic properties, Coastal bermudagrass is rapidly becoming the dominant forage throughout much of the southern United States, already occupying several million acres. However, milk production of cows fed Coastal bermudagrass as the principal feed generally has not been satisfactory (6). The poor performance was probably due to low consumption (10). Grinding and pelleting Coastal has been shown to increase its consumption by dairy calves (8) and beef steers (2).

Feeding all finely ground forage, whether pelleted or not, or severely restricting the amount fed, will drastically reduce the fat content of milk (9). Romning et al. (11) have shown that the effect of feeding ground and pelleted forage on milk fat content increases as higher levels of concentrates are fed.

Feeding an all-pelleted ration has produced rumen parakeratosis, but feeding small amounts of long forage with the pellets eliminated this abnormal rumen condition and improved feed consumption and gains of beef steers (5).

The purpose of the study herein presented was to determine whether grinding and pelleting Coastal bermudagrass would increase forage consumption and milk production of dairy cows and whether any adverse effects on milk composition or animal health would be exhibited when Coastal bermudagrass pellets were fed along with a very limited amount of long hay and a low level of concentrates over an extended period of time. A secondary purpose was to determine the performance of cows fed Coastal bermudagrass in the two forms relative to that of those fed commercial alfalfa hay of a quality available to dairymen in the area.

EXPERIMENTAL PROCEDURE

The Coastal bermudagrass was grown on Norfolk sandy loam, fertilized with 500 lb of 0-10-20 and 82 lb of N per acre 30 days before being harvested in August at an average age of 40 days, after the previous cutting, field-cured and baled. Alternate bales were ground with a hammer mill, using a 3/16-inch screen and pelleted with steam through a 1/4-inch die. The Coastal hay graded U.S. No. 2. The baled alfalfa was purchased from Midwestern sources in three lots, in an effort to obtain hay typical of that sold commercially in the Southeastern area. Hay from the three lots was fed at ran-

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dom. On an average, the alfalfa contained 84.5% alfalfa, 11.5% timothy, and 4.0% other forages and foreign material and graded U.S. No. 2 green alfalfa-light timothy hay.

In a continuous type design with a preliminary standardization period, the following forages were fed to 12 Holstein, six Jersey, and three Guernsey cows: (a) baled Coastal bermudagrass hay ad libitum; (b) pelleted Coastal bermudagrass hay, ad libitum, plus 2.0 lb of baled hay per cow daily; and (c) commercial alfalfa hay ad libitum. The forages were fed twice per day and were available to the cows continuously except for the time required for milking.

Following calving, the cows were adjusted to a high level of concentrates. From the 3rd through the 7th wk they were fed Coastal bermudagrass hay and concentrates ad libitum as a standardization diet (7).

The experimental feeds were each fed to four cows for the 8th-37th wk in Replicate One and to three cows for the 8th-27th wk in Replicate Two. When the treatments were initiated (8th wk) concentrates were reduced to 1 lb for each 5 lb of 4% FCM produced during the standardization period. Thereafter, the amount was decreased 6% each 4 wk. The concentrate mixture consisted of 69.6% ground snapped corn, 27.4% cottonseed meal, 1.0% NaCl, 1.5% defluorinated rock phosphate, and 0.5% vitamin A concentrate (3.0 million I.U. per lb).

Milk production was weighed five days per week throughout the study. Five-day composite samples were analyzed for milk fat by Babcock procedures (1) and solids-not-fat by Watson Lactometer (3) each of the last 3 wk of the standardization period and for alternate weeks during the treatment period. Protein was determined by the Kjeldahl method (1) on samples composited for two alternate week periods. At bi-weekly intervals three to five bales of hay or bags of pellets were sampled for proximate analyses (1). Feed intakes were determined by weighing the amounts fed and refused with forage being measured on a group basis, within replications, and grain on an individual cow basis. The cows were weighed on three consecutive days at the end of the standardization period, after adjusting to the experimental diets, at monthly intervals, and at the end of the treatment periods.

RESULTS AND DISCUSSION

During the last 3 wk of the standardization period there was very little average change in milk production (Figure 1). However, when the cows were changed from the standardization diet of ad libitum hay and concentrates to the experimental forages and limited concentrates there was a sharp drop in the level of milk produced (Figure 1). After 3 wk on the treatments this decline averaged 26, 19, and 19% for groups fed the Coastal hay, Coastal pellets, and alfalfa hay, respectively. Following this, the decline in milk production was approximately the same for all treatment groups and at a more normal rate.

Cows fed Coastal pellets plus 2.0 lb of long hay per day produced more milk and 6.8 lb more FCM per day than those fed baled Coastal. The higher milk production when pelleted Coastal was fed is attributed to the large increase in the amount of feed consumed (Table 1). Somewhat more feed was required per pound of milk when pellets were fed, as contrasted to Coastal hay. The milk production of those cows fed alfalfa was not significantly different from that of the other two groups. The average amounts of forage dry matter consumed per 100 lb of body weight per day were: 3.08, 2.10, and 2.73 for the cows fed Coastal pellets, Coastal hay, and alfalfa hay, respectively. Average initial weights of the corresponding groups of cows were: 1,136, 1,039, and 1,092 lb.

Milk from cows fed the pelleted Coastal bermudagrass contained significantly more fat, solids-not-fat, and protein than that from those fed the same grass as hay (Table 1). The 2.0 lb of baled hay was fed with the pellets in a deliberate attempt to alleviate the reduction in milk fat content (9) and rumen parakeratosis which have been observed previously (5). It is apparent that any depressing effect which the pellets might have had on fat content was eliminated. The fat test of milk from cows fed the alfalfa hay was intermediate between that

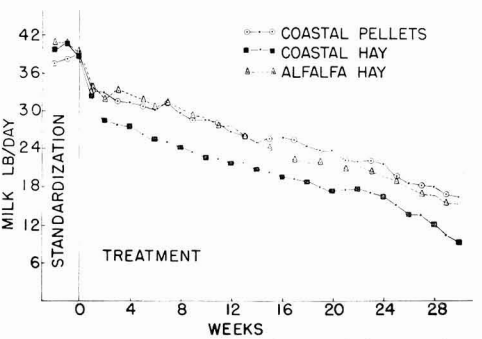

FIG. 1. Milk production of cows fed three forages during the standardization and treatment periods.

TABLE 1

Milk and FCM production, feed consumption, weight gains, and milk composition of cows fed the various forages

	Coastal pellets	Alfalfa hay	Coastal hay
	(lb/cow/day)		
Milk production [a]	27.0 [b]	26.1	22.4
FCM production [a]	28.3	25.9	21.5
Forage consumed [c]	37.6	32.2	23.6
Concentrates consumed [d]	6.5	6.7	6.7
lb FCM/lb concentrates [d]	4.2	4.0	3.2
lb FCM/lb total feed [d]	0.62	0.68	0.71
Weight gains	0.1	−0.1	−0.6
	(%)		
Fat [a]	4.27	3.98	3.78
Solids-not-fat in milk [a]	8.94	8.58	8.49
Protein in milk [a]	3.56	3.37	3.21

[a] Adjusted for slight differences in the standardization period by covariance procedures (4).
[b] Values underlined by the same line are not significantly different ($P = 0.05$).
[c] Differences in forage consumption were highly significant statistically ($P = 0.01$).
[d] Values not analyzed statistically.

of the other two groups and not significantly different from either (Figure 2). Some of the differences in per cent milk fat between those fed the two forms of Coastal bermudagrass may have been due to the low plane of nutrition of the cows fed the hay. The low level of milk production and the 0.6 lb of body weight lost per day in this period when cows normally gain, as well as the low forage consumption, indicate that they did not eat enough Coastal hay to maintain a satisfactory plane of nutrition.

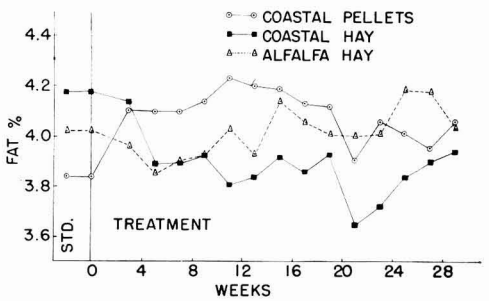

FIG. 2. Milk fat content of cows fed three forages during the standardization and treatment periods.

The higher protein and solids-not-fat content of milk from cows fed the pelleted Coastal relative to those fed this hay unpelleted is attributed primarily to the higher nutrient intake, as this has been shown to have considerable influence on these values (13). The intermediate protein and solids-not-fat percentages from those fed alfalfa hay may also be related to differences in level of nutrient intake. However, the effect of the change in physical form on rumen fermentation may tend to increase solids-not-fat and protein as suggested by Rook et al. (12).

The chemical composition of the Coastal pellets, Coastal hay, alfalfa hay, and the concentrates on a dry matter basis was: protein 12.6, 12.4, 17.2, and 19.1; crude fiber; 30.6, 33.9, 35.0, and 12.0; ether extract; 1.7, 1.6, 1.5, and 4.0; and ash; 4.7, 4.9, 7.2, and 5.3.

In this study pelleting Coastal bermudagrass, when fed with a small amount of long hay and a relatively low level of concentrates, greatly increased the consumption of forage, resulted in higher milk production, and did not adversely affect milk composition or apparent cow health, even over extended periods. The differences in performance between the group fed pelleted Coastal bermudagrass and those fed the same hay baled might have been smaller if a higher level of grain had been fed to all the cows. The exact amount of long hay needed or required has not been conclusively established. However, in the light of other research it seems probable that the long hay does have beneficial effects. The 2.0 lb of long hay per day given the cows fed pellets in this experiment was eaten with relish.

Whether pelleting Coastal bermudagrass will prove to be a profitable practice will depend on many factors, including the cost of producing pellets relative to competitive feeding systems which result in comparable milk production. The commercial alfalfa used in this study was a better forage for supporting milk production than was the unpelleted Coastal hay. However, it was not superior to the pelleted Coastal.

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REFERENCES

(1) ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS. Official Methods of Analysis. 9th ed. Washington, D. C. 1960.

(2) BEATY, E. R., MCCREERY, R. A., AND BROOKS, O. L. Relationship Between the Physical Form of Roughage and Animal Performance. Proc. 8th Intern. Grassland Congr., 708. 1960.

(3) CASTILLO, L. S., TRIMBERGER, G. W., HERRINGTON, B. L., HENDERSON, C. R., AND TURK, K. L. Factors Affecting Chloride Content and Relationship of Chloride, Protein, and Solids-not-fat in Guernsey Milk. J. Dairy Sci., 44: 1961. 1961.

(4) COCHRAN, W. G., AND COX, G. M. Experimental Designs. John Wiley and Sons, Inc., New York. 1950.

(5) CULLISON, A. E. Effect of Physical Form of the Ration on Steer Performance and Certain Rumen Phenomena. J. Animal Sci., 20: 478. 1961.

(6) KING, W. A., BRANNON, C. C., AND WEBB, H. J. Comparison of Coastal Bermuda, Common Bermuda, and Oat Hays with Alfalfa Hay for Milking Cows. South Carolina Agr. Expt. Sta., Bull. 490. 1961.

(7) LUCAS, H. L. Critical Features of Good Dairy Feeding Experiments. J. Dairy Sci., 43: 193. 1960.

(8) MILLER, W. J. Comparison of Lard, Tallow, Butter and Hydrogenated Cottonseed Oil in Starters and of Pelleted vs. Nonpelleted Coastal Bermudagrass Hay for Calves. J. Dairy Sci., 45: 759. 1962.

(9) POWELL, E. B. One Cause of Fat Variation in Milk. p. 40. Proc. Am. Soc. Animal Production. 1938.

(10) REID, J. T. Problems of Feed Evaluation Related to Feeding of Dairy Cows. J. Dairy Sci., 44: 2122. 1961.

(11) RONNING, M., MEYER, J. H., AND CLARK, G. T. Pelleted Alfalfa Hay for Milk Production. J. Dairy Sci., 42: 1373. 1959.

(12) ROOK, J. A. F., AND BALCH, C. C. The Effects of Intraruminal Infusions of Acetic and Propionic Acids on the Yield and Composition of the Milk of the Cow. Proc. Nutrition Soc., 18: XXXIV. 1959.

(13) ROOK, J. A. F., LINE, C., AND ROWLAND, S. J. The Effect of Plane of Energy Nutrition of the Cow During the Late Winter-Feeding Period on the Changes in the Solids-not-fat Content of Milk During the Spring-Grazing Period. J. Dairy Research, 27: 427. 1960.

RELATION OF DAYS OPEN AND DAYS DRY TO LACTATION MILK AND FAT YIELDS [1]

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SUMMARY

The relationships between days open, days dry, and 90-day and 305-day production were studied, using 4,385 lactation records from nine North Carolina Institutional Holstein herds from June, 1950, through December, 1958. Relationships were studied separately for first, second or later, and all lactations to determine if adjustment of 305-day lactation records for days open and days dry are warranted in compiling sire summaries.

The intra-herd-year-season phenotypic correlations between 90-day production and days open were not significant and ranged from 0.05 to 0.08, suggesting that level of production had very little influence on this meeasure of fertility. The heritability estimates for days open were also very low, ranging from 0.01 in first lactations to 0.09 for all records.

Length of the previous dry period had very little influence on production, accounting for only 0.6 and 0.3% of the variation in 90-day and 305-day fat yield and less than 0.1% of the variation in the two measures of milk production.

Number of days open during the lactation significantly influenced production and accounted for 6.5, 4.3, and 4.2% of the variation in 305-day milk yield for first, second or later, and all lactations, respectively. Factors for adjusting 305-day production for differences in days open are presented.

Early conception and short dry periods are reported to have depressing effects on lactation milk yields (4, 12, 13, 21, 22). Some dairymen and researchers also are of the opinion that high production is antagonistic to early conception following parturition (5, 18). These observations suggest a dual cause and effect relationship between lactation yield and reproductive efficiency. High-producing cows may not conceive as readily as low producers; and cows with more days open may have less interference of pregnancy on lactation milk yield.

The opinion that high-producing cows do not conceive as readily as low producers lacks conclusive experimental evidence (25). Most of the studies concerning the relation between reproductive efficiency and production have utilized measures of complete lactation yield with some lactations as long as 500 days. Carman (5) and Lewis and Harwood (18) attributed the correlation between lactation production and reproductive efficiency to the depressing influence of high production on fertility. However, Lee et al. (17) and Sanders (22) attributed the correlation between production and reproductive efficiency to the inhibitory action of gestation on production. The influence of level of production on reproductive efficiency must be examined using a measure of initial level of production before gestation could exert an inhibitory influence. Boyd et al. (3) correlated the first 120-day yield with services per conception and obtained a coefficient of −0.04. A study conducted on records taken from the Guernsey Herd Register by Gaines (11) revealed a correlation of only 0.039 between the first full month's production and the service period.

Investigations of the relation between production and reproductive efficiency should take into account the major environmental factors that may influence the observed correlations. Herd, year, season, age, and length of the previous dry period are among the factors that should be considered. Sanders (21) observed that the seasonal differences in length of dry period and service period were responsible for part of the seasonal differences in milk yield. Differences in production associated with age

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and the length of the previous dry period have received considerable attention. However, the relation of age to the length of the previous dry period and days open in the current lactation, and the possible interrelationships between previous dry period and days open, have not been investigated extensively.

This study was undertaken to determine the existing relationships between production, days open, and days dry, and to determine if adjustment of 305-day lactation records for these variables is warranted in compiling sire summaries.

DATA AND METHODS

Data collected in nine state-owned Holstein herds in the North Carolina Institutional Breeding Program from June, 1950, through December, 1958, were studied. Since June, 1949, these nine Holstein herds have been organized as one breeding unit, with an attempt to maximize genetic progress, within practical limits, using progeny testing and selection. Good managemental practices have been emphasized since the beginning of the program. As a general rule, heifers initially are inseminated when they are 15 months old and attain a body weight of 750 lb. For subsequent lactations, cows are initially inseminated on the first heat 60 to 70 days postpartum.

The number of days open during the 305-day lactation period was used as the measure of reproductive efficiency in this study. Days open during the lactation provides essentially the same information as days carried calf. Both of these measures are superior to calving interval, in that they permit the inclusion of data on the final lactation of cows leaving herds in a barren condition. Days open also has the advantage in herd management, in that it focuses on the need for conception by a specified time after parturition. Days open is determined at the time of conception; whereas, days carried calf is directly influenced by the number of days in milk.

Complete records of less than 305 days in milk were included only if the cow remained in the herd for the full 305-day period. The number of days open for all lactations followed by a normal calving were computed by subtracting a 280-day gestation period from the calving interval (6). There were 755 complete lactation records not followed by a normal calving. Most of these records were for the last lactations of cows removed from the herds. By using all available information, such as records of breeding, heat dates, pregnancy diagnosis and disposal, the number of days open was determined for 90% of these lactations. Actual means for traits included in the study and their age-adjusted standard deviations are presented in Table 1.

The production during the first 90 days, as determined by the first three test days, was used as the measure of production during the initial breeding period.

The data were analyzed in three groupings, first, second and later, and all lactation records. There were 1,314 first lactations and 3,071 second and later lactations, or a total of 4,385 lactation records meeting all requirements for inclusion in this study. In general, the analyses were the same for each grouping, except that the length of the previous dry period could be included as an independent variable only in the second and later records. The data were adjusted for age effects based on the age influences, indicated by the linear and the curvilinear regression of the several variables on age at freshening in months.

Components of variance and covariance were estimated from the age-adjusted data by adapting Henderson's (1953) Method II to a model with covariates and extending it to adjust covariances between traits for fixed effects. The

TABLE 1

Actual mean values for traits by groups

Trait	Record grouping		
	First	Second or later	All
Age in months	32	70	59
Days dry		72 (35)	
Days open	143 (77) [a]	146 (77)	145 (77)
90-Day milk (lb)	3,670 (559)	5,016 (739)	4,613 (681)
90 Day fat (lb)	129 (21)	177 (30)	163 (27)
305-Day milk (lb)	10,370 (1,683)	12,706 (2,180)	12,007 (1,970)
305-Day fat (lb)	368 (58)	445 (78)	422 (70)

[a] Age adjusted within-herd-year-season-sire standard deviations.

model for a given character including the p fixed effects is:

$$Y_{ijk} = \mu + a_i + s_{ij} + e_{ijk} + \sum_{m=1}^{p} b_m (x_m)_{ijk}$$

Where: Y_{ijk} is the observation on the k^{th} daughter of the j^{th} sire in the i^{th} herd-year-season group.

μ is the general mean.

a_i is the effect common to all records in the i^{th} herd-year-season group.

s_{ij} is the effect common to all daughters of the j^{th} sire in the i^{th} herd-year-season group.

e_{ijk} is an effect peculiar to the k^{th} daughter of the j^{th} sire in the i^{th} herd-year-season group, including measurement and random errors.

The b_m are coefficients for the regression of the Y_{ijk} on the independent x_m on an among cow in sire in herd-year-season basis, and the means for the a_i, s_{ij}, and e_{ijk} are zero, and their variances are σ_a^2, σ_s^2, and σ_e^2, respectively.

RESULTS AND DISCUSSION

Age at freshening. The regression of 90-day and 305-day milk and fat on age followed the well-established pattern. Linear regression adequately accounted for changes in production associated with age at freshening in first-lactation records. In the other two groupings of the data, the regression of yield on age was distinctly curvilinear. The age effects were larger in the 90-day records than in the 305-day records. From the analyses combining all records, 29 and 28% of the variance was attributable to age in 90-day records for milk and fat, respectively; whereas, only 17% of the variance was attributable to age in 305-day milk and fat records.

The regressions of days open and days dry on age are given in Table 2. In these data, only a small percentage of the variation in days open and days dry can be attributed to age. This low relation between days open and age is in close agreement with the findings of Lewis and Harwood (18). The regression of days dry on age is in agreement with the findings of Dickerson (7), who reported that the length of the dry period increased slightly with age. Johansson and Hansson (16), however, stated that the dry period between the first and second lactation was shorter than the subsequent dry periods which showed no trend with advancing age.

TABLE 3

Intra-herd-year-season phenotypic correlations between days open and 90-day milk and fat yield

Lactation	90-day milk	90-day fat
First	0.08 ± 0.04	0.08 ± 0.04
Second or later	0.06 ± 0.03	0.05 ± 0.03
All	0.06 ± 0.02	0.06 ± 0.02

Relation between days open and 90-day production. The intra-herd-year-season phenotypic correlations between days open and 90-day milk and fat yield are presented in Table 3. These correlations of 0.05 to 0.08 are so small that there is little evidence that high production during the first 90 days of lactation delayed conception. These estimates compare favorably with the correlation of 0.04 between the first month's production and the service period reported by Gaines (11). The phenotypic regression of days open on 90-day milk yield for all lactations indicated that as 90-day milk

TABLE 2

Regression coefficients [a] for the effect of age (A) and age squared (A^2) on days open and days dry

Character	A [b]	A^2	Per cent of variation due to regression
Days open [c]	0.36 ± 0.09		0.61
	0.17 ± 0.30	0.001 ± 0.002	0.63
Days dry [d]	0.21 ± 0.05		0.99
	0.72 ± 0.20	−0.003 ± 0.001	1.39

[a] Partial regression coefficients where values for A and A^2 are given.
[b] Age in months.
[c] All lactation records.
[d] Second and later lactation records.

yield increased from two standard deviations below the mean to two standard deviations above the mean (i.e., 2,868 lb) only an 18-day increase in days open would be expected.

These correlations and regressions include contributions from both the genetic and environmental influences. Intentional managemental delays in breeding high producers could have added a positive increment to these relationships. However, the managemental programs in these herds include regular breeding of all cows at the first estrus 60 to 70 days postpartum. The correlations obtained indicate that there was neither an important influence of initial yield on days open, nor was their evidence of a differential delay in breeding higher producers. As was previously stated, only records where the cows remained in the herds for 305-days post-partum could be included in this study. If a higher proportion of the low-producing cows which were open were removed from the herds prior to 305-days post-partum, as compared to the high-producing cows which were open, some additional positive bias might be included in the values given in Table 3.

The length of the previous days dry could not have exerted much influence on days open, either directly or through its influence on 90-day yield. In these data the correlation between preceding dry period and days open was only 0.07 ± 0.03 and, as later results point out, days dry accounted for less than 1% of the variance in 90-day yield.

Relation of days dry to yield. Variation in production and days open associated with the length of the previous dry period could be estimated only in second and later lactation records. Figure 1 shows the curvilinear regression of 90-day and 305-day milk yield on days dry. As the number of days dry increased from 0 to 100, the predicted milk yield increased 100 and 200 lb in 90-day and 305-day records, respectively. These regressions accounted for less than 0.1% of the variance in milk yield. The

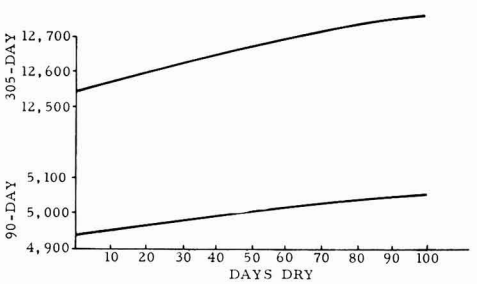

FIG. 1. Intra-herd-year-season-sire regression of milk yield on days dry.

length of the dry period was slightly more important in the two measures of fat yield, accounting for 0.6 and 0.3% of the variation in 90-day and 305-day records, respectively. The flat curves indicated in Figure 1 are not consistent with the often-reported sharp decline in production for dry periods of less than 40 days (1, 2, 9, 16, 23). In these data, however, only 9.4% of the dry periods were less than 40 days and 25% were more than 80 days. Hence, the records with under 40 days dry had little impact on the regression equation. In addition, the importance of the dry period may vary with the level of feeding. Dickerson (7) found that the length of the dry period was more important in low-producing herds.

Relation between days open and yield. Relationships that exist between 305-day yield and days open may be interpreted to be due largely to the influence of gestation on production, since the correlations between days open and 90-day yield were small and not significant, and the length of the previous dry period appeared to be unimportant and not significantly correlated with days open. The regressions of 305-day milk yield on days open are shown in Figure 2. The estimated regressions for milk yield on days open are in close agreement for each grouping of the data accounting for 6.5, 4.3, and 4.2% of the age-adjusted variance in first, second or later, and all lactation records. Similar analyses indicated that 6.0, 4.0, and 3.6% of the variance in 305-day fat yield could be attributed to days open. The corresponding multiple correlation coefficients for fat are 0.24, 0.20, and 0.19, respectively. Touchberry et al. (26) found a simple correlation of 0.12 between days open and fat yield, and the comparable correlation in these data of 0.18 was not significantly different.

Gavin (13) and Sanders (22) found that production seemed to decline more rapidly 16 to 20 wk following conception. One of the early theories held that this decrease in milk production was due to the drain of nutrients imposed by the developing fetus (4, 8, 14, 19). However, it is doubtful if the nutrient requirements of the fetus are great enough to account for all of the observed decline in milk yield. This is especially true in view of the data presented by Swett et al. (24), indicating that more than half of the fetal growth takes place during the last 60 days of gestation. Many other theories involving various types of hormone action also have been presented. However, in an extensive review on the subject, Reece (20) indicates that none of the theories presented thus far has been substantiated. The one fact that

seems well established is that pregnancy, especially during the later half, is accompanied by a marked depression in milk yield.

The variation in production attributable to days open in these data was to some degree influenced by lactation length. Thirty per cent, or 1,304 of the lactations, were less than 305 days in length. During the majority (843) of these lactations the cows conceived prior to 85 days post-partum, and during these lactations sufficient time was not available for a complete 305-day lactation and also a 60-day dry period. These differences in production that are associated with lactation length and, in turn, associated with reproductive status should be included as effects of days open on production. The short lactations of cows that have inherently low persistency would be expected to be associated with an average number of days open, since days dry and days open are essentially independent. Hence, these short lactations should not have contributed to the regression of production on days open.

Adjustment for days open. The regression of 305-day milk yield on days open, for each grouping of the data, shown in Figure 2, indicated that there was about a 1,000 lb difference in the production of contemporary paternal half sibs that conceived at 60 and 160 days post-partum. Such differences in production appear to be closely associated with the depressing effect of gestation. There is little evidence that conception was more difficult in inherently high producers than in the lower producers, since the correlations between 90-day milk yield and days open, shown in Table 3, were low. These results are supported by the findings of Boyd et al. (3) and Gaines (11). Also, genetic differences in days open in these data were unimportant. Heritability estimates for days open, derived from the sire components of variance, were 0.01, 0.05, and 0.09 in first, second and later, and all lactations, respectively. In addition, the regression of daughter on dam for 449 daughter-dam pairs was −0.001. Even if it were assumed that the genetic correlation between days open and milk yield was unity, adjustment of records for days open would not involve genetic influence for yield, since the heritability of days open is essentially zero.

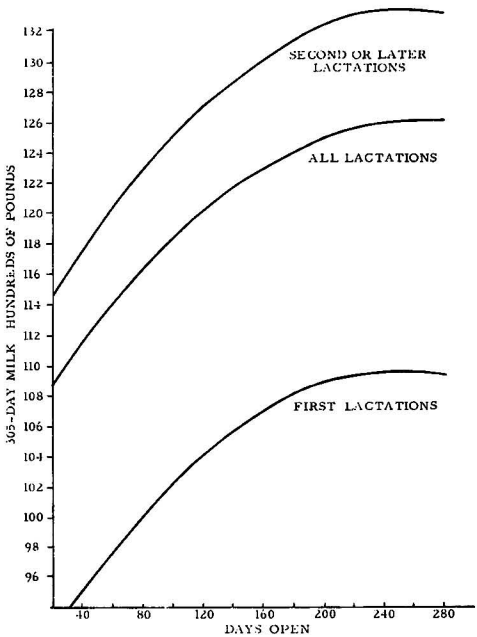

FIG. 2. Intra-herd-year-season-sire regression of 305-day milk yield on days open.

The adjustment of lactation records for various environmental factors poses several difficult problems. Adjustments derived from one set of data might not be appropriate for records in different herds or future records in the same herds. In fact, they can not be expected to fit precisely all records in the data from which they were derived. It is impossible to actually control many of the environmental factors that influence production; thus, it is necessary to resort to certain statistical controls to enhance the accuracy of estimates of the breeding values of dairy animals.

Since most of the bulls are at least conditionally selected or rejected on the basis of the first lactations of a reasonably small group of daughters, these first-sire summaries would be most seriously affected by differences in days open. This would be especially true where the proof was from a single herd that had encountered reproductive irregularities. Many of the environmental effects of management on sire proofs are side-stepped by using contemporary comparisons. These comparisons, however, remove only variation associated with herds, years, and seasons. At the present time, age conversion factors are widely used to adjust milk yield for differences due to age. This type of adjustment is possible because age at freshening is a measurable character assumed to have no direct genetic correlation with production. The age and genetic variation in days open appears to be unimportant; hence, adjustment of lactation production for differences in days open may be of considerable value.

Multiplicative factors derived from the intra-herd-year-season-sire regression of milk yield on days open should provide an appropriate adjustment for days open. Sanders (22) suggested multiplicative factors in his report concerning the effect of gestation on complete lactations. In his data there were only slight differences in factors derived from low- and high-producing cows. Factors derived from the three regression equations, shown in Figure 2, were in very close agreement. The equations for second or later and all lactations provided the same factors. Those for first lactations ranged from 0.02 higher to 0.01 lower than those for the two other groupings of the data, as days open increased from 20 to 220 days. Factors developed from all lactations using 100 days open as the base are shown in Table 4. This base of 100 days open

TABLE 4

Multiplicative factors for adjusting 305-day production for variation in days open

Days open	Factor	Days open	Factor
24 or less	1.09	86– 95	1.01
25–30	1.08	96–105	1.00
31–35	1.07	106–115	.99
36–45	1.06	116–135	.98
46–55	1.05	136–155	.97
56–65	1.04	156–175	.96
66–75	1.03	176–205	.95
76–85	1.02	206 or more	.94

was chosen, as it is between the mean and the mode and a high percentage of the data requires only a small correction. In addition, when conception occurs with no more than an average of 100 days open, a twelve- to thirteen-month calving interval can be maintained.

REFERENCES

(1) Arnold, P. T. D., and Becker, R. B. Influence of Preceding Dry Period and of Mineral Supplement on Lactation. J. Dairy Sci., 19: 257. 1936.

(2) Bayley, N. D., and Heizer, E. E. Herd Data Measures of Effect of Certain Environmental Influences on Dairy Cattle Production. J. Dairy Sci., 35: 540. 1952.

(3) Boyd, L. J., Seath, D. M., and Olds, D. Relationship Between Level of Milk Production and Breeding Efficiency in Dairy Cattle. J. Animal Sci., 13: 89. 1954.

(4) Brody, S., Ragsdale, A. C., and Turner, C. W. The Effect of Gestation on the Rate of Decline of Milk Secretion with the Advance of the Period of Lactation. J. Gen. Physiol., 5: 777. 1923.

(5) Carman, G. M. Interrelations of Milk Production and Breeding Efficiency in Dairy Cows. J. Animal Sci., 14: 753. 1955.

(6) DeFries, J. C., Touchberry, R. W., and Hays, R. L. Heritability of the Length of the Gestation Period in Dairy Cattle. J. Dairy Sci., 42: 598. 1959.

(7) Dickerson, G. E. Estimates of Producing Ability in Dairy Cattle. J. Agr. Research, 61: 561. 1940.

(8) Eckles, C. H. The Nutrients Required to Develop the Bovine Fetus. Missouri Agr. Expt. Sta., Research Bull. 26. 1916.

(9) Erb, R. E., Goodwin, M. M., Morrison, R. A., and Shaw, A. O. Lactation Studies. I. Effect of Gestation. J. Dairy Sci., 35: 224. 1952.

(10) Etgen, W. M. The Effect of Gestation on Milk and Butterfat Production in Dairy Cattle. Ph.D. thesis, Ohio State University. 1958.

(11) Gaines, W. L. Milk Yield in Relation to Recurrence of Conception. J. Dairy Sci., 10: 117. 1927.

(12) Gaines, W. L., and Davidson, F. A. The Effect of Advance in Lactation and Gestation on Mammary Activity. J. Gen. Physiol., 9: 325. 1926.

(13) Gavin, W. Studies in Milk Records: The Influence of Foetal Growth on Yield. J. Agr. Sci., 5: 309. 1913.

(14) Gowen, J. W. Intrauterine Development of the Bovine Fetus in Relation to Milk Yield in Guernsey Cattle. J. Dairy Sci., 7: 311. 1924.

(15) Henderson, C. R. Estimation of Variance and Covariance Components. Biometrics, 9: 26. 1953.

(16) Johansson, I., and Hansson, A. Causes of Variation in Milk and Butterfat Yield of Dairy Cows. Kgl. Lantbruksakad. Tidskr. Nr. 6 1/2: 1. 1940.

(17) Lee, J. E., Fosgate, O. T., and Carman, J. L. Some Effects of Certain Environmental Inherited Influences upon Milk and Fat Production in Dairy Cattle. J. Dairy Sci., 44: 296. 1961.

(18) Lewis, R. C., and Harwood, R. E. The Influence of Age, Level of Production and Management on Calving Interval. Michigan Agr. Expt. Sta., Quart. Bull., 32: 546. 1950.

(19) Ragsdale, A. C., Turner, C. W., and Brody, S. The Effect of Gestation upon Lactation in the Dairy Cow. J. Dairy Sci., 7: 24. 1924.

(20) Reece, R. P. Mammary Gland Development and Function. The Endocrinology of Reproduction. (J. T. Velardo, ed.) pp. 213-240. Oxford University Press, Inc., New York. 1958.

(21) Sanders, H. G. The Variations in Milk Yield Caused by Season of the Year, Service Age and Dry Period, and Their Elimination. Part I. Season of the Year. J. Agr. Sci., 17: 339. 1927.

(22) SANDERS, H. G. The Variations in Milk Yield Caused by Season of the Year, Service, Age and Dry Period and Their Elimination. Part II. Service. J. Agr. Sci., 17: 502. 1927.

(23) SANDERS, H. G. The Variations in Milk Yields Caused by Season of the Year, Service, Age, and Dry Period, and Their Elimination. Part IV. Dry Period, and Standardization of Yields. J. Agr. Sci., 18: 209. 1928.

(24) SWETT, W. W., MATTHEWS, C. A., AND FOHRMAN, M. H. Development of the Fetus in the Dairy Cow. USDA, Tech. Bull. 964. 1948.

(25) TOUCHBERRY, R. W. The Association Between the Level of Butterfat Production and the Number of Services Required per Conception. Estratto da Genetica Agraria, 10: 89. 1958.

(26) TOUCHBERRY, R. W., ROTTENSTEN, K., AND ANDERSEN, H. Associations Between Service Interval, Interval from First Service to Conception, Number of Services per Conception, and Level of Butterfat Production. J. Dairy Sci., 42: 1157. 1959.

EFFECT OF FREQUENT FEEDING ON WEIGHT GAIN RESPONSE IN YOUNG DAIRY HEIFERS [1]

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SUMMARY

In four successive trials, weight gain was observed on growing Holstein heifers as a result of feeding 24 × or 10 × vs. 2 × daily. Both restricted and unrestricted systems for governing feed intake were employed. Frequent feeding induced faster weight gain only when the rations were quantitatively restricted, and only for a period of 30 to 38 days. There were no significant differences in feed consumption between treatment and control animals.

In recent years, considerable interest has been focused on the proposal that more frequent feeding of the growing ruminant will produce faster weight gains. If this proposal is correct, then by the simple expedient of offering portions of the daily ration at intervals more frequent than the traditional once or twice daily, the livestockman could cause his animals to grow faster and come into production sooner, thus increasing his efficiency of production.

Work has been reported using sheep (2, 13), dairy heifers (8, 12), and beef cattle (9, 11) which indicates that these animals will show weight gain response to frequent feeding. Other workers (4) reported no advantage to frequent feeding with lambs. Earlier workers (8) concluded that there was no advantage to frequent feeding with respect to milk production, while more recent work (1) has shown, largely on the basis of a greater feed intake, that feeding dairy cows four or seven times daily increased milk production.

The study reported here was undertaken to determine if a weight gain response to frequent feeding could be maintained in growing ruminants over a longer period of time than had been reported from other stations, and if so, to determine the optimum number of intervals that feed should be offered.

EXPERIMENTAL PROCEDURE AND RESULTS

The feeding of the frequently fed animals was accomplished by the use of an electrically controlled conveyor running over the mangers. A day's supply of feed was placed in the conveyor in the morning and was delivered to the animals automatically at the desired intervals. The hay was chopped for all animals, to facilitate its use in the conveyor. Concentrate feed was distributed uniformly on top of the chopped hay in the conveyor for the frequently fed animals, and was hand-fed along with the hay to the controls. Water was provided by automatic drinking cups, and trace-mineralized salt was placed before the animals at all times. Analyses of variance were applied to the data according to the method of Snedecor (14).

Trial 1. Twelve Holstein heifer calves, ranging in weight from 235 to 325 lb, were paired according to age and weight. One member of each pair was randomly selected to receive its daily ration in 24 hourly allotments, while the controls were fed twice a day, at 7 AM and 4 PM. The ration was composed of medium-quality grass hay, predominantly timothy, and a 17% commercial dairy concentrate mixture. The hay was furnished at a level which allowed approximately 10% refusal, and this level was adjusted daily. Concentrate consumption was kept constant at 2.5 lb daily per animal. Weights of all animals were recorded initially and on three consecutive days at monthly intervals. The animals were individually fed, and daily records of feed consumption and refusal were kept.

After eight months, during which the animals showed normal gains and general good health, no statistically significant differences in weight gains were apparent. There were no statistically significant differences between treatments at the end of any of the monthly intervals during the trial. Total feed consumption by the two groups was essentially the same. The data are summarized in Table 1.

Results of this trial were not in agreement with those of Gordon and Tribe (2), Mochrie

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TABLE 1

Body weight gain and feed consumption by heifer calves fed 2 × and 24 × daily in Trial 1 [a, b]

No. times fed	No. animals	Total weight gain	Total feed consumed
		(lb)	
2 ×	6	2,223	15,234
24 ×	6	2,214	15,319
Difference		9	85

[a] Length of trial = 245 days.
[b] Rations adjusted daily to 10% refusal allowance.

et al. (8), Mohrman et al. (9), Putnam et al. (11), and Rakes et al. (12, 13). These workers, however, reported having fed their respective animals considerably fewer times daily. In the present study it was noted, in the case of the frequently fed animals, that inasmuch as they received a small portion of their ration each hour, with no break in the sequence, there was always present some uneaten feed. Thus, it was reasoned that perhaps the number of feeding intervals for the test group was too many and so continuous as to approximate a regimen not essentially different from that of the control group.

Trial 2. The procedure of Trial 1 was used with respect to the hay fed, method of feeding, and data collected. The same group of twelve animals as in Trial 1 was used, again paired according to weight. They were allowed a 21-day preliminary feeding period to establish the daily hay intake. This level was adjusted within pairs and then held constant throughout the trial, which lasted 60 days. One member of each pair was randomly assigned to receive its daily ration in equal portions at ten hourly intervals during the daytime, beginning at 7 AM. The controls were fed twice a day as in Trial 1. One-half pound of soybean oil meal per day per animal was substituted for the 17% concentrate mixture used in Trial 1. Because of a shift in weighing schedules the animals in this trial were weighed at the end of the first 38 days instead of the usual 30 days. Since the daily ration was not increased during the course of this trial, there was very little accumulation of feed in the mangers throughout the day, so that essentially no feed was available to the animals during the night.

After 38 days of this treatment, the frequently fed group had outgained their controls by 102 lb, or 17 lb per animal. This difference was significant ($P < .05$). After 60 days, however, the difference had decreased to 44 lb (7.3 lb per animal) and was not statistically significant. Feed consumption at the end of the 60-day period was nearly the same for both groups. The data are presented in Table 2.

TABLE 2

Body weight gain and feed consumption by heifer calves fed nonincreasing ration 2 × and 10 × daily

No. times fed	No. animals	Total weight gain		Total feed consumed
		38 days	60 days	
		(lb)		
2 ×	6	119	268	6,191
10 ×	6	221	312	6,178
Difference		102[a]	44	13

[a] Significant at the 5% level of probability.

These results indicated a decreasing difference in weight gain with time, and also suggested the possibility that when an animal has gained to a point beyond which a constant daily ration fails to supply nutrients in adequate amounts to maintain the added weight, the rate of gain declines.

Trial 3. In this trial, instead of restricting the daily hay intake to a constant level as in Trial 2, the animals were all fed at a level which was governed by an approximate 10% refusal. This level was adjusted daily, and the refusal was weighed back in the AM. The hay was similar in kind and quality to that used in the previous trials, and was fed 2 × and 10 × daily to respective members of two groups of animals. The animals used were a different group of 12 Holstein heifers, and ranged in weight from 382 to 522 lb. They were paired according to weight and previous treatment. Three-quarters of a pound of soybean oil meal was fed to each animal daily.

Although this experiment was continued for only 60 days in comparison to eight months for Trial 1, results were similar. At the end of the first 30 days there were no statistically significant differences in weight gain between the two groups, and when 60 days of this treatment were completed the two groups were still approximately even. The data for this trial are presented in Table 3.

A greater difference in total feed consumption appeared in this trial, with the control group having consumed 48.5 lb more hay per animal than the frequently fed group, but statistical analysis showed this difference to be nonsignificant.

TABLE 3

Body weight gain and feed consumption by heifer calves fed nonrestricted ration [a] 2 × and 10 × daily

No. times fed	No. animals	Total weight gain 30 days	Total weight gain 60 days	Total feed consumed
		(lb)		
2 ×	6	332	651	4,742
10 ×	6	341	641	4,450
Difference		9	10	292

[a] Ration adjusted daily to 10% refusal allowance.

It was suggested from these findings, and from the results obtained in Trial 2, that restriction of feed intake may have a bearing on the problem. It was reasoned that a response to frequent feeding may accrue in a situation wherein the method (frequency) of feeding resulted in a more or less continuous nonsatisfied appetite on the part of the animals. This condition may exist when a constant daily ration is maintained over the length of a trial period, but not when the daily ration is allowed to change. This possibility is supported by results obtained in Trial 2, and from the work reported from other stations (2, 8, 12, 13), for in those trials in which advantage was shown for frequent feeding, the animals were maintained on a nonincreasing daily ration.

Trial 4. This trial was conducted to investigate the possibility of feed restriction being a factor, and was conducted in a manner similar to Trial 3 with respect to kind and quality of ration, number of treatments, and number of feeding intervals within the respective treatments. The same group of animals was used as in the previous trial. The hay fed to the frequently fed animals was offered at a level in which only a bare minimum refusal from each animal was allowed. This refusal was composed of undesirable and coarse stems. Close daily scrutiny was maintained, in order to make adjustments in the rations to insure that this refusal remained minimal. The control animals were allowed approximately 10% refusal in their rations. The trial lasted for 60 days. Data for this trial are presented in Table 4.

The data for the first 30 days showed a weight gain advantage of 106 lb for the frequently fed group. This difference was highly significant ($P < .01$). When the trial had run for 60 days, all of this advantage had disappeared. The two groups ended the trial nearly even in weight, with the control group weighing nearly 10 lb more per animal. As in the preceding trial, feed consumption was higher in the control group but, again, this was found to be nonsignificant statistically.

DISCUSSION

Results obtained in these trials indicated that when growing ruminants were fed their diets on an unrestricted regimen, no advantage was obtained by feeding at frequent intervals. Although Mohrman et al. (9) have reported weight gain advantage for frequent feeding on an unrestricted feed intake regimen, it is noted that 17% more feed was consumed by the frequently fed animals.

On the other hand, when the feed was restricted, the data showed a significant advantage in frequent feeding for a 4- or 5-wk period, after which all advantage disappeared. The initial advantage observed was in agreement with work reported by others (2, 8, 11-13), but was of shorter duration. Thus, it is suggested that the change from a once- or twice-a-day feeding routine to several times a day leads to conditions which alter the rumen microbial population and/or change the amount or ratio of end products of rumination sufficiently to cause a temporary response which may last for a few weeks, after which the animals' adjustment to the new routine is completed and a response no longer accrues. The work of Moir and Somers (10) has shown a highly significant increase in numbers of ruminal protozoa when sheep were fed 4 × as compared to 2 × daily. More recently, Knox and Ward (3) have reported that total volatile fatty acid concentration was significantly increased, acetic and propionic acids were decreased and increased, respectively, and the acetic/propionic ratio was lowered in the ingesta of cows fed 8 × as compared to 2 ×

TABLE 4

Body weight gain and feed consumption by heifer calves fed semirestricted ration [a] 2 × and 10 × daily

No. times fed	No. animals	Total weight gain 30 days	Total weight gain 60 days	Total feed consumed
		(lb)		
2 ×	6	182	595	5,231
10 ×	6	288	536	4,916
Difference		106[b]	59	315

[a] Minimum daily refusal of coarse stems allowed.
[b] Significant at the 1% level of probability.

daily. Putnam et al. (11) were unable to confirm this result.

Results indicating that response to frequent feeding may be only temporary in nature are not inconsistant with other reports, when it is considered that in those experiments that showed weight gain advantage (2, 8, 11-13) the reversal type of trial was employed. If the response is temporary, then it could be expected that results obtained after a reversal of treatment had been made at a time in the experiment when the frequently fed animals may have reached their peak in advantage would reflect an accumulation of temporarily accelerated weight gains from two groups of animals successively given the same treatment. Such results would not be a true indication of the long-term performance of animals on a frequent feeding schedule.

No direct evidence is available to offer an explanation of possible mechanisms involved in the present study. Although Rakes et al. (13) discussed possible explanations for the weight gain response, these do not suggest why the effect could not be maintained. Indirect evidence which may provide a clue in this respect comes from the West Virginia station. Welch et al. (15) noted that the utilization of nonprotein nitrogen (NPN) by lambs increased with time and reached a maximum in 35 days. This work was confirmed and extended by McLaren et al. (6, 7), who indicated that an adaptation response took place in the tissues when the dietary source of nitrogen was changed from natural rations to NPN compounds. Subsequent work by McLaren et al. (5) has further suggested that specific enzymic pathways of metabolism in the tissues are affected. In light of these findings, it seems tenable to suggest that as a result of frequent feeding specific enzymic pathways for the metabolism of end products of rumination may be altered because of a change in the concentration ratios of these metabolites, and once the new pathways are established any favorable response induced by the change in feeding regimen levels off.

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REFERENCES

(1) Campbell, J. R., and Merilan, C. P. Effects of Frequency of Feeding on Production Characteristics and Feed Utilization in Lactating Dairy Cows. J. Dairy Sci., 44: 664. 1961.

(2) Gordon, J. G., and Tribe, D. E. Importance of Frequency of Feeding to Sheep. Brit. J. Nutrition, 6: 89. 1952.

(3) Knox, K. L., and Ward, G. M. Rumen Concentrations of Volatile Fatty Acids as Affected by Feeding Frequency. J. Dairy Sci., 44: 1550. 1961.

(4) Leffel, E. C., and Komarek, R. J. Effects of Frequency of Feeding upon Rumen Volatile Fatty Acids and Weight Gains in Lambs. Proc. Am. Soc. Animal Production, No. Atlantic Sec., 21: 1. 1960.

(5) McLaren, G. A., Anderson, G. C., Martin, W. G., and Cooper, W. K. Fixation of Ammonia Nitrogen by Rumen Mucosa. J. Animal Sci., 20: 942. 1961.

(6) McLaren, G. A., Anderson, G. C., Welch, J. A., Campbell, C. D., and Smith, G. S. Diethylstilbestrol and Length of Preliminary Period in the Utilization of Crude Biuret and Urea by Lambs. I. Digestion and Nitrogen Retention. J. Animal Sci., 18: 1319. 1959.

(7) McLaren, G. A., Anderson, G. C., Welch, J. A., Campbell, C. D., and Smith, G. S. Diethylstilbestrol and Length of Preliminary Period in the Utilization of Crude Biuret and Urea by Lambs. II. Various Aspects of Nitrogen Metabolism. J. Animal Sci., 19: 44. 1960.

(8) Mochrie, R. R., Thomas, W. E., and Lucas, H. L. Influence of Feeding Equalized Intakes on Animal Response. J. Animal Sci., 15: 1256. 1956.

(9) Mohrman, R. K., Neumann, A. L., Mitchell, G. E., and Albert, W. W. Effect of Hand-Feeding, Self-Feeding, and Frequent-Interval Feeding on Performance of Beef Cattle. University of Illinois Cattle Feeders Day, p. 15, September, 1959.

(10) Moir, R. J., and Somers, M. Ruminal Flora Studies. VIII. The Influence of Rate and Method of Feeding a Ration upon Its Digestibility, upon Ruminal Function, and upon the Ruminal Population. Australian J. Agr. Research, 8: 253. 1957.

(11) Putnam, P. A., Gutierrez, J., and Davis, R. E. Effects of Frequency of Feeding upon Rumen Volatile Acids, Protozoal Population, and Weight Gains in Angus Heifer Calves. J. Dairy Sci., 44: 1364. 1961.

(12) Rakes, A. H., Hardison, W. A., Albert, J., Moore, W. E. C., and Graf, G. C. Response of Growing Dairy Heifers to Frequency of Feeding. J. Dairy Sci., 40: 1621. 1957.

(13) Rakes, A. H., Lister, E. E., and Reid, J. T. Some Effects of Feeding Frequency on the Utilization of Isocaloric Diets by Young and Adult Sheep. J. Nutrition, 75: 86. 1961.

(14) Snedecor, G. W. Statistical Methods. 5th ed. The Iowa State College Press, Ames, Iowa. 1956.

(15) Welch, J. A., Anderson, G. C., McLaren, G. A., Campbell, C. D., and Smith, G. S. Time, Diethylstilbestrol and Vitamin B_{12} in the Adaptation of Lambs to NPN Utilization. J. Animal Sci., 16: 1034. 1957.

EFFECT OF LEVEL OF INTAKE AND PHYSICAL FORM OF THE DIET ON PLASMA GLUCOSE CONCENTRATION AND VOLATILE FATTY ACID ABSORPTION IN RUMINANTS [1]

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SUMMARY

An attempt was made to investigate some of the factors affecting plasma glucose concentration in sheep. A randomized complete block design was used to study the effects on plasma glucose concentrations of three feeds each fed at three levels of intake to sheep either 15 or 27 months old. The three rations were chopped hay, pelleted finely ground hay, and the pelleted mixture of 45% corn meal and 55% finely ground hay. At the medium and high levels of intake the plasma glucose concentrations of the animals receiving hay pellets or grain-hay pellets were higher ($P<0.01$) than those of sheep fed chopped hay. The lambs (15 months old) on the medium or high level of intake had a significantly higher ($P<0.01$) plasma glucose value than the low-level-of-intake lambs. The plasma glucose concentrations of the 27-month-old sheep did not respond to increased levels of intake.

To try to explain some of the differences in plasma glucose concentrations reported here, glucose and volatile fatty acid (VFA) absorption studies were conducted on six sheep fed either chopped or pelleted, ground hay. The method to measure the absorption of a given metabolite involves the serial sampling of blood from the portal vein and the carotid artery and the measurement of the portal blood flow rate during the same experimental period by a thermodilution method. There was essentially no glucose absorption from the gastrointestinal tract. In the rumen ingesta, there was a trend for the ratio of acetic to propionic acid to be lower when the test rations were fed in the pelleted, ground form. The relative proportions of VFA in the portal blood were markedly different from those in the rumen ingesta. Although the data are not sufficient to make definite conclusions concerning the effect of physical form of hay on the amounts of fatty acids absorbed, they demonstrated that appreciable amounts of formic acid, 47 to 13.4 g per day, were absorbed. The total quantities of VFA absorbed per day ranged from 73 to 89 g per day.

Ruminants present in their carbohydrate metabolism certain peculiarities which distinguish them from simple-stomached animals. Mature ruminants absorb practically no glucose from their digestive tract [Schambye (25-27), Annison (2), Phillipson (20), and Elsden (9)] and depend almost entirely on gluconeogenesis for their glucose supply. Although it has been shown that propionic acid is a precursor of blood glucose in ruminants [Johnson (15), Clark and Malan (7)], to our knowledge it has not been proved that propionic acid is the main precursor.

Fermentable carbohydrates of the diet are broken down to short-chain fatty acids in the rumen. The importance of these metabolites in the energy economy of the animal has been recognized (2, 6, 10). Less attention has been given to the quantitative inter-relationships between the fatty acids absorbed and glucose metabolism. An understanding of these relationships in the normal animal might provide leads to the causes of certain baffling metabolic disorders such as ketosis. In these studies it is of prime importance to consider a range of nutritional levels with extreme conditions without

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[2] A part of the data reported here was recorded in the Ph.D. thesis presented by A. Bensadoun to the Graduate School, Cornell University, 1960.

which the values of certain parameters in marginal metabolic situations would be missed.

It is generally believed that plasma glucose concentrations are not subject to variations caused by feed consumption, level of intake, and kinds of feed (21). However, recently Rook et al. (23) showed in one of their experiments with dairy cows that changes in plasma glucose concentrations occurred with varying levels of intake.

This report is concerned with the changes in plasma blood glucose concentration due to changes in the plane of nutrition, physical form of the diet, and the concomitant changes in the quantities of volatile fatty acids (VFA) absorbed from the gastro-intestinal tract of sheep.

EXPERIMENTAL PROCEDURE

Experiment 1. Plasma glucose concentrations were determined in the jugular blood of 27 sheep and 18 lambs used in a feeding experiment. The sheep were 1 yr older than the lambs. A randomized complete block design was used to study the effect of physical form of the diet and level of intake on the energy utilization of a mixed timothy-alfalfa hay. The three rations were chopped hay, pelleted finely ground hay, and a pelleted mixture of 45% corn meal and 55% finely ground hay. Each of these was fed at three levels of intake designated as low, medium, and high. Three sheep and two lambs were assigned at random to each of the treatment groups. The low level of intake animals received enough feed to show positive energy balance. The animals on the high level of intake were fed ad libitum. The medium level of intake was adjusted every week in order that it should be intermediate between the low and the high levels of intake. The average feed intakes over the 196-day feeding period are given in Table 1.

TABLE 1

Average feed intakes

Level		Feed intakes		
		Chopped	Pelleted	Corn-hay pellets
		——(*g/kg of B.W.*$^{.75}$)——		
Low	Sheep	46.9	46.5	41.1
	Lamb	49.9	47.5	39.4
Medium	Sheep	56.2	55.3	52.0
	Lamb	59.3	58.4	56.0
High	Sheep	70.2	82.4	69.1
	Lamb	70.6	97.3	90.2

After the animals had been fed experimental diets for at least 150 days, blood samples were taken for glucose analysis. The samples were taken at 2 to 3 PM. Data were examined by an analysis of variance. The Duncan's new multiple-range test was used to compare the among-treatment means.

Experiment 2. In an experiment complementary to the preceding one, the amounts of glucose and VFA's absorbed by conscious animals were determined. Six sheep equipped with an exteriorized carotid loop and a rubber rumen cannula were fed either an early-cut timothy hay or a mixed alfalfa-timothy hay in either chopped or pelleted, finely ground form. Sheep 1, 2, and 3 received the timothy hay and Sheep 4, 5, and 6 were fed the mixed alfalfa-timothy hay. Hay in the following amounts and form were fed in one meal per day: Sheep 1, 1,000 g of pellets; Sheep 2 and 3, 1,000 g of chopped hay; Sheep 4, 1,300 g of pellets; Sheep 5, 1,000 g of pellets; and Sheep 6, 500 g of chopped hay.

The method (4) used to study absorption involves the serial sampling of blood from the portal vein and carotid artery for glucose and VFA analyses and the measurement of the portal blood flow rate during the same experimental period. The amount of a metabolite absorbed during a given interval of time is then obtained as follows:

Amount of metabolite absorbed in interval $t = (M_p - M_c)\ F_t$; where,

M_p = average concentration of metabolites in the portal vein and

M_c = average concentration of metabolites in the carotid artery during the interval of time, t; and

F_t = Flow of blood in the portal vein during the interval of time, t.

In these studies the amounts of metabolites absorbed during 2-hr intervals were calculated for periods of 8 to 15 hr after the feeding of the test diets. To estimate the fatty acid absorption for the remaining 9 to 16 hr average absorption rates were calculated using the blood concentrations and the portal blood flow rates during the last hour of observation and the hour before the beginning of a run. Arterial and portal blood samples for metabolite analyses were withdrawn every 2 hr during the sampling period. One to 12 blood-flow rate measurements were carried out during each 2-hr period.

The portal blood samples were drawn through a polyethylene catheter anchored in the wall of the portal vein (4). The method of determining

TABLE 2

Effect of physical form of diet and level of intake on plasma glucose concentrations [a] in the jugular blood of sheep and lambs

Level of intake	Animal	Type of feed: Chopped hay	Hay pellets	Corn-hay pellets	Average
		(mg per 100 ml of plasma)			
Low	Sheep [b]	62.9	63.2	65.5	63.8
	Lamb [c]	63.0	60.1	61.9	61.7
Medium	Sheep [b]	59.1	68.7	67.3	65.0
	Lamb [c]	62.6	75.1	73.9	70.5
High	Sheep [b]	57.3	65.0	71.1	64.5
	Lamb [c]	65.8	76.1	78.7	73.5
Average		61.8	68.0	69.7	

[a] The plasma glucose concentration for each sheep was determined by taking two blood samples on successive days, analyzing them in duplicates, and averaging the four values.
[b] Each plasma glucose value represents the average of the plasma glucose levels of three sheep.
[c] Each plasma glucose value represents the average of the plasma glucose levels of two lambs.

portal blood flow was the Fegler (11) thermodilution technique. A known amount of cold saline was injected in a branch of the mesenteric vein and the corresponding changes in temperature were recorded downstream in the portal vein. At least a week before the data were collected the surgery necessary for blood-flow measurements and blood sampling was performed. Details concerning the surgery and portal blood-flow measurements have already been published (4).

Total VFA concentrations were determined by steam-distillation. Acetic, propionic, butyric, and valeric acids were separated by gas-chromatography (3). Formic acid was analyzed by colorimetry (12). Plasma glucose concentrations were determined by the Somogyi iodometric titration method (28).

RESULTS AND DISCUSSION

Experiment 1. Effect of pelleting on plasma glucose concentrations. The mean glucose concentrations for each experimental group are given in Table 2 and the results of analyses of variance are shown in Tables 3, 4, and 5. At the medium and high levels of intake the plasma glucose concentrations of the animals receiving hay pellets were higher ($P < 0.01$) than those of sheep fed chopped hay. The plasma glucose concentrations of the animals fed hay pellets were not significantly different from those fed corn-hay pellets at any level of intake. At the low level of intake the plasma glucose level was the same for all treatments.

No changes in blood glucose concentration due to nature of diet were reported by Reid (21) in his comprehensive study of carbohydrate metabolism in sheep. Reynolds et al. (22) obtained small but significant differences in the plasma glucose concentrations of calves (16 wk of age) fed pelleted hay versus calf starter. In a different trial the same authors found differences between hay pellet-fed calves and calves on a whole milk diet.

TABLE 3

Analysis of variance in levels of plasma glucose of sheep

Source of variation	d.f.	Mean square	F
Level of intake	2	157.9	8.4**
Kind of feed	2	254.0	13.4**
Age	1	183.3	9.7**
Level × kind	4	75.4	4.0*
Level × age	2	119.1	6.3**
Kind × age	2	1.5	.1
Level × kind × age	4	5.5	.3
Residual	27	18.9	

* $P < .05$.
** $P < .01$.

TABLE 4

Interaction of level of intake × kind of feed

Level	Kind of feed		
	Long hay	Hay pellets	Corn-hay pellets
	(mg per 100 ml of plasma)		
Low	62.9	61.6	63.7
Medium	60.8	71.9	70.6
High	61.5	70.5	74.9

Any two means not underscored by the same line are significantly ($P < .01$) different.

Effect of level of intake and age. The lambs which were about 15 months old at the time of blood sampling responded markedly to changes in intake level (Figure 1). The lambs on the medium or high level of intake had a significantly higher ($P < .01$) plasma glucose (Table 5) value than the low-level-of-intake lambs. The sheep which were 27 months old at the time of sampling did not respond to increased levels of intake and at the high level of intake had significantly ($P < .01$) lower plasma glucose levels than the corresponding younger lambs on the same level of intake. Sampson and Boley (24) also reported an increase in blood glucose with increased levels of intake in nonpregnant, 18-month-old ewes. These results are at variance with Reid's (21) findings. Reid (21) did not give the specific ages of his animals in his report. A difference in age might explain the apparent discrepancy of the results.

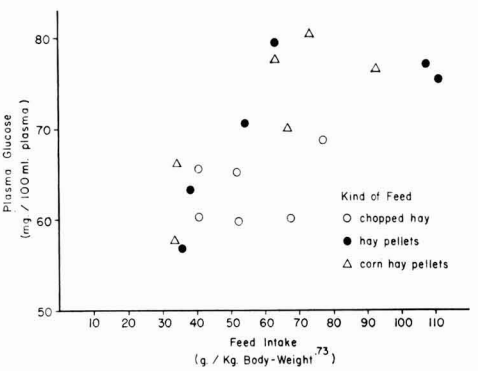

FIG. 1. Plasma glucose concentrations of 15-months-old lambs receiving chopped hay, hay pellets, or corn-hay pellets at various levels of intake.

Experiment 2. Absorption studies. The results of absorption studies (Tables 6 and 7) are reported here in an attempt to explain some of the differences in peripheral plasma glucose concentrations reported above. Blood glucose concentrations in the portal vein were not significantly different from the corresponding arterial samples in all cases except for Sheep 6. Sheep 6 showed significantly ($P < .01$) higher arterial concentrations. These results as a whole confirm the findings of Schambye (25-27) and Annison et al. (1). On most diets no appreciable amounts of glucose are absorbed from the gastro-intestinal tract of adult ruminants. The higher plasma glucose in the pelleted hay-fed animals, therefore, cannot be explained on the basis of increased glucose absorption per se.

The concentrations of VFA in rumen ingesta showed a trend for the acetic to propionic acid ratio to be lower when the test rations were fed in the pelleted, ground form (Table 6). This confirms the results of previous experiments in which the effects of pelleting on rumen VFA were studied (14). The portal blood analyses revealed marked changes in the relative proportions of the volatile fatty acids absorbed compared to the distribution in the rumen. These changes are even more accentuated when formic acid is included in the group of fatty acids considered. Formic acid was found in very small proportions in the rumen liquor (less than 1% of the total VFA in all cases) and was not included in Table 6. In the portal blood, however, its proportion was found to be as high as 35% of total VFA. Rumen VFA concentrations can be translated in terms of VFA production and absorption only if the mechanisms of absorption are known. The

TABLE 5

Interaction on plasma glucose concentration of level of intake and age of sheep

Animals	Age	Animals per group	Level of intake		
			Low	Medium	High
	(months)		*(mg per 100 ml of plasma)*		
Sheep	27	9	63.8	65.0	64.5
Lambs	15	6	61.7	70.5	73.5

Any two means not underscored by the same line are significantly ($P < .01$) different.

TABLE 6

Average concentrations and molecular percentages of various constituents in blood and rumen samples taken over 8-hr periods after feeding of chopped or pelleted hay

Sheep no.	Molecular percentages of VFA										Portal formic acid	Plasma glucose	
	Rumen ingesta					Portal blood							
	A	P	B	V	A/P	A	P	B	V	A/P		Portal	Arterial
Feed = Timothy hay											*(m-moles/liter)*	*—(mg/100 ml)—*	
1 (1,000 g HP [a])	72.0	18.6	9.4		3.9	86.0	14.1			6.1	.15	69.4	.....
2 (1,000 g CH [b])	80.1	14.5	5.4		5.5	89.1	10.9			8.2	.35	43.6	.....
3 (1,000 g CH [b])	73.3	16.9	7.9		4.5	87.1	12.0	0.9		7.3	.39	54.1	53.9
Feed = Mixed Alfalfa – Timothy hay													
4 (1,300 g HP [a])	53.0	24.5	16.5	6.0	2.2	86.3	11.4	1.3	1.0	7.6	.23	78.7	77.5
5 (1,000 g HP [a])	52.5	23.7	19.0	4.7	2.2	73.8	11.3	5.6	9.2	6.5	.20	88.3	89.2
6 (500 g CH [b])	69.2	21.0	7.7	2.1	3.3	81.0	9.6	3.4	6.0	8.4	.10	74.4	75.6

[a] Hay pellets.
[b] Chopped hay.

TABLE 7

Response of a flame ionization detector to various fatty acids

Formic	Acetic	Propionic	Butyric	Iso-valeric	Valeric
		(Response/mole relative to acetic acid)			
.006	1	2.03	2.63	2.42	2.96

results of studies of relative VFA absorption rates are conflicting (13, 16, 18) and the discrepancies between conclusions reflect differences in the physiological conditions under which the observations were made. Until these mechanisms of absorption are established in the conscious, fed animal, it will be difficult to give quantitative meaning to VFA concentrations in the rumen.

Annison et al. (2) have established in very critical analyses the existence of formic acid in the blood of ruminants. Its presence in rabbit blood (8) had been established as early as 1932. Because of its labile properties, formic acid can be lost very easily in the course of an analysis. When a mixture of VFA in aqueous solution is separated in a gas chromatographic analysis employing thermo-conductivity detection, the water peak obscures the formic acid peak. If a flame ionization detector is used, it is difficult to estimate formic acid because of the poor sensitivity of this detector for formic acid (Table 7). This explains the authors' choice of analyzing formic acid by a colorimetric procedure.

The results of rumen perfusion experiments led McCarthy et al. (19) and Brown et al. (5) to conclude that the proportions of VFA were nearly the same in the rumen and the blood leaving the rumen. The discrepancy between the results of that study and those reported here reflects essentially a difference in the technique employed. The isolated rumen seems to have an altered physiological behavior.

Estimates were made of the total amounts of the individual VFA's absorbed by sheep fed chopped or pelleted, ground hay. The method of measuring VFA absorption is based upon portal-arterio differences and the measurement of the portal blood-flow rate. In Figure 2 are given, for Sheep 2, the arterial and portal blood VFA concentrations, the portal blood flow rates, and the amounts of VFA absorbed during each 2-hr interval after feeding. Chiefly because of the formation of clots at the tips of the injection and sampling catheters, the application of the method in conscious, healed specimens is difficult and tedious. In the present experiments, the patterns of portal blood flow in three sheep (no. 2, 4, and 6) were obtained during sufficiently long periods of time to allow the amounts of the VFA's absorbed to be computed. These are summarized in Table 8. Although the data are not sufficient to make definite conclusions concerning the effect of the physical form of hay on the amounts of fatty acids absorbed, they demonstrate that appreciable quantities of formic acid are absorbed. The fatty acid mixture absorbed by Sheep 4, receiving pelleted, ground hay, contained a higher proportion of propionic acid and a lower proportion of formic acid, than the mixture absorbed by the sheep ingesting chopped hay. Relative to the amounts of butyric acid in ruminal ingesta, those absorbed were quite small.

It would seem improbable that the higher levels of plasma glucose observed in animals fed high levels of pelleted feeds than in those fed chopped hay can be explained entirely by the absorption of a greater amount of propionic acid. The relative proportions of glu-

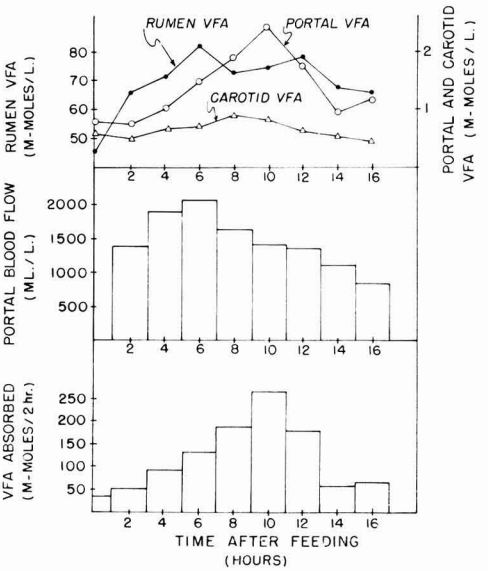

FIG. 2. Concentrations of VFA in rumen ingesta, carotid blood, and portal blood, portal blood flow rates, and total VFA absorbed at various intervals after the ingestion of 1,000 g of chopped hay (Sheep 2).

TABLE 8

Total volatile fatty acids absorbed per day on various diets

Sheep no.		Amounts of VFA absorbed				
		F	A	P	B	V
		(g)				
2	Timothy hay (1,000 g CH)	13.4	45.0	14.7		
4	Mixed timothy-alfalfa (1,300 g HP)	4.7	58.1	21.6	2.7	1.6
6	Mixed timothy-alfalfa (500 g CH)	7.1	57.4	9.1	4.2	4.6

HP = Hay pellets.
CH = Chopped hay.

cogenic amino acids absorbed might also contribute to the higher levels of plasma glucose. On the other hand, the concentration of glucose in plasma does not necessarily reflect the size of the glucose pool (17). Further study is needed to determine whether the increased plasma concentration of glucose associated with the ingestion of high levels of pelleted feeds reflects a shrinking of the glucose space and/or a reduced rate of glucose utilization, rather than an increased production of glucose within the body.

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REFERENCES

(1) ANNISON, E. F. Studies on the Volatile Fatty Acids of Sheep Blood with Special Reference to Formic Acid. Biochem. J., 58: 675. 1954.

(2) ANNISON, E. F., HILL, K. J., AND LEWIS, D. Studies on the Portal Blood of Sheep. 2. Absorption of Volatile Fatty Acids from the Rumen of the Sheep. Biochem. J., 66: 592. 1957.

(3) BENSADOUN, A. Direct Determination of the Absorption of Volatile Fatty Acids from the Gastrointestinal Tract of Ruminants. Part I. Short Chain Volatile Fatty Acid Analysis in Biological Materials. Ph.D. thesis, Cornell University, Ithaca, New York. 1960.

(4) BENSADOUN, A., AND REID, J. T. Estimation of Portal Blood Flow in Ruminants: Effect of Feeding, Fasting, and Anaesthesia. J. Dairy Sci., 45: 540. 1962.

(5) BROWN, R. E., DAVIS, C. L., STAUBUS, J. R., AND NELSON, W. O. Production and Absorption of Volatile Fatty Acids in the Perfused Rumen. J. Dairy Sci., 43: 1788. 1960.

(6) CARROLL, E. J., AND HUNGATE, R. E. The Magnitude of the Microbial Fermentation in the Bovine Rumen. Appl. Microbiol., 2: 205. 1954.

(7) CLARK, R., AND MALAN, J. R. Alterations in the Blood Sugar and Ketone Levels Caused by Dosing Acetate, Propionate and Butyrate into the Rumen of the Sheep. Onderstepoort J. Vet. Research, 27: 101. 1956.

(8) DROLLER, H. Zum Nachweis geringer Mengen von Ameisensaure in Blut und Geweben, insbesondere in der Haut. Z. physiol. Chem., 211: 57. 1932.

(9) ELSDEN, S. R. The Fermentation of Carbohydrates in the Rumen of the Sheep. J. Exptl. Biol., 22: 51. 1945.

(10) EMERY, R. S., SMITH, C. K., AND HUFFMAN, C. F. The Amounts of Short Chain Acids Formed During Rumen Fermentation. J. Animal Sci., 15: 854. 1956.

(11) FEGLER, G. The Reliability of the Thermodilution Method for Determination of the Cardiac Output and Blood Flow in the Central Veins. Quart. J. Exptl. Physiol., 42: 254. 1957.

(12) GRANT, W. M. Colorimetric Microdetermination of Formic Acid Based on Reduction to Formaldehyde. Anal. Chem., 20: 267. 1948.

(13) GRAY, F. V. The Absorption of Volatile Fatty Acids from the Rumen. J. Exptl. Biol., 25: 135. 1948.

(14) HEMKEN, R. W., AND KING, R. L. The Effect of Pelleted Hay and Heated Corn on Milk Composition, Rumen Volatile Fatty Acids, and Milk Production. Proc. Maryland Nutr. Conf., p. 45. 1962.

(15) JOHNSON, R. B. The Effects of Administration of Acetic, Propionic and n-Butyric Acids upon the Blood Glucose and the Ketone Body Levels of Goats. Cornell Vet., 45: 273. 1955.

(16) KIDDLE, P., MARSHALL, R. A., AND PHILLIPSON, A. T. A Comparison of the Mixtures of Acetic, Propionic and Butyric Acids in the Rumen and in the Blood Leaving the Rumen. J. Physiol., 113: 207. 1951.

(17) KRONFELD, D. S., TOMBROPOULOS, E. G., AND KLEIBER, M. Glucose Biokinetics in Normal and Ketotic Cows. J. Appl. Physiol., 14: 1026. 1959.

(18) MASSON, M. J., AND PHILLIPSON, A. T. The

Absorption of Acetate, Propionate and Butyrate from the Rumen of Sheep. J. Physiol., 113: 189. 1951.

(19) McCarthy, R. D., Shaw, J. C., McCarthy, J. L., Lakshmanan, S., and Holter, J. B. Production and Absorption of Organic Acids by the Perfused Goat Rumen. Proc. Soc. Exptl. Biol. Med., 99: 556. 1958.

(20) Phillipson, A. T., and McAnally, R. A. Studies on the Fate of Carbohydrates in the Rumen of Sheep. J. Exptl. Biol., 19: 199. 1942.

(21) Reid, R. L. Studies on the Carbohydrate Metabolism of Sheep. I. Range of Blood-Sugar Values Under Several Conditions. Australian J. Agr. Research, 1: 182. 1950.

(22) Reynolds, P. J., Warner, R. G., and Loosli, J. K. Unpublished data, Cornell University. 1962.

(23) Rook, J. A. F., and Line, C. The Effect of the Plane of Energy Nutrition of the Cow on the Secretion in Milk of the Constituents of the Solids-not-fat Fraction and on the Concentrations of Certain Blood-Plasma Constituents. Brit. J. Nutrition, 15: 109. 1961.

(24) Sampson, J., and Boley, L. E. Studies on the Total Ketone Bodies, Sugar and Calcium of the Blood of Non-pregnant, Non-lactating Ewes. J. Am. Vet. Med. Assoc., 96: 480. 1940.

(25) Schambye, P. Volatile Acids and Glucose in Portal Blood of Sheep. I. Nord. Vet. Med., 3: 555. 1951.

(26) Schambye, P. Volatile Acids and Glucose in Portal Blood of Sheep. 2. Sheep Fed Hay and Hay Plus Crushed Oats. Nord. Vet. Med., 3: 748. 1951.

(27) Schambye, P. Volatile Acids and Glucose in Portal Blood of Sheep. 3. The Influence of Orally Administered Glucose. Nord. Vet. Med., 3: 1003. 1951.

(28) Somogyi, M. Determination of Blood Sugar. J. Biol. Chem., 160: 69. 1945.

RELATION OF RATION COMPOSITION TO RUMEN DEVELOPMENT IN EARLY-WEANED DAIRY CALVES WITH OBSERVATIONS ON RUMINAL PARAKERATOSIS [1]

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SUMMARY

A calf starter containing VFA salts was compared with a control starter in terms of growth, health, ruminating activity, and rumen development of dairy calves. There was no significant difference ($P > 0.05$) between the respective starters regarding weight gain and rumen development of calves to 39 days of age. The amount of feed consumed was closely associated with rumen development as indicated by statistically significant regressions ($P < 0.025$) of various criteria of rumen development (weight of total rumen, musele and mucosa, and per cent mucosa) on feed consumption to different periods of the experiment.

All of the calves ruminated at 11 days of age; however, there was no significant difference ($P > 0.05$) between groups of calves in the amount of time spent ruminating at either 11 or 25 days of age.

Five out of eight calves receiving the starter containing the VFA salts had some degree of ruminal parakeratosis as compared to one out of eight calves receiving the control starter. The cause of this condition was not determined.

During recent years it has been shown that young dairy calves may be raised successfully using an early weaning system (3, 13, 20). However, the success of such a system of raising calves depends on early rumen development, since it is necessary for the calves to subsist entirely on dry feed at an early age.

The rumen of a new born calf is essentially nonfunctional, having a smooth, stratified, squamous epithelium with no prominent papillae, and the microbial population has not been established (1, 16). Therefore, considerable development with respect to capacity, mucosal and muscular tissue, and microbial population is necessary before dry feeds of plant origin can be utilized effectively. It has been established that the stimulus for development of the rumen tissue does not come entirely from the physical nature of the feed, but is due partly to the volatile fatty acids (VFA) which result from rumen fermentation (5, 7, 14).

The objective of the work reported herein was to determine the effect of adding salts of propionic and butyric acids to a calf starter on rumen development and growth of early-weaned dairy calves. In addition, the relation of the composition of the starter ration to the onset of rumination in young dairy calves was investigated.

EXPERIMENTAL PROCEDURE

Twenty-four Holstein bull calves were grouped into three blocks of eight calves, each on the basis of season of birth. Each calf received colostrum for three days and was started on experiment the fourth day after birth. Whole milk was fed at the daily rate of 10% of initial body weight during the first 14 days on experiment and then 8% of initial weight for an additional ten days. Chlortetracycline [3] was fed twice daily in the milk and 25 mg per pound was included in the starter ration. The calves were housed in individual tie stalls with expanded metal screen floors, so that no bedding was required.

The calves within each block were randomly assigned to either an experimental or control ration. The experimental ration consisted of a basal ration [4] plus a mixture of fatty acid

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[3] Chlortetracycline supplied as Aurofac-D in the milk and as Aurofac-10 in the starter ration.

[4] Ingredients in basal ration expressed as per cent of final mixture: cubed corn, 20; crimped oats, 20; wheat bran, 6; corn distillers solubles, 5; dried molasses, 4; soybean meal, 12; dehydrated alfalfa crumbles, 20; dicalcium phosphate, 1; trace-mineral salt, 1; antibiotic-vitamin premix, 1.

TABLE 1

Composition of mixture of volatile fatty acid salts

Fatty acid salt	Molecular weight	Per cent of mixture	Per cent of final ration
Sodium propionate	96	10.5	1.05
Potassium propionate · H_2O	130	14.2	1.42
Calcium propionate · H_2O	204	22.3	2.23
Sodium butyrate	110	12.0	1.20
Potassium butyrate · H_2O	144	15.7	1.57
Calcium butyrate · H_2O	232	25.3	2.53

salts, whereas the control ration was made up of the basal ration with glucose added as a readily available source of energy. The mixture of fatty acid salts (Table 1) and the glucose each made up 10% of the respective ration in which it was included. On a molar basis equal amounts of propionate and butyrate were used and the amounts of calcium, potassium, and sodium salts were calculated to give an equal amount of each in the mixture. The respective rations were fed free-choice throughout the experiment and constituted the sole diet after the calves were weaned abruptly at 28 days of age. An effort was made to get each calf to consume dry feed at an early age.

Observations were made on the ruminating activity of 16 calves during the experiment. The amount of time spent ruminating, eating, and drinking was determined during two separate 24-hr periods, when the calves were 11 and 25 days of age, respectively.

Upon sacrifice of each calf at 39 days of age, the digestive tract was removed for study. Each compartment of the stomach was separated from its omenta and stripped of its larger blood vessels, lymph nodes, and fat. The different stomach compartments and intestines were weighed before and after the removal of ingesta to determine the weight of ingesta contained therein. A 100-cm^2 section was taken from the ventral wall of the cranial dorsal sac of the rumen and the muscular and mucosal layers were separated. Each layer was weighed and then dried at 90 C for 48 hr to obtain the dry weight. The remainder of the rumen was also separated into mucosal and muscular layers and the respective weights added to the fresh tissue weights of the 100-cm^2 section to calculate the percentage of mucosa for the entire rumen.

During the early part of the experiment an abnormal ruminal wall condition, similar to that described by Jensen et al. (10), was observed in some calves. Since the condition appeared to occur more frequently in the group of calves fed the ration containing VFA salts, its incidence and severity were recorded for the last 16 calves on the experiment. Observations were made on the gross appearance of the entire rumen, and photographs were made of a section of the ventral wall of the cranial dorsal sac using a Graflex-type camera. Histological sections were also prepared from the same area to determine any differences in the ruminal mucosa from calves of the two groups.

Two additional calves were assigned to each group to obtain data on the pH and proportion of VFA in the rumen fluid. Rumen samples were taken at 18, 25, 32, and 39 days of age by means of a stomach tube. The analyses for rumen VFA were made using the method described by Keeney (11).

RESULTS AND DISCUSSION

The average weight gain and feed consumption of the calves receiving the control ration surpassed that of those fed the ration containing the fatty acid salts (Table 2); however,

TABLE 2

Average feed consumption and weight gains of calves to 39 days of age

Ration	Feed consumed		Weight gain
	Milk	Calf starter	
	(lb)		
Control	195	35.2	27.2
VFA salts	195	24.8	21.4

there was sufficient variation among calves within each group that the differences between groups were not statistically significant ($P > 0.05$). The addition of the VFA salts was not expected to have any direct effect on the growth of the calves as the two rations were calculated to have approximately the same energy content; and, since the calves receiving the VFA ration neither started consuming dry feed at an earlier age nor consumed more of it, no advantage in growth rate was obtained. Similarly, Martin et al. (12) found no significant difference in live-weight gains of calves fed a ration containing fatty acid salts vs. those fed a purified diet.

With respect to the weight of different stomach compartments, only the average combined weight of the omasum and abomasum of the VFA group surpassed that of the control group (Table 3). The differences between groups were not statistically significant ($P >$

TABLE 3

Average fresh tissue weights of different stomach compartments and percentage of ruminal mucosa of calves sacrificed at 39 days of age

Ration	Omasum-abomasum	Reticulum	Total rumen			Cranial-dorsal sac of rumen [a]	
			Muscle	Mucosa		Fresh tissue	Dry
	(g)				(%)	(% mucosa)	
Control	382	140	269	338	55.3	60.9	67.7
VFA salts	409	133	239	279	52.7	56.8	65.3

[a] Measurements based on a 100-cm^2 section taken from the cranial-dorsal sac.

0.05), whether the values were expressed in terms of total organ weight or in relation to ingesta-free body weight. More extensive papillary development was evident in the control than in the VFA group; however, the difference in average percentage of ruminal mucosa in favor of the control group was not statistically significant ($P > 0.05$). Thus, addition of VFA salts to the ration did not increase development of the rumen as determined by measurements at 39 days of age.

A partial explanation for the above results may be found in a comparison of the relative amounts of VFA available from the two rations. If one assumes that fermentation of 10 lb of feed in the rumen results in the production of 34 equivalents of volatile acids, as indicated by Carroll and Hungate (2), an average of 120 equivalents of VFA would have been produced by the calves fed the control diet in the present study. Analyses of rumen fluid from calves fed this ration revealed that propionic and butyric acids represented approximately one-half of the total VFA; thus, it was estimated that about 60 M of these acids were produced during the 7-wk period. In comparison, an average of approximately 45 M of propionic and butyric acids were available to the calves fed the VFA ration, including 7.4 M consumed directly as VFA salts. Thus, the approach of accelerating rumen development by the addition of VFA salts to the ration proved unsatisfactory, due to failure of the calves to consume sufficient quantities of this ration at an early age.

Although the total amount of VFA administered has been observed (5, 16) to be a critical factor determining the extent of papillary development in the calf, the exact level required for this development has not been determined. Tamate et al. (16) reported that typical papillary growth did not occur in calves given approximately 30 M (2.5 kg) of volatile acid, either as a mixture of acetic and propionic acids or as butyric acid, over an 8-wk period, while papillary growth was observed in calves given 61 and 63 M of butyric and propionic acid, respectively. In the present study, a moderate degree of papillary development occurred in two calves which consumed a total of only 20.8 and 25.3 lb of the control ration, respectively. On the basis indicated above, an estimated 35 and 43 M of propionic and butyric acids were available to the respective calves for stimulation of papillary growth over the 7-wk period. The presence of absorptive material in the rumen which would prolong the exposure of the ruminal wall to the VFA has been observed to be a factor influencing papillary development (5), and this factor along

TABLE 4

Average feed consumption and ruminating activity of calves at 11 and 25 days of age

Age of calves	Ration	Dry feed intake [a]	Time spent in 24 hr		
			Ruminating	Eating	Drinking
(days)		(lb)	(min)		
11	VFA salts	0.25	12.0	15.0	9.0
	Control	0.84	14.0	24.0	6.0
25	VFA salts	4.50	154.0	45.0	27.0
	Control	8.57	187.0	51.0	7.0

[a] Total pounds of dry feed consumed from 4-11 days of age and from 4-25 days of age, respectively.

with increasing levels of rumen VFA with advancing age, as observed in this study, may account for the apparent divergence of these results and those previously reported (5, 16). Obviously, more work is needed to clarify this point.

Variation in rumen development among calves within the two groups of calves in the present study was associated with differences in feed consumption during different periods of the experiment. Regressions of various criteria of rumen development (weight of total rumen, muscle, and mucosa, and per cent mucosa) on the amount of feed consumed to 18, 28, or 39 days of age were statistically significant ($P < 0.025$), except for the regression of weight of rumen muscle on feed consumed to 18 days of age.

Calves fed the control ration spent slightly more time ruminating at 11 and 25 days of age than those fed the VFA (Table 4); however, the differences between groups were not statistically significant ($P > 0.05$). All calves were observed to ruminate at 11 days of age, even though there was a considerable amount of variation among calves in the amount of time spent in this activity. Other workers (15, 16) have likewise observed that the onset of rumination in the calf occurs at a very young age.

At least part of the observed difference between groups in ruminating time at 25 days of age appeared to be related to a difference in amount of feed consumed to that age. A correlation coefficient of 0.46, which approached statistical significance (P *ca.* 0.05), was found between ruminating time and feed consumption to 25 days of age. Similarly, Swanson and Harris (15) obtained a highly significant correlation of 0.51 between rumination time and feed consumption of calves 6 to 16 wk of age.

Five out of eight calves receiving the ration containing VFA salts had some degree of ruminal parakeratosis as compared to one of eight calves receiving the control ration. The rumens of the majority of the calves in the control group had well-developed papillae with no incrustation (Figure 1). In contrast, the calves diagnosed as having ruminal parakeratosis had less well-developed papillae, which were incrusted with dark, keratinized material (Figure 2). A furfuraceous desquamation of the incrusted, keratinized material occurred very rapidly when handled. Regardless of the degree of papillary development most of the larger papillae in each rumen were arranged in folds with very short papillae located between the folds.

The histological appearance of the ruminal wall of all calves compared favorably with the description set forth by Habel and Biberstein (6). Microscopically, some degree of desquamation of the stratum corneum was observed in all calves of both groups, and was very extensive in the calves with ruminal parakeratosis. In the calves with ruminal parakeratosis the stratum corneum was thickened in those papillae in which desquamation was not complete (Figure 3). Where desquamation had occurred it was difficult to demonstrate microscopically the incrusted cap of keratinized material, because it had separated from the papilla proper either before or during the histological preparation of the tissues.

Although ruminal parakeratosis has been observed in cattle and sheep by a number of workers under various conditions (4, 8-10, 17, 19), its cause has not been determined. Vidacs and Ward (19) suggested a low acetate-to-propionate ratio in the rumen as the cause of this condition in dairy cows; however, in a later report (18) the presence of a heat-sensitive or protein-bound factor in the rumen fluid was suggested as the causative agent. Some workers (4, 8) have observed a lower rumen pH in animals fed rations which resulted in ruminal parakeratosis. However, Hinders et al. (8) found that parakeratosis was not eliminated when the pH of the rumen was raised from 6.0 to 6.65 by the addition of Na_2HPO_4.

In the present study, the pH of the rumen contents of calves receiving the ration with VFA salts was only slightly different from that of calves receiving the control ration, and there were no consistent differences between groups with respect to the proportion of VFA in the rumen fluid. Therefore, the reason for a greater incidence of ruminal parakeratosis in the calves receiving the VFA ration in this experiment was not established. An attempt is being made presently to clarify the relationship between the incidence of ruminal parakeratosis and the production and/or absorption of volatile fatty acids within the rumen.

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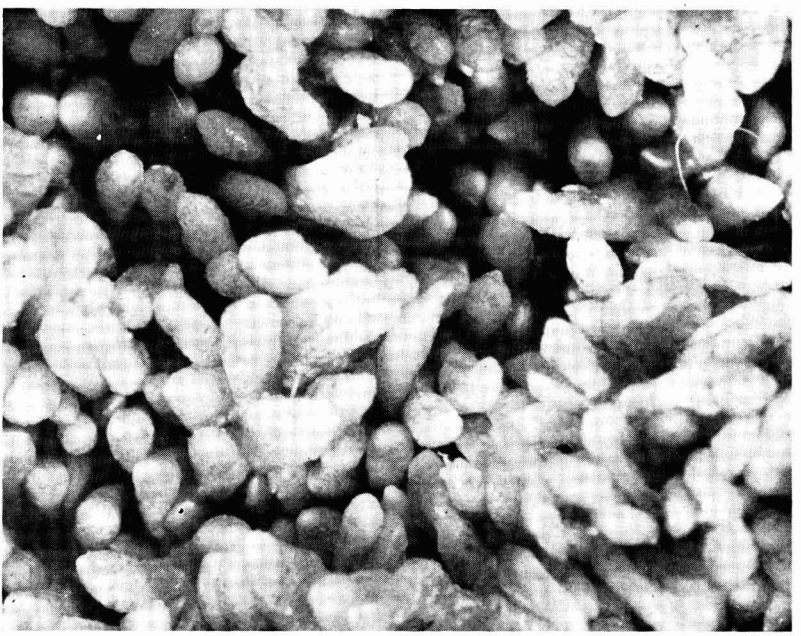

FIG. 1. Well-developed papillae with no incrustation from calf fed control starter. × 10.

FIG. 2. Papillae incrusted with dark keratinized material indicating ruminal parakeratosis. × 10.

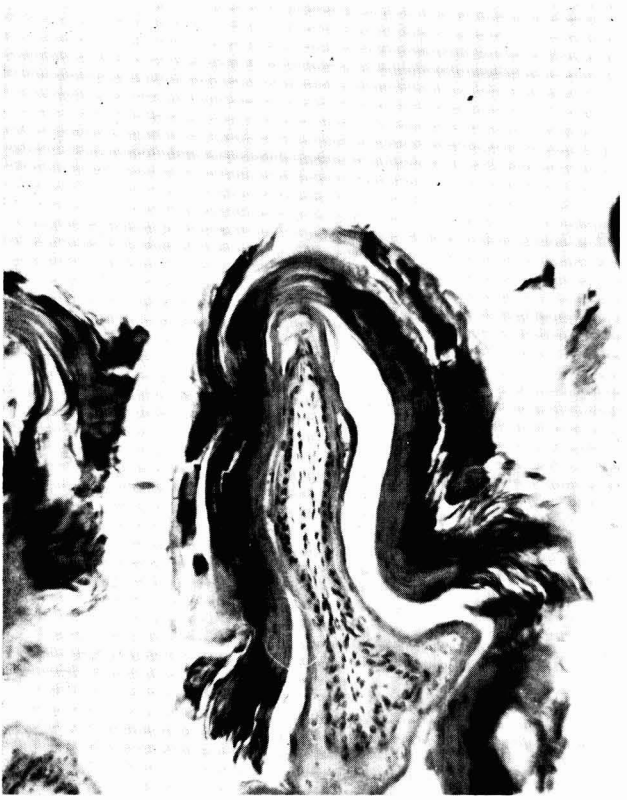

FIG. 3. A papilla with the stratum corneum thickened and a partially desquamated cap of incrusted keratinized material. × 100.

REFERENCES

(1) BRYANT, M. P., SMALL, N., BOUMA, C., AND ROBINSON, I. Studies on the Composition of the Ruminal Flora and Fauna of Young Calves. J. Dairy Sci., 41: 1747. 1958.

(2) CARROLL, E. J., AND HUNGATE, R. E. The Magnitude of the Microbial Fermentation in the Bovine Rumen. Appl. Microbiol., 2: 205. 1954.

(3) CLARK, R. D., AND WHITING, F. Further Studies on Raising Calves with Limited Amounts of Milk. Can. J. Agr. Sci., 41: 16. 1961.

(4) CULLISON, A. E. Effect of Physical Form of the Ration on Steer Performance and Certain Rumen Phenomena. J. Animal Sci., 20: 478. 1961.

(5) FLATT, W. P., WARNER, R. G., AND LOOSLI, J. K. Influence of Purified Materials on the Development of the Ruminant Stomach. J. Dairy Sci., 41: 1593. 1958.

(6) HABEL, R. E., AND BIBERSTEIN, E. L. Fundamentals of the Histology of Domestic Animals. 426 pp. Comstock Publishing Associates, Ithaca, New York. 1957.

(7) HARRISON, H. N., WARNER, R. G., SANDER, E. G., AND LOOSLI, J. K. Changes in the Tissue and Volume of the Stomachs of Calves Following the Removal of Dry Feed or Consumption of Inert Bulk. J. Dairy Sci., 43: 1301. 1960.

(8) HINDERS, R. G., VIDACS, G., AND WARD, G. M. Effects of Feeding Dehydrated Alfalfa Pellets as the Only Roughage to Dairy Cows. J. Dairy Sci., 44: 1178. 1961.

(9) HOPKINS, H. A., FONTENOT, J. P., AND MESTANZA, W. M. Effect of Grinding and Pelleting on Feedlot Performance, Digestibility and Incidence of Rumen Parakeratosis in Lambs. (Abstr.) J. Animal Sci., 19: 652. 1960.

(10) JENSEN, R., FLINT, J. C., UDALL, R. H., DEEM, A. W., AND SEGER, C. L. Parakeratosis of the Rumens of Lambs Fattened on Pelleted Feed. Am. J. Vet. Research, 19: 277. 1958.

(11) KEENEY, M. Direct Chromatographic Determination of C_1 to C_4 Fatty Acids in Rumen Fluid. Maryland Agr. Expt. Sta., Misc. Publ., 238: 23. 1955.

(12) MARTIN, W. G., RAMSEY, H. A., MATRONE, G., AND WISE, G. H. Responses of Young Calves to a Diet Containing Salts of Volatile Fatty Acids. J. Dairy Sci., 42: 1377. 1959.

(13) NOLLAR, C. H., DICKSON, I. A., AND HILL, D. L. Value of Hay and Rumen Inoculation in an Early-Weaning System for Dairy Calves. J. Dairy Sci., 45: 197. 1962.

(14) SANDER, E. G., WARNER, R. G., HARRISON, H. N., AND LOOSLI, J. K. The Stimulatory Effect of Sodium Butyrate and Sodium Propionate on the Development of Rumen Mucosa in the Young Calf. J. Dairy Sci., 42: 1600. 1959.

(15) SWANSON, E. W., AND HARRIS, J. D., JR. Development of Rumination in the Young Calf. J. Dairy Sci., 41: 1768. 1958.

(16) TAMATE, H., MCGILLIARD, A. D., JACOBSON, N. L., AND GETTY, R. Effect of Various Dietaries on the Anatomical Development of the Stomach in the Calf. J. Dairy Sci., 45: 408. 1962.

(17) THOMPSON, G. B., KINTNER, L. D., AND PFANDER, W. H. Some Effects of Ration Preparation on Alterations of the Rumen Mucous Membrane. (Abstr.) J. Animal Sci., 17: 1220. 1958.

(18) VIDACS, G., HINDERS, R. G., AND WARD, G. M. The Use of the Rat to Study Changes of Rumen Epithelium (Parakeratosis) Occurring on Dehydrated Alfalfa Pelleted Diet. J. Dairy Sci., 44: 1178. 1961.

(19) VIDACS, G., AND WARD, G. M. Parakeratosis Condition of Rumen Epithelium Produced by an All-Concentrate Ration. J. Dairy Sci., 43: 875. 1960.

(20) YANG, M. G., BUSH, L. J., AND ODELL, G. V. Enzyme Supplementation of Rations for Dairy Calves. J. Agr. Food Chem., 10: 322. 1962.

RELIABILITY OF SCORE-CARD JUDGING OF HAY[1, 2]

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SUMMARY

A total of 129 samples of alfalfa or alfalfa-brome mixtures (55 first-crop, 65 later-crop, and nine additional samples) were each scored twice by seven judges, using a score-card based on stage of growth, leafiness, and color and condition. Cutting dates were recorded for the first-crop samples. These dates provided a means, other than the judges' estimates, of scoring stage of growth on the first-crop samples.

Repeatability in the use of the score-card was studied on a within-judge basis by computing the appropriate intraclass correlation. These repeatability estimates ranged from .42 to .80. Between-judge repeatabilities were estimated by averaging simple between-judge correlations. These repeatability estimates ranged from .38 to .78. All estimates of repeatability were highly significant.

Simple correlation and multiple regression techniques were used to study the relationship of the score-card and its components to various indicators of nutritive value. Simple correlations were averaged for the seven judges over both scorings. These analyses indicated that the score-card was most effective in reflecting nutritive value when used among later crop samples low in grass content. Among these samples the average correlations between final score and crude protein, crude fiber, estimated TDN, and percentage of cellulose were .50, −.43, .43, and −.51, respectively, and were highly significant. However, among first-crop samples and among later-crop samples which were high in grass content nonsignificant correlations were obtained.

The multiple regression analyses indicated that dates of cutting for first-crop samples and leafiness scores for later-crop samples were the most useful criteria of the score-card for predicting nutritive value. However, even among the later-crop samples, where the score card appeared most effective, it accounted for only 20-30% of the variation in estimated TDN.

The need for defining and measuring hay quality has been long recognized by research workers, extension personnel, and feeders of livestock. Actual feeding and digestion trials have helped to answer many of the questions in this area, but there is still a need for a simple, yet accurate measure of hay quality.

Many simple methods have been proposed. The proximate chemical analysis, proposed by Henneberg and Stohman in 1863 (3), is still the basis of the official method of feed analyses today. Some methods of estimating digestible energy (DE) or total digestible nutrients (TDN) from various components of the proximate chemical analysis are (a) the use of Morrison's digestion coefficients (9), (b) regression equations as developed by Schneider et al. (14), and (c) the Axelsson-Reid-Swift formula (7). Work by Pigden and Bell (11), Quicke et al. (12), Hershberger et al. (7), and Baumgardt (1) has led to the development of artificial rumen techniques which can be used to estimate DE or TDN. Canadian workers (4, 6) have further used such techniques to study rate of digestion, and these workers have related this work to the Nutritive Value Index, which appraises both the digestible energy content of a hay and the rate of voluntary consumption. Reid et al. (13) have shown that simple indexes such as percentage of leaves or date of cutting can be used to estimate the digestibility of hay.

In 1957, Werner, of Wisconsin, developed a score-card for hay to be used as an educational tool in extension programs. The score-card was designed to encourage farmers (a) to cut

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hay earlier, (b) to do a better job of saving leaves, and (c) to handle and cure the hay properly, so as to prevent losses from excessive heating and mold development. It was felt that these three items should be important in determining over-all feeding value of which both palatability and digestibility are important components.

Nichols (10) evaluated this score-card in 1959 and found that it was repeatable and that it did reflect nutritive value to some extent. However, Nichols felt that (a) his samples lacked sufficient range in color and condition, that (b) the score-card was not compared with an adequate measure of hay quality, and that (c) dates of cutting were inaccurate. Therefore, the present study was undertaken to appraise the same score-card with the above-mentioned difficulties in mind.

DATA AND METHODS

Hay samples were collected in the fall of 1959 from 49 southern Wisconsin dairy farms cooperating with the University of Wisconsin and the USDA in an environmental project study. The hay samples were predominantly alfalfa and alfalfa-brome mixtures, and they included 55 first-crop, 65 later-crop, and nine additional samples. The nine additional samples were included in order to obtain greater variation in color and condition scores, and represented hay which had severely weathered or had been left in the mow from the previous year.

An attempt was made to obtain samples of the first-crop and a later-crop from each farm. To secure representative samples of each crop, five or six different probes were made with a core sampler and the contents of these probes were then placed together in a large paper bag to make a composite sample for later judging and testing. Samples from first, second, and third crops were not mixed, and within a crop, hays with obvious species or weathering differences were not mixed.

Dates of cutting for the first-crop samples had been recorded by the farmers at the time of cutting. However, under farm conditions each crop of hay is normally cut on more than one day. Therefore, it was necessary to assign to each first-crop sample an average cutting date based on the farmer's recordings. These average cutting dates ranged from May 29 to July 10.

At the time of judging, the samples were placed on tables under fluorescent lighting and labelled only as to first or later crop. The samples were scored independently by seven judges on two different occasions. The score-card used is shown in Table 1. The average score of the seven judges for both scorings was 28.07, 30.35, 26.73, and 84.76 for stage of growth, leafiness, color and condition, and final score, respectively.

In scoring stage of growth there is less range allowed for later-crop samples than for first-crop samples. This is in accordance with observations made by Reid et al. (13), that the digestibility of later-crop hays is less variable than that of first-crop hays, regardless of stage of growth. Cutting dates were also used for scoring stage of growth on first-crop samples by the use of the alternate scoring system as shown in Table 1.

Repeatability in the use of the score-card was studied both on a within-judge and a between-judge basis. Within-judge repeatabilities were estimated by computing the appropriate intraclass correlation. Between-judge repeatability values were estimated by averaging simple between-judge correlations.

To determine the effectiveness of the score-card as a method for measuring nutritive value of the hay, it was necessary to obtain the best estimate of TDN or DE from the chemical analysis available. For this determination 49 test samples [3] were used to compare TDN or DE calculated by different methods to the actual values from feeding trials. The data on these 49 samples included actual digestibility values, values of the proximate chemical analysis, and in vitro cellulose digestibility values. On a dry matter basis these samples contained an average of 15.58% crude protein and 29.12% crude fiber. The average animal digestibility values were 62.5% TDN and 2,834 cal/g of DE. These values are included to give some idea of the quality of these samples.

The methods of estimating either TDN or DE, which were compared, were (a) the use of Morrison's digestion coefficients (9), (b) the Axelsson-Reid-Swift formula (7), (c) regression equations introduced by Schneider and coworkers (14), and (d) an in vitro cellulose digestion procedure as proposed by Baumgardt (1). Data on 39 additional samples [4] were also available for a further comparison of Schneider's (14) regression equations and the Axelsson-Reid-Swift formula (7). Simple correlations were computed, to study the relationships

[3] These samples had been obtained from Pennsylvania State University, Rutgers University, and the Pasture Research Lab, State College, Pennsylvania, for in vitro digestibility studies.

[4] The necessary information on these samples was obtained through correspondence with Wright of Rutgers University.

TABLE 1

Hay score-card

I. Stage of growth when cut	Points: First crop	Points: Later crops (2nd, 3rd, _____)
1. Initial bloom or earlier	35_____	33_____
2. 1/10 bloom	33_____	32_____
3. 1/4 bloom	31_____	31_____
4. 1/2 bloom	28_____	29_____
5. 3/4 bloom	24_____	27_____
6. Full bloom	20_____	24_____
7. Late bloom to seed stage	15_____	20_____

Alternate scoring: Score full score for first-crop hay cut on June 1 or earlier, and subtract ¾ point for each day later. Minimum score, 12 points.

Score_____

II. Leafiness, % Leaves	Points
1. 50 or more	35_____
2. 45-49	34_____
3. 40-44	33_____
4. 35-39	31_____
5. 30-34	28_____
6. 25-29	25_____
7. 20-24	22_____
8. 15-19	19_____
9. Less than 15	15_____

III. Color and condition	Points
1. Bright green	30_____
2. Medium green	29_____
3. Faded green	26_____
4. Faded green, sl. musty	24_____
5. Slight brownish	23_____
6. Slight grayish, some mold	21_____
7. Medium brown	16_____
8. Pronounced mold and gray	10_____
9. Deep brown to black	6_____
10. Very moldy and white	5_____

	Score
Stage of growth	_____
Leafiness	_____
Color and condition	_____
Final score	_____

between the various estimates of TDN or DE and actual digestibility values.

The 129 samples involved in the evaluation of the score-card were analyzed by the use of the proximate chemical analysis, and percentage of cellulose was determined on these samples by a method proposed by Crampton and Maynard (5) and later modified by Matrone (8). From these analyses four indicators of nutritive value were selected to study the relationship of the score-card to nutritive value. These indicators were crude protein, crude fiber, Schneider's TDN, and percentage of cellulose. On a dry-matter basis the average crude protein content and the average crude fiber content of these 129 samples were 17.47 and 28.98%, respectively. The mean values for Schneider's TDN and percentage of cellulose were 57.17 and 33.30%, respectively. Simple correlation and multiple regression techniques were then used to study the relationship of the score-card and its components to these indicators of nutritive value.

RESULTS AND DISCUSSION

Judge repeatability. A three-way factorial design was used for a series of analyses of variance to study score-card judging. The three main sources of variation included differences between samples, between judges, and between replicates (i.e., between the two scorings). All effects were considered as random. Following this study, the sources of variation in the three-way factorial design were combined to give the following breakdown: between judges (J), between samples/within judge (S/J), and error

TABLE 2

Within-judge repeatabilities as estimated by intra-class correlations [a]

	Stage of growth	Leafiness	Color and condition	Final score
55 First-crop samples	.44	.42	.66	.51
65 Later-crop samples	.45	.60	.69	.61
120 First- and later-crop samples	.48	.54	.70	.60
129 Total samples	.52	.70	.80	.74

[a] All within-judge repeatability estimates were highly significant.

(E). The variance components of the mean squares were then calculated, and the variance component ratio, $\frac{S/J}{S/J + E}$, or the intraclass correlation, was used to express within-judge repeatability.

Estimates of within-judge repeatability are shown in Table 2. Color and condition appeared to be the most repeatable component of the score-card; whereas, stage of growth, as scored by the judges, appeared to be the least repeatable. However, it should be remembered that stage of growth is estimated by such criteria as blossoms and seed pods which are difficult to see in chopped and core samples. Repeatability estimates for leafiness scores were higher among the later-crop samples than among first-crop samples. The estimates of within-judge repeatability for leafiness, color and condition, and final score increased markedly when all 129 hay samples were considered. The higher repeatabilities in this over-all study were probably due to the greater variation provided by the nine samples included originally to provide a greater range for scoring color and condition.

Simple correlation coefficients between the scores of individual judges were averaged to obtain estimates of repeatability between judges. These repeatability estimates are shown in Table 3. These values followed much the same pattern and were of about the same magnitude as the estimates of within-judge repeatability. However, for leafiness scores the estimates of between-judge repeatability were generally higher than similar estimates of within-judge repeatability. It should be noted that within-judge repeatability estimates would be lowered by the presence of a sample-×-replicate interaction, which would indicate that certain samples had been scored consistently (over-all judges) higher or lower in the first or second scoring. On the other hand, estimates of between-judge repeatability would be decreased by the presence of a sample-×-judge interaction, which would indicate that certain judges had scored some samples consistently (over both scorings) higher or lower than had other judges. The original three-way factorial analysis of variance showed a relatively large sample-×-replicate interaction and a small sample-×-judge interaction in the scoring of leafiness, which would explain the higher between-judge repeatability estimates for leafiness scores.

All estimates of repeatability, both within- and between-judges, were highly significant. However, it should be pointed out that before the nine additional samples were included in the analysis none of the repeatability estimates exceeded .70 and many of them were considerably lower, particularly for stage of growth scores.

Comparison of four methods of estimating TDN or DE. Before studying the relationship of the score-card to nutritive value, 49 test samples were used to compare four methods of estimating TDN or DE. The data on these test samples and the four methods compared have already been described. Correlations between

TABLE 3

Between-judge repeatabilities as estimated by averaging simple interclass correlations between seven judges over two scorings [a]

	Stage of growth	Leafiness	Color and condition	Final score
55 First-crop samples	.44	.59	.65	.59
65 Later-crop samples	.39	.71	.64	.69
120 First- and later-crop samples	.45	.67	.64	.66
129 Total samples	.50	.76	.76	.78

[a] All between-judge repeatability estimates were highly significant.

the estimates of TDN or DE and actual TDN or DE values were computed and results were as follows:

Method of estimating TDN or DE	Correlation with Actual TDN	Actual DE
Schneider's	.49**	.54**
Axelsson's	.29*	.51**
Morrison's	−.03	−.12
In vitro Cellulose Digestibility	.75**	.85**

* = P < .05.
** = P < .01.

In vitro cellulose digestibility values exhibited a closer relationship with either actual TDN or actual DE than did any of the three estimates of digestibility based on components of the proximate chemical analysis. Therefore, it appeared that in vitro cellulose digestibility values would serve as a useful test for the score-card, and a further investigation is planned to study the relationship of the score-card to cellulose digestibility.

The correlations involving Morrison's estimated TDN with actual digestibility values were not significantly different from zero, whereas the correlations involving Schneider's and Axelsson's methods were statistically significant and of comparable value. As mentioned previously, information on 39 additional samples was available for a further comparison of Schneider's and Axelsson's methods. Using this information, it was found that the correlation between TDN values calculated by Schneider's equation and actual TDN was highly significant ($r = .529$), whereas the correlation between TDN values by Axelsson's formula and actual values was not quite significant ($r = .306$). These results were in agreement with those involving actual TDN values on the 49 test samples. Therefore, Schneider's regression equation appeared to be more useful than Axelsson's formula for testing the relationship of the score-card to nutritive value.

Relationship of the score-card to nutritive value. As already explained, four indicators of nutritive value (crude protein, crude fiber, percentage of cellulose, and Schneider's estimated TDN) were used in simple correlation analyses, to study the relationship of the score-card to nutritive value. These results appear in Table 4. The cutting date scores in Table 4 refer to stage of growth scores derived from actual cutting dates by use of the alternate scoring system, as shown in Table 1. The remainder of the correlation coefficients in Table 4 refer to correlations averaged for the seven judges over both scorings.

TABLE 4
Correlations between score-card values and four indicators of nutritive value

	Crude protein	Crude fiber	Schneider's TDN	Percentage of cellulose
Cutting date scores				
55 First-crop samples	.54**	−.27*	.33*	−.38**
St. of growth scores				
55 First-crop samples	.26*	−.15	.10	−.26*
65 Later-crop samples	.35**	−.24*	.25*	−.29*
120 First and later	.37**	−.22*	.18*	−.32**
129 Total samples	.43**	−.32**	.31**	−.41**
Leafiness scores				
55 First-crop samples	.11	−.15	.01	−.40**
65 Later-crop samples	.32**	−.32**	.34**	−.43**
120 First and later	.32**	−.30**	.20*	−.46**
129 Total samples	.42**	−.46**	.44**	−.59**
Color and condition scores				
55 First-crop samples	−.13	.11	−.22	−.21
65 Later-crop samples	.16	−.28*	.26*	−.37**
120 First and later	.13	−.17	.06	−.36**
129 Total samples	.26**	−.35**	.35**	−.48**
Final scores				
55 First-crop samples	.18	−.12	.01	−.39**
65 Later-crop samples	.36**	−.35**	.36**	−.45**
120 First and later	.37**	−.30**	.20*	−.48**
129 Total samples	.46**	−.46**	.45**	−.60**

* = P < .05.
** = P < .01.

TABLE 5

Correlations between final scores and four indicators of nutritive value within samples grouped according to grass content

	Crude protein	Crude fiber	Schneider's TDN	Percentage of cellulose
55 First-crop samples				
Low grass content (30 samples)	.33	−.06	−.05	−.46*
High grass content (25 samples)	.07	−.20	.24	−.22
65 Later-crop samples				
Low grass content (42 samples)	.50**	−.43**	.43**	−.51**
High grass content (23 samples)	.15	−.21	.13	−.30

* = $P < .05$.
** = $P < .01$.

The correlations involving the first-crop samples indicated that the alternate stage of growth scores based on actual cutting dates were more closely related to the four indicators of nutritive value than were the judges' scores for estimated stage of growth. However, the correlations involving scores based on cutting dates were not as high as might have been expected, considering the results of Reid et al. (13). The fact that most of the cutting dates represented an average date for the days over which the crop was cut could explain why the correlations were not higher. Also, Baumgardt (2) has pointed out that the relationship between cutting date and stage of growth and between cutting date and digestibility can vary from year to year. This would suggest that a single alternate scoring system for stage of growth based on actual cutting dates might not be suitable over all years.

The correlation analyses in Table 4 suggested that among first-crop samples actual cutting dates would be more useful than judges' estimates of stage of growth. Therefore, in subsequent multiple regression analyses, cutting date scores were substituted for the judges' estimated stage of growth scores.

Results in Table 4 also indicated that all of the score-card components were more closely associated with nutritive value among later-crop samples than among first-crop samples. In fact, among the first-crop samples most of the correlations did not reach significance. Among the later-crop samples all but one of the correlations were either significant or highly significant, but none of the correlation coefficients was greater than .45. In general, the correlations involving the 120 first- and second-crop samples were not high enough to suggest that the score-card could be used to accurately predict nutritive value. The highest correlations resulted when all 129 samples were considered. As in the repeatability analysis, this again indicated that the nine added samples contributed greater variation to the over-all analysis.

In further analyses similar correlations were computed within samples grouped according to high or low grass content. The results of these analyses, Table 5, indicated that the score-card had a closer relationship to nutritive value among samples low in grass content. Among the first-crop samples, one of the correlations was significantly different from zero, whereas among the later-crop samples, highly sifinificant correlations were obtained when those samples, which were low in grass content, were involved in the analyses. The fact that the score-card had a closer relationship to the indicators of nutritive value among samples low in grass content could be explained in part by the fact that the score-card had been designed primarily for judging legume hays. The lack of association between the score-card and estimated nutritive value over all types of samples indicated that the score-card could not be used satisfactorily to estimate nutritive value. Even among the 42 later-crop samples which were low in grass content, the highest correlation between final score and an indicator of nutritive value reached a magnitude of only .51.

The first scorings of four judges were then used in multiple regression analyses in an attempt to study the independent influence of the score-cards' components on the indicators of nutritive value. The results of these analyses were difficult to interpret, because the results varied among the groups of samples, among the four judges, and among the different indicators of nutritive value. To avoid some confusion, only those analyses involving Schneider's TDN as the dependent variable are shown in Table 6. Schneider's TDN was chosen because it seemed to be a more comprehensive indicator of over-all nutritive value than crude protein, crude fiber, or percentage of cellulose. In these multiple regression analyses the scores for stage

TABLE 6

Multiple regression analyses involving Schneider's TDN on the score-card components (SG = stage of growth, L = leafiness, CC = color and condition)

	Judge 1 $b' \pm Sb'$ [a]	Judge 2 $b' \pm Sb'$	Judge 3 $b' \pm Sb'$	Judge 4 $b' \pm Sb'$
55 First-crop samples				
SG	−.02 ± .13	.22 ± .15	.15 ± .15	.21 ± .14
L	.32 ± .15	−.31 ± .16	.12 ± .17	.06 ± .16
CC	−.43 ± .15	−.04 ± .15	−.18 ± .15	−.19 ± .15
R^2 [b]	.15	.09	.07	.08
AS [c]	.30 ± .12	.31 ± .14	.31 ± .14	.33 ± .13
L	.26 ± .14	−.12 ± .15	.07 ± .15	.03 ± .15
CC	−.40 ± .14	−.08 ± .15	−.16 ± .15	−.18 ± .15
R^2	.24	.14	.13	.14
65 Later-crop samples				
SG	.02 ± .13	.05 ± .19	.20 ± .11	.04 ± .14
L	.30 ± .13	.34 ± .17	.37 ± .12	.41 ± .13
CC	.21 ± .13	.07 ± .14	.19 ± .11	.15 ± .12
R^2	.19	.18	.31	.26

[a] $b' \pm Sb'$ = Standard partial regression coefficient plus or minus its corresponding standard error.
[b] R^2 = Multiple coefficient of determination.
[c] AS = Alternate stage of growth score based on cutting date.

of growth, leafiness, and color and condition were considered as the independent variables. The standard partial regression coefficients (b′) for the different independent variables can be compared to determine the relative importance of the components of the score-card in predicting Schneider's TDN. This type of comparison is possible because the standard partial regression coefficients are expressed in the same units, standard deviations. The multiple coefficients of determination (R^2) indicate the percentage of variation in the dependent variable (Schneider's TDN), accounted for by the three independent variables.

Among the 55 first-crop samples, the alternate scores (AS) for stage of growth, based on actual cutting dates, were more closely related to Schneider's TDN than were the judges' scores for stage of growth (SG). The substitution of the alternate scores in place of the judges' scores for stage of growth increased the R^2 values in all cases, but for three of the four judges these R^2 values reached a magnitude of only about .14.

Among the later-crop samples leafiness consistently (for all four judges) appeared to be the most useful component of the score-card. In the analyses involving the later-crop samples, most of the R^2 values were higher than they had been for the first-crop samples. In other words, the score-card was generally able to account for more of the variation in Schneider's TDN among later-crop samples than among first-crop samples. However, even among the later-crop samples the score-card components were able to account for no more than 20-30% of the variation in Schneider's TDN. As was true for the simple correlation analyses, the multiple regression analyses also indicated that the score-card could not be used to predict nutritive value with reliability.

It is concluded that the score-card is somewhat repeatable both within and between judges, but it appears to reflect nutritive value only to a limited extent. The score-card appears most effective when used among later-crop samples low in grass content. Dates of cutting for first-crop samples and leafiness scores for later-crop samples seem to be the most useful criteria of the score-card.

It should be noted that the lack of an adequate measure of nutritive or feeding value with which to test the score-card was a limiting aspect of this study. Acceptability or palatability, which was not taken into consideration here, is an important determinant of actual feeding value of hay. It is possible that the evaluation of the effectiveness of the score-card and its components could change considerably, if different indicators of nutritive value were considered.

REFERENCES

(1) BAUMGARDT, B. R. Comparison and Development of Laboratory Methods of Forage Evaluation. Ph.D. thesis, Rutgers University. 1959.

(2) BAUMGARDT, B. R. Private communication. University of Wisconsin. 1961.

(3) BROWNE, C. A. The Origin and Application of the Term Nitrogen-Free-Extract in the Valuation of Feeding-Stuffs. J. Assoc. Offic. Agr. Chemists, 23: 102. 1940.

(4) CRAMPTON, E. W., DONEFER, E., AND LLOYD, L. E. A Nutritive Value Index for Forages. J. Animal Sci., 19: 538. 1960.

(5) CRAMPTON, E. W., AND MAYNARD, L. A. The Relation of Cellulose and Lignin Content to the Nutritive Value of Animal Feeds. J. Nutrition, 15: 383. 1938.

(6) DONEFER, E., CRAMPTON, E. W., AND LLOYD, L. E. Prediction of the Nutritive Value Index of a Forage from in Vitro Rumen Fermentation Data. J. Animal Sci., 19: 545. 1960.

(7) HERSHBERGER, T. V., LONG, T. A., HARTSOOK, E. W., AND SWIFT, R. W. Use of the Artificial Rumen Technique to Estimate the Nutritive Value of Forages. J. Animal Sci., 18: 770. 1959.

(8) MATRONE, G. A Study of Lignin and Cellulose Methods for the Chemical Evaluation of Feeds. M.S. thesis, Cornell University, Ithaca, New York. 1944.

(9) MORRISON, F. B. Feeds and Feeding. 22nd ed. Morrison Publ. Co., Ithaca, New York. 1956.

(10) NICHOLS, C. W. Repeatability and Evaluation of Score Card Judging in Estimating Nutritive Value of Hay. M.S. thesis, University of Wisconsin. 1959.

(11) PIGDEN, W. J., AND BELL, J. M. The artificial Rumen as a Procedure for Evaluating Forage Quality. J. Animal Sci., 14: 1239. 1955.

(12) QUICKE, G. V., BENTLY, O. G., SCOTT, H. W., AND MOXON, A. L. Cellulose Digestion in Vitro as a Measure of the Digestibility of Forage Cellulose in Ruminants. J. Animal Sci., 18: 275. 1959.

(13) REID, J. T., KENNEDY, W. K., TURK, K. L., SLACK, S. T., TRIMBERGER, G. W., AND MURPHY, R. P. Effect of Growth Stage, Chemical Composition, and Physical Properties upon the Nutritive Value of Forages. J. Dairy Sci., 42: 567. 1959.

(14) SCHNEIDER, B. H., LUCAS, H. L., CIPOLLONI, MARY ANN, AND PAVLECK, HELEN M. The Prediction of Digestibility of Feeds for which There Are Only Proximate Chemical Data. J. Animal Sci., 11: 77. 1952.

HERITABILITY OF BIRTH WEIGHT AND ITS RELATIONSHIP WITH PRODUCTION IN DAIRY CATTLE

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SUMMARY

The data included in this study were taken from the University of Illinois dairy herd and consisted of 1,234 birth weights representing the five major breeds of dairy cattle. A comparison of the confidence intervals demonstrates significant breed differences at the 0.001 level of probability for all possible comparisons. The fixed effects of sex of calf and calving sequence were estimated by the method of least-squares. These combined effects were found to be significant at the 0.01 level of probability in all five breeds. The adjustment factors so obtained were applied to the birth weights before the estimates of heritability and genetic correlations were derived. The differences associated with the sires of the calves were found to be significantly different from zero in all breeds except Guernseys.

The pooled within-breed estimates of heritability of birth weight obtained by multiplying, respectively, by 4, 2, and 2, the paternal half-sib correlation, the intra-sire regression of offspring on dam, and the full-sib correlation were 0.38, 0.48, and 0.51. Theoretically, it should be relatively easy to change the average birth weight of a population.

The differences between estimates by different methods could be due to sampling errors, epistatic and dominance effects, to additive genetic effects of birth weight as a characteristic of the dam, or to maternal effects of the dam on birth weight of the calf. The small magnitude of the differences between the three estimates indicates that the within-breed maternal effect was small and that birth weight was mostly a characteristic of the calf.

The correlations between the birth weight of the calf and the subsequent milk and fat production of the dam were calculated on a within-sire basis for the five breeds and for a within-breed pooled estimate. The absence of correlations significantly different from zero indicated that the birth weight of the calf and the immediately subsequent lactation production of the dam were essentially independent.

Intra-service-sire correlations between the birth weight of a calf and its subsequent production were not significantly different from zero in any of the five breeds or for a within-breed pooled estimate.

The pooled within-breed estimates of the genetic correlations between birth weight and milk and fat productions were small and not significantly different from zero. It appears that birth weight and production are genetically independent.

Weight at birth is one of the first characteristics of an animal that can be easily measured and, in addition, weight does not change appreciably during the first few days of life (12). For beef cattle, investigators have shown the importance of birth weight in predicting weaning weight (5, 11), weight at 1 yr (11), rate of gain to weaning, rate of gain during fattening, or rate of gain from birth to slaughter (3, 5, 8, 11, 14), weight at six months (7), fattening performance (16), and final weight (15, 21). Hammond (6) reported that within a number of species an association has been found between birth weight and viability. Most of the studies of the heritability of the birth weight in cattle are relative to beef cattle (2, 3, 9-11, 18, 20). The estimates are quite variable and range from 0.11 to 1.00; however, the majority of them fall within the range of from 30 to 50%. On the other hand, relatively few investigators (1, 14, 15) have studied the rela-

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tion between birth weight and production in dairy cattle.

Because birth weight is rather closely correlated with beef characteristics, and dairy beef is likely to become more important in the world, it is pertinent to know its heritability and its relationships with production. The primary objectives of this study were to determine the heritability of birth weight in dairy cattle and its relationships with milk and fat yields.

SOURCE OF DATA

The data included in this study were obtained from breeding records of dairy cattle in the University of Illinois herd, for the years from 1940 to 1959. The 1,234 birth weights in the sample consisted of 213 Ayrshires, 163 Brown Swiss, 154 Guernseys, 587 Holsteins, and 117 Jerseys. The calves were weighed within the 24 hr following birth. All birth weights of single, normal, living calves were included, regardless of their magnitude, whereas twins and stillbirths were excluded from the data. The calves were sired by 15 Ayrshire, 16 Brown Swiss, 14 Guernsey, 57 Holstein, and 13 Jersey bulls. All available and pertinent milk and fat yields were adjusted for age of the cow at calving and length of the lactation period according to the most recent DHIA conversion factors.

ANALYSIS OF DATA AND RESULTS

Variation due to breed, sex, and calving sequence. All available birth weights were grouped by sex and calving sequence for each of the five breeds, in order to determine the appropriate effects of these causes of variation, to derive valid adjustment factors and, finally, to estimate with more precision the part of the phenotypic variance that could be attributed to genetic effects. The fourth and subsequent calves were grouped in the same class.

The average birth weights for breeds, sex within breed, calving sequence within breed, and the confidence intervals, respectively, at the .001, .01, and .05 levels are given in Table 1. The standard deviation for the whole breed was used in computing confidence intervals for the different subclasses of the same breed; consequently, the confidence intervals for sex and calving sequence subclass are only approximate. The breed averages are significantly different from each other at the .001 level of probability. The bull calves are significantly heavier than the heifers at the .01 level of probability in the Ayrshire, Brown Swiss, Guernsey, and Holstein breeds and at the .05 level in the

TABLE 1

Average birth weights and confidence intervals for breeds (.001 level), sex (.01 level), and calving sequence (.05 level)

Breed		Ayrshire	Brown Swiss	Guernsey	Holstein	Jersey
Number		213	163	154	587	117
Average birth weight *(lb)*		80.4	102.6	71.1	94.7	54.4
Confidence interval (C.I.)		±2.1	±3.0	±3.2	±1.6	±2.4
Standard deviation		9.56	11.86	11.93	11.82	8.01
Males	No.	107	87	84	302	62
	Avg	84.3	106.9	76.2	98.1	56.9
	C.I.	±2.4	±3.3	±3.3	±1.7	±2.7
Females	No.	106	76	70	285	55
	Avg	76.5	97.6	67.5	91.1	51.6
	C.I.	±2.4	±3.5	±3.7	±1.8	±2.9
First calving	No.	98	68	56	207	34
	Avg	77.8	97.5	70.1	90.0	50.0
	C.I.	±1.9	±2.9	±3.2	±1.6	±2.8
Second calving	No.	54	42	46	158	37
	Avg	81.0	106.7	71.8	97.7	56.8
	C.I.	±2.6	±3.7	±3.5	±1.8	±2.7
Third calving	No.	28	25	27	100	19
	Avg	84.2	105.3	69.7	98.5	57.3
	C.I.	±3.7	±4.9	±4.7	±2.3	±3.8
Fourth and later calving	No.	33	28	25	122	27
	Avg	84.2	106.2	73.6	95.4	54.7
	C.I.	±3.4	±4.6	±4.9	±2.1	±3.2

TABLE 2

Effect of sex and calving sequences within breed on birth weights of dairy calves obtained by least-squares analysis

	Ayrshire	Brown Swiss	Guernsey	Holstein	Jersey
Number	213	163	154	587	117
Effect of sex					
S_1	4.12	4.45	3.62	3.46	2.35
S_2	−4.12	−4.45	−3.62	−3.46	−2.35
Effect of calving sequence O_1	−3.76	−7.19	−1.90	−4.99	−5.21
O_2	− .33	1.53	+1.25	2.50	2.02
O_3	1.81	4.15	− .89	2.37	2.92
O_4	2.28	1.51	+1.53	.12	.27
F value of the test of significance of the combined effects of sex and calving sequence	18.21**	18.23**	4.06**	30.99**	18.80**

** Significant at the .01 level of probability.

Jersey breed. Average birth weights for the second, third, and fourth calvings are not significantly different from each other for any of the possible comparisons for the five breeds. In Brown Swiss and Holsteins, the first calves are significantly lighter than all the following ones. The first calves are only significantly lighter than the third and fourth calves in Ayrshires, and the second and third calves in Jerseys. The calving sequence does not have a significant effect on birth weight in the Guernseys.

Although the data covered a 20-yr period, there was no apparent trend in the yearly birth weight means after the data were adjusted for sex of calf and sequence of calving and the sire of the calf. This would be expected, since there has been no conscious effort to change birth weight.

Adjustment for sex and calving sequence. Because of disproportionate subclass numbers, the effects associated with sires, sex, and calving sequence cannot be separated directly by considering each independently; therefore, a simultaneous consideration of all effects has to be made and this is most conveniently done by the method of least-squares. The following mathematical model was assumed to represent the birth weights for each breed:

$$Y_{ijkl} = \mu + B_i + S_j + O_k + e_{ijkl} \qquad (1)$$

in which Y_{ijkl} is the birth weight of the l^{th} calf of the k^{th} calving sequence of the j^{th} sex of the i^{th} sire. μ is the over-all mean birth weight. B_i is the effect of the i^{th} sire expressed as a deviation from the over-all mean. S_j is the effect of the j^{th} sex expressed as a deviation from the over-all mean. O_k is the effect of the k^{th} calving sequence expressed as a deviation from the over-all mean μ, and e_{ijkl} represents the random errors which are assumed to be independent. All the variables were assumed to be fixed except B_i and e_{ijkl} which were assumed to be random variables with means of zero and variances of σ^2_B and σ^2_e, respectively. The sire equations were absorbed into the sex of calf and calving sequence equations so that a system of six least-squares equations with six unknowns resulted. Because the determinant of this 6 × 6 coefficient matrix was equal to zero, two restrictions were imposed in the equations:

$$\sum_{j=1}^{2} S_j = \sum_{k=1}^{4} O_k = 0 \qquad (2)$$

The reduced system of four equations was then solved by an interactive method. The constants S_1, O_1, O_2, O_3 were obtained directly and the two remaining constants, S_2 and O_4, were derived from Equation (2). The results for the five breeds are listed in Table 2, where the effects are expressed as deviations from the mean. Thus, the second Holstein calves are $(4.99 + 2.50) = 7.49$ lb heavier than the first ones and male Holstein calves are 6.92 lb heavier than females. The effects for sex and calving sequence were both significant at the 5% level for all breeds, with the exception of sequence of calving for the Guernsey breed. The F values listed in Table 2 are those for the combined effects of sex and calving sequence. These combined effects were significant at the 0.01 level of probability for all five breeds.

Heritability of birth weight. After adjusting the birth weights for the combined effects of sex and calving sequence, the correlations between the birth weights of paternal half-sibs and between full-sibs and the intra-sire regression of offspring's birth weight on dam's birth weight were used as bases for calculating heritability of birth weight, the values of which are listed in Table 3. Only the paternal half-sib estimate for Guernseys and the full-sib estimate for Holsteins failed to be significantly different from zero.

To the extent that birth weight is a characteristic of the calf, its heritability can be obtained by multiplying the paternal half-sib correlation by four. The groups of full-sibs have not been accounted for because of the small number of full-sib comparisons relative to the number of half-sib comparisons; consequently, the half-sib estimates of the heritability of birth weight are larger than they should be for heritability as defined in the narrow sense.

As far as the estimates of the heritability of birth weight obtained by multiplying the full-sib correlation by two are concerned, only the estimate for Holsteins failed to be significantly different from zero.

All available pairs were used in calculating the regression of birth weight of calves on the birth weight of dams for each breed and for a within-breed pooled estimate. Any yearly variations would be appreciably removed by working on an intra-sire basis. The same adjustment values were applied to the birth weights of dams as to the birth weights of calves, since all the dams were from the same herd and most of them were involved in the study as calves. When a dam was remated to the same sire, the resulting calf's birth weight was included in the calculation with a replicate of the birth weight of the dam. This could be a cause for the estimate of heritability of birth weight obtained by doubling the regression of offspring on dam being larger than the half-sib correlation estimate.

When dealing with the paternal half-sib estimates of heritability, it has been shown (9) that

$$h^2 = \frac{4\,(F-1)}{K+F-1} \qquad (3)$$

where F is the value obtained by dividing the between sire mean square by the within sire mean square and K is the appropriate coefficient of the component of variance for sires in the mean square associated with differences between sires. Shown in Table 4 are the values for K and F.

It can be shown that $F = \frac{1+(K-1)\,r}{1-r}$ where r is the intraclass correlation. Fisher (4) has shown that $Z = \frac{1}{2}\log_e \left[\frac{1+(K-1)\,r}{1-r}\right] = \frac{1}{2}\log_e F$ and that $\sigma_Z = \sqrt{\frac{K}{2\,(K-1)\,(n_1-2)}}$, where n_1 represents the number of groups. In the present case, n_1 represents the number of paternal half-sib groups.

By transforming F to Z and deriving the standard errors of Z, it can be seen that the paternal half-sib estimates of heritability for the different breeds are not significantly different from each other.

The pooled within-breed paternal half-sib estimate is considered to be the most valid of the half-sib estimates of heritability. By mak-

TABLE 3

Heritability of birth weight in dairy breeds

	4 × pat. half-sib. corr.			2 × intra-sire reg. of off. on dam			2 × full-sib corr.		
	No. of calves	No. of sires	Heritability	No. of pairs	Heritability	σ_b[a]	No. of calves	No. of parental groups	Heritability
Ayrshire	213	15	.37*	213	.81**	.08	102	44	.67**
Brown Swiss	163	16	.41*	163	.49**	.08	60	27	.94**
Guernsey	154	14	.21	154	.72**	.10	68	29	.64[a]
Holstein	587	37	.39**	587	.30**	.04	150	71	.12
Jersey	117	13	1.17*	117	.70**	.09	52	22	1.19**
Pooled within-breed estimate	1,234	95	.38**	1,234	.47**	.03	432	193	.51**

* Significant at the .05 level of probability.

** Significant at the .01 level of probability.

[a] Standard errors of the regression coefficients which were doubled to obtain an estimate of heritability.

TABLE 4

Between and within-group components of variance, the values for K. F, Z, and σ_Z for paternal half-sibs and full-sibs

	Paternal half-sib estimates						Full-sib estimates					
	σ^2_B	σ^2_W	F	K	Z	σ_Z	σ^2_B	σ^2_W	F	K	Z	σ_Z
Ayrshire	10.3	101.3	2.35	13.3	.43	.20	46.0	90.6	2.17	2.31	.39	.13
Brown Swiss	10.5	91.3	2.11	9.7	.37	.19	41.9	47.3	2.96	2.22	.54	.17
Guernsey	6.8	123.7	1.60	10.9	.21	.21	36.5	77.9	2.09	2.34	.37	.15
Holstein	11.1	103.8	2.67	15.6	.49	.12	8.4	130.9	1.13	2.11	.06	.11
Jersey	15.0	36.3	2.35	8.5	.43	.22	33.2	21.6	4.61	2.35	.76	.18
Pooled within breed estimates	10.3	98.0	2.35	12.9	.43	.08	30.0	87.2	1.77	2.24	.29	.07

$$F = \frac{\sigma^2_W + K\,\sigma^2_B}{\sigma^2_W} = \frac{\text{Between-group mean square}}{\text{Within-group mean square}}.$$

$$Z = \frac{1}{2}\log_e\left[\frac{1 + (K-1)\,r}{1-r}\right];\ r = \frac{\sigma^2_B}{\sigma^2_B + \sigma^2_W}.$$

$$\sigma_Z = \sqrt{\frac{K}{2\,(K-1)\,(n_1-2)}}.$$

ing a transformation of the F value of 2.35 shown in Table 4 to Z, one can place confidence intervals on Z. The 95% confidence interval is:

$$[Z - 1.96\,\sigma_Z \leqslant Z \leqslant Z + 1.96\,\sigma_Z]$$
or
$$[.27 \leqslant .43 \leqslant .58]$$

Since $h^2 = \frac{4\,(F-1)}{K + F - 1}$, the 95% confidence interval for Z above can be transformed back into terms of F, thus placing a confidence interval on heritability. For the pooled within-breed paternal half-sib estimate of heritability, the 95% confidence interval is $[.21 \leqslant .38 \leqslant .58]$.

The estimates based on full-sib relationships can be treated in the same way except that $h^2 = \frac{2\,(F-1)}{K + F - 1}$. By making the transformation to Z and deriving σ_Z, it can be seen from Table 4 that the full-sib estimate for Holsteins is significantly smaller than the estimates for the other breeds. No other differences are significant. The 95% confidence interval for the pooled within-breed full-sib estimate of heritability is $[.27 \leqslant .51 \leqslant .74]$. The difference between the pooled within-breed half-sib estimate and the pooled full-sib estimate is significant at the 10% level.

Shown in Table 3 are the standard errors of the regression coefficients which were doubled to provide the intrasire regression estimates of heritability. By observing these standard errors, it can be seen that the intrasire regression of offspring on dam for Holsteins is significantly smaller than those for the four other breeds. No other differences were significant.

To put a confidence interval on the estimate obtained from the pooled within-breed, intrasire regression of offspring on dam, and to compare this estimate with the full-sib and paternal half-sib estimates, a logical procedure is to make a transformation to Z. In this case, $Z = \frac{1}{2}[\log_e (1+r) - \log_e (1-r)]$ and $\sigma_Z = \sqrt{\frac{1}{N-3}}$, where r is the intra-sire correlation between parent and offspring and N is the total number of pairs less the number of sires.

In these data, the pooled within-breed intrasire regression of offspring on dam is .234, and the corresponding correlation is .221. Because the above regression and correlation coefficients are so nearly equal, it appears legitimate to use the regression coefficient as an estimate of the correlation coefficient, make a transformation to Z, and place confidence intervals on the estimate of heritability. The 95% confidence intervals are $[.358 \leqslant .468 \leqslant .588]$. When the standard error of the regression coefficient itself was used in deriving a 95% confidence interval, the following result was obtained: $[.348 \leqslant .468 \leqslant .588]$.

With the paternal half-sib, the full-sib and the parent offspring pooled estimates of heritability all expressed in terms of Z, it is possible to make tests of significance between the three estimates. Such tests indicated that the difference between the paternal half-sib estimate and the regression of offspring on parent

estimate was significant at the 0.05 level of probability and that between the half-sib and full-sib estimates was significant at the 0.10 level. The difference between the full-sib and parent offspring estimates was not significant.

Relationship between birth weight and production. All available milk and fat yields for the lactations immediately following the birth of calves that had birth weight recorded were adjusted for age of the cow at calving and length of the lactation period. DHIA conversion factors were used to adjust for age and to reduce records made in more than 305 days to a 305-day basis, and to extend incomplete records to a 305-day basis. The lactations were only considered as incomplete in case of death or casualty and were not extended if they were just short lactations. However, only those incomplete lactations longer than 100 days were included.

The birth weights were adjusted for sex and calving sequence according to the adjustment values previously derived. When a cow had several calves for which birth weight had been recorded, each of them was included in the study and paired with the milk and fat yields following its birth. When the birth weight of the cow was compared to her future production, there were as many replicates of this birth weight as she had calves with a birth weight that could be paired with her production in the subsequent lactation.

The correlations between birth weight of calf and immediately subsequent lactation production of dam were calculated on a within-sire of the calf basis, to remove environmental variations. These correlations should indicate whether there are factors affecting both the birth weight of the calf and the immediately subsequent lactation production of the dam. Correlations between the birth weight of a calf and her subsequent production records were computed on a within-service-sire basis, to remove environmental variations. These correlations could be helpful in predicting the milking potential of any young dairy heifer. The pooled estimates were computed on a within-breed within-sire basis. The results listed in Table 5 show that none of these correlations for either milk or fat was significant at the 0.05 level of probability; thus seemingly indicating that birth weight and production are independent traits.

Shown in Table 6 are estimates of the genetic correlations between birth weight and milk production and birth weight and milk fat production. By regrouping the data from which the correlations in Table 5 were derived, paternal half-sib estimates of the genetic correlations between birth weight and production were derived as shown by Robertson (17). The estimates of $0.137 \pm .094$ and $.026 \pm .096$ between birth weight and milk and birth weight and fat production, respectively, indicate that birth weight and production are essentially independent genetically. There is the possibility that the genetic correlations between birth weight and production differ among breeds. Those for Ayrshire and Jersey were approximately -0.50, but with only 13 Ayrshire and 14 Jersey sires these correlations were not significant. Those for Brown Swiss, Guernsey, and Holstein were approximately equal to the

TABLE 5

Correlations between birth weight and production [a]

Breed	No. of pairs	No. of sires	r_{WM}	r_{WF}	r'_{WM}	r'_{WF}
Ayrshire	172	14	+.094	+.035	−.088	−.049
Brown Swiss	123	15	−.065	+.014	−.012	−.056
Guernsey	124	13	+.104	+.078	+.137	+.087
Holstein	475	36	−.050	−.032	+.012	+.003
Jersey	93	10	+.046	+.044	+.043	+.026
Pooled estimate [b]	987	88	+.006	+.007	+.015	−.007

r_{WM} = Intra-service-sire correlation between the birth weight of the calf and the immediately subsequent lactation milk production of its dam.

r_{WF} = Intra-service-sire correlation between the birth weight of the calf and the immediately subsequent lactation milk fat production of its dam.

r'_{WM} = Intra-service-sire correlation between the birth weight of a calf and her subsequent milk production.

r'_{WF} = Intra-service-sire correlation between the birth weight of a calf and her subsequent milk fat production.

[a] = None of these correlations is significantly different from zero at the .05 level of probability.

[b] = The pooled correlations were estimated on a within-breed within-sire basis.

TABLE 6

Genetic correlations between birth weight and milk production and birth weight and milk fat production

	No. sires	No. daughters	$r_{G_W G_M}$	$r_{G_W G_F}$
Pooled within-breed paternal half-sib estimate	109	987	.137 ± .094	.026 ± .096
Pooled within-sire, within-breed, daughter-dam estimate	88	987	.040 ± .148	.047 ± .145

pooled within-breed estimates and at least 20 sires were involved for each of these breeds.

Daughter-dam estimates of the genetic correlations were derived by making use of the correlations in the last line of the middle two columns of Table 5 and the within-breed intrasire correlations between dam and daughter for birth weight (.221), milk production (.11), and milk fat production (.10). The daughter-dam estimates of the genetic correlations are shown in the last line of Table 6 and are not significantly different from zero.

DISCUSSION OF RESULTS

The pooled estimates of heritability of birth weight obtained by multiplying respectively by 4, 2, and 2, the paternal half-sib correlation (A), the within-sire regression of offspring on dam (B), and the full-sib correlation (C) were equal to 0.380, 0.478, and 0.511, respectively. In addition to sampling errors, the difference between the estimates (A) and (B) of 0.088 may be attributed to a fraction of the epistatic variance included in (B) and not in (A) and also to a fraction of the additive genetic variance of birth weight as a characteristic of the dam and to so-called maternal effects. The difference between the estimates (A) and (C) may be attributed to a fraction of the dominance variance, and a small fraction of the epistatic variance that is included in (C) but not in (A), and to a fraction of the additive variance of birth weight as a characteristic of the dam. The maternal effect is in addition to the dam's genetic contribution to the factors, but these estimates of heritability by different methods indicate that birth weight is mostly a characteristic of the calf and that permanent maternal effects are small.

Including fractions of the epistatic variance tends to increase the three estimates of the heritability as far as it is considered in the narrow sense as defined by Lush (13); moreover, estimates (A) and (B) are also slightly biased upwards because the full-sibs were not removed from the data. However, the additive genetic effects may be assumed to represent at least 35% of the phenotypic variance and this magnitude of heritability is large enough to make selection efficient. Selection for increasing birth weights might have some serious disadvantages after a few generations. In fact, if heavier calves make calving more difficult, the results might be deleterious for both dam and calf. This fact has been particularly noticed in Europe, where the practice of crossing dairy cows with beef sires is used. For example, Charolais sires giving average-size calves at birth are preferred, in spite of some lack in subsequent growth rate and weaning weight. Increasing birth weight would tend to increase length of gestation period too and, consequently, the calving interval. On the other hand, selection for lighter weight at birth could tend to weaken the breed after a few generations.

The absence of a significant correlation between the birth weight of a calf and the immediately subsequent milk or fat lactation production of its dam indicates independence of these characteristics. Consequently, it would be possible to select for birth weight without influencing milk and fat production of the dams of the calves. The results agree with the nonsignificant correlation of +0.05 reported by Blackmore et al. (1) obtained from 287 daughter-dam pairs with Holsteins.

Phenotypic correlations of approximately zero were found between the birth weight of calves and their subsequent production and this agrees with the results obtained by Munkacsi (15); however, Martin (14) reported a significant but small positive correlation between birth weight and milk production.

The genetic correlations of approximately zero between birth weight and milk and fat production indicate that birth weight and production are genetically independent. Indeed, the results are not surprising, since Touchberry (19) and Blackmore et al. (1) found that milk and fat production seemed to be genetically independent of weight and we have also seen previously (3, 5, 7, 8, 11, 14, 15, 21) that birth weight was positively correlated with weight and growth rate.

In concluding, it can be said that birth weight of the calf is a heritable characteristic not significantly correlated with the immediately subsequent lactation production of the dam of the calf nor with the calf's own subsequent production. The genetic correlations of essentially zero indicate that birth weight and production are genetically independent.

REFERENCES

(1) BLACKMORE, D. W., MCGILLARD, L. D., AND LUSH, J. L. Genetic Relations Between Body Measurements at Three Ages in Holstein. J. Dairy Sci., 41: 1045. 1958.

(2) BURRIS, M. J., AND BLUNN, C. T. Some Factors Affecting Gestation Length and Birth Weight of Beef Cattle. J. Animal Sci., 11: 34. 1952.

(3) DAWSON, W. M., PHILLIPS, R. W., AND BLACK, W. H. Birth Weight as a Criterion of Selection in Beef Cattle. J. Animal Sci., 6: 247. 1947.

(4) FISHER, R. A. Statistical Methods for Research Workers. 12th ed. Sec. 39. Oliver and Boyd. 1954.

(5) GREGORY, K. E., BLUNN, C. T., AND BAKER, M. L. A Study of Some of the Factors Influencing the Birth and Weaning Weights of Beef Calves. J. Animal Sci., 9: 338. 1950.

(6) HAMMOND, D. J. Physiological Factors Affecting Birth Weight. Nutrition Soc. Proc., 2: 8. 1944.

(7) JORDAO, L. P., AND ASSIS, F. DE P. The Study of Flemish Cattle in Brazil, Gestation Period, Birth Weight, Weight of Cows After Calving and the Increase in Weight of the Calves. Animal Breeding Abstr., 18: 949. 1947.

(8) KNAPP, B., BAKER, A. L., QUESENBERRY, J. R., AND CLARK, R. T. Record of Performance in Hereford Cattle. Montana Agr. Expt. Sta., Bull. 397: 30. 1941.

(9) KNAPP, B., AND NORDSKOG, A. W. Heritability of Growth and Efficiency in Beef Cattle. J. Animal Sci., 5: 62. 1946.

(10) KOCH, R. M. Heritability of Economic Characteristics in Beef Cattle. Abstr. in Iowa State Coll. J. Sci., 1955: 445. 1953.

(11) KOCH, R. M., AND CLARK, R. T. Genetic and Environmental Relationships Among Economic Characteristics in Beef Cattle. I. Correlations Among Paternal and Maternal Half-sibs. II. Correlations Between Offspring and Dam and Offspring and Sire. III. Evaluating Maternal Environment. J. Animal Sci., 14: 775; 14: 786; 14: 979. 1955.

(12) KOCH, R. M., SCHLEICHER, E. W., AND ARTHAUD, V. H. Weight Changes in Beef Calves Following Birth. J. Animal Sci., 14: 792. 1955.

(13) LUSH, J. L. Intra-sire Correlations or Regressions of Offsprings on Dam as a Method of Estimating Heritability of Characteristics. Proc. Am. Soc. Animal Production, 1940: 293. 1940.

(14) MARTIN, T. G. Factors Affecting Growth Rate of Dairy Calves. Iowa State Coll. J. Sci., 30: 412. 1956.

(15) MUNKACSI, F. The Effect of Birth Weight on Some Economic Characteristics. Animal Breeding Abstr., 23: 1058. 1955.

(16) PIERCE, C. D., AVERY, H. G., BURRIS, M., AND BOGART, R. Rate and Efficiency of Gains in Beef Cattle. Some Factors Affecting Performance Testing. Oregon Agr. Expt. Sta., Tech. Bull., 33: 32. 1954.

(17) ROBERTSON, A. The Sampling Variance of the Genetic Correlation Coefficient. Biometrics, 15: 469. 1959.

(18) SHELBY, C. E., CLARK, R. T., AND WOODWARD, R. R. The Heritability of Some Economic Characteristics of Beef Cattle. J. Animal Sci., 14: 372. 1955.

(19) TOUCHBERRY, R. W. Genetic Correlations Between Five Body Measurements, Weight, Type and Production in the Same Individual Among Holstein Cows. J. Dairy Sci., 34: 242. 1950.

(20) TYLER, W. J., CHAPMAN, A. B., AND DICKERSON, G. E. Sources of Variation in the Birth Weight of Holstein-Friesian Cattle. J. Dairy Sci., 30: 483. 1947.

(21) WILLARD, H. S. Effect of Holstein Birth Weight on Calf Gain and Final Weight. Wyoming Agr. Expt. Sta., Bull., 286: 14. 1948.

VARIATION IN CONCEPTION RATES OF GUERNSEY CATTLE [1]

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SUMMARY

Data from 42 Guernesy herds served by one A.I. stud, and 37 Guernsey herds served by another were analyzed to determine heritability of conception rate. The data included records of 2,470 daughters of bulls in these studs, and breedings occurred during the period of 1950 to 1959. Heritability estimates from intrasire correlations of paternal half-sib groups were 0.08 and 0.02 based on an average of from one to four records per daughter, while those based on heifer records only were 0.06 and zero, respectively. Regression of daughters on dams yielded heritability estimates of 0.03 and 0.10 for the two studs.

Early studies found little evidence for genetic variability in conception rates of dairy cattle. Dunbar and Henderson (2) estimated heritability of nonreturn to first service at 180 days of pregnancy to be near zero in production-tested Holstein cows. Rottensten and Touchberry (5) found similar results in dairy heifers at Danish progeny-testing stations.

However, daughter-dam comparisons of the number of services required (1 or > 1) in two purebred Holstein herds and one commercial herd indicated that the condition of repeat-breeding was slightly heritable (1).

Studies in an experimental Holstein herd (4) indicated highly significant differences in the conception rates of cows from different sire lines as diagnosed by rectal palpation 35-41 days after breeding. There were also significant differences between sire lines in the effect of inbreeding on conception rate in both cows and heifers.

A field study on Holsteins by Inskeep et al. (3) yielded a heritability estimate for conception rate (birth of a live calf as a result of first service) of 0.085 on the basis of intra-sire correlation of paternal half-sib groups.

More recently, Shannon and Searle (6) estimated heritability of nonreturn rates for the male on the basis of son-sire regression to be 0.55 ± 0.26.

The present study was designed to estimate heritability of conception rate at first service in the Guernsey breed.

DATA AND METHODS

Data were taken from barn breeding records and calf record books of 42 Guernsey herds, both grade and purebred, served by Badger Breeders Cooperative, Shawano, Wisconsin (Stud I) and 37 herds served by Tri-State Breeders Cooperative, Westby, Wisconsin (Stud II). These data provided information on 1,237 and 1,233 daughters of bulls in the two studs, respectively, for years 1950 through 1959. Cows were considered to have conceived if the first insemination of a service period resulted in the birth of a live calf. Fertile inseminations were coded 1; infertile ones were coded 0. For each daughter and her dam, records were taken for as many of the following four service periods as were available: nulliparous, primiparous, and the first two recorded multiparous service periods. From one to four records were obtained for each daughter and zero to four records for each dam. These records were based on breedings to all Guernsey service sires in the studs.

Analysis of variance of the differences in fertility among paternal half-sib groups was calculated in order to estimate heritability of conception rate. Two analyses were made: one was of mean conception rates based on all available service periods (a maximum of four) of each daughter, and the second was of the nulliparous records only. In both, only progeny groups composed of a minimuum of ten daughters were included. Estimates of heritability

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were obtained from the intra-sire correlation of paternal half-sisters.

Analysis was made to determine whether significant differences existed in fertility levels among herds. Regressions of daughters' conception rate on dams' conception rate were then calculated on a within herd basis, and tests for heterogeneity of the individual herd regressions were made. Mean conception rates of daughters and dams were used in these analyses. These regressions provided further estimates of heritability.

RESULTS

The number of sires in Stud I represented by groups of ten or more daughters totalled 34. Included in these groups were 1,117 of the 1,237 available daughter records. The over-all mean conception rate at first service of these daughters was 53.1% (based on the average of a maximum of four service periods). Similarly, Stud II provided records on ten or more daughters for each of 32 sires. These groups included 1,097 of 1,233 available daughter records with an over-all mean conception rate of 54.2%. The half-sib groups had fertility ranges of 8 to 67% ($\bar{x} = 52.4\%$) and 35 to 73% ($\bar{x} = 54.8\%$) for the two studs, respectively.

Significant differences ($P < 0.05$) were found among paternal half-sib groups in the Stud I area (Table 1). The intrasire correlation provided a heritability estimate for conception rate (additive genetic variance) of 0.08. Records from Stud II, however, yielded a heritability estimate of 0.02 from statistically nonsignificant differences among paternal half-sib groups.

When only nulliparous records were considered, 33 half-sib groups with a total of 981 daughters from Stud I sires and 27 half-sib groups with 839 daughters from Stud II sires were analyzed. The over-all mean fertility levels were 52.1 and 54.5%, respectively. Group conception rates ranged from 8.3 to 75.9% ($\bar{x} = 51.5\%$) and 40.7 to 81.8% ($\bar{x} = 56.1\%$), respectively. In Stud I records, significant differences ($P < 0.05$) in conception rates were again found among sire groups and heritability was estimated at 0.06 (Table 2). The Stud II records showed no significant differences and the estimate was approximately zero.

There were, on the average, 18.7 and 16.6 herds, respectively, per paternal half-sib group in the parous records. Thus, it was assumed in the foregoing analyses that any effect due to herd would be random among the daughters of the various sires. All available daughter records were included in an analysis for herd differences in conception rate before studying the daughter-dam comparisons. The number of records per herd ranged from nine to 67 in Stud I and five to 105 in Stud II. The ranges of herd mean conception rates were 30.9 to 75.5% and 29.6 to 80.0%, respectively. Herd differences were highly significant ($P < 0.01$) in both studs (Table 3).

Since significant herd differences existed, the

TABLE 1

Analysis of variance of conception rates among paternal half-sib groups based on average records

	Stud I			Stud II		
Source	d.f.	Mean square	Variance	d.f.	Mean square	Variance
Among sires	33	.2420*	.0029	31	.1837[a]	.0009
Within sires	1,083	.1479	.1479	1,065	.1537	.1537

* $P < 0.05$.
[a] $0.20 < P < 0.25$.

TABLE 2

Analysis of variance of conception rates among paternal half-sib groups based on nulliparous records

	Stud I			Stud II		
Source	d.f.	Mean square	Variance	d.f.	Mean square	Variance
Among sires	32	.3656*	.0041	26	.2500[a]	.0001
Within sires	948	.2459	.2459	812	.2482	.2482

* $P < 0.05$.
[a] $P > 0.25$.

TABLE 3
Analysis of variance of conception rates among herds

Source	Stud I		Stud II	
	d.f.	Mean square	d.f.	Mean square
Among herds	41	.2814**	36	.3235**
Within herds	1,195	.1450	1,196	.1497

** $P < 0.01$.

regression of daughters' conception rate on dams' conception rate was calculated on a within herd basis. A total of 1,060 daughter-dam pairs from Stud I and 1,047 pairs from Stud II were available for analysis. The intra-herd regressions were 0.01 and 0.05 for Studs I and II, respectively, and individual herd regressions were not heterogeneous. These non-significant regressions yielded heritability estimates of 0.03 and 0.10 which are of the same general magnitude as those obtained in other studies. It should be noted that regressions of this magnitude could not be statistically significant with the number of records available for this study.

The heritability estimates obtained in this study are somewhat higher than they would have been, had the mean conception rates been adjusted to a single observation basis. However, adjustment did not seem warranted, since the estimates were low.

REFERENCES

(1) CASIDA, L. E. Present Status of the Repeat-Breeder Cow Problem. J. Dairy Sci., 44: 2323. 1961.

(2) DUNBAR, R. S., AND HENDERSON, C. R. Heritability of Fertility in Dairy Cattle. J. Dairy Sci., 36: 1063. 1953.

(3) INSKEEP, E. K., TYLER, W. J., AND CASIDA, L. E. Hereditary Variation in Conception Rate of Holstein-Friesian Cattle. J. Dairy Sci., 44: 1857. 1961.

(4) MARES, S. E., MENGE, A. C., TYLER, W. J., AND CASIDA, L. E. Genetic Factors Affecting Conception Rate and Early Pregnancy Loss in Holstein Cattle. J. Dairy Sci., 44: 96. 1961.

(5) ROTTENSTEN, K., AND TOUCHBERRY, R. W. Observations on the Degree of Expression of Estrus in Cattle. J. Dairy Sci., 40: 1457. 1957.

(6) SHANNON, P., AND SEARLE, S. R. Heritability and Repeatability of Conception Rate of Bulls in Artificial Breeding. J. Dairy Sci., 45: 86. 1962.

MOTILITY AND FERTILITY OF BULL SEMEN EXTENDED AT HIGH RATES IN YOLK EXTENDER CONTAINING CATALASE

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SUMMARY

Sperm survival in 20% yolk extender (CUE and CU-16) during a 12-day storage period was improved by catalase. The improvement was greater for semen extended to five million motile sperm per milliliter than for semen extended to ten million ($P < .01$), thus reducing the dilution effect on motility. However, fertility tests of semen used for insemination within a few days of collection showed no beneficial effect of catalase on fertility. In two trials the control CUE extender averaged 71.0 and 76.9% 60- to 90-day nonreturns compared with 71.5 and 74.6% nonreturns for CUE containing 20 μg of catalase per milliliter. When bull semen was extended to five million motile sperm vs. ten million motile sperm per milliliter of CUE extender, no difference in fertility was observed ($P > .05$). In two trials totaling 64,930 inseminations, the 60- to 90-day percentage of nonreturns for 5×10^6 motile sperm were 74.6 and 75.2, and for the control 10×10^6 motile sperm were 76.1 and 76.1. These results clearly indicate that the usefulness of genetically superior sires can be increased by reducing the number of sperm per insemination.

Numerous reports (2-5, 11, 12, 14-17, 20-22) have indicated that fertility of bull semen declines with increasing extension rates, particularly when the number of sperm inseminated appears to become critical. Bratton et al. (3) found that the 60- to 90-day nonreturns declined 4.2% when sperm numbers were reduced from ten million to five million motile sperm per milliliter. Willett (21) reported that the decline in fertility was accelerated when fewer than six million total sperm per milliliter were used, but also observed a decline when the number of sperm used was less than 12 million per milliliter. Recently, New Zealand workers (13) have reported only one percentage unit difference in fertility between 12.5 and 6.25 million sperm per insemination when the extension rate was kept constant and sperm numbers varied by using 1 ml vs. 0.5 ml of extended semen per insemination.

Catalase is known to prolong sperm survival (7, 19). Andersen and Rottensten (1) observed that the beneficial effect of catalase in yolk-citrate and CUE extenders was more pronounced at high extension rates. This effect also was noted in a preliminary report by Foote (6).

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The objectives of the experiments reported here were to establish the relationship between catalase and fertility of semen when different numbers of sperm were inseminated, and to more reliably establish the relationship between sperm numbers and fertility.

EXPERIMENTAL PROCEDURE

Semen for laboratory studies and for the field trials was obtained from bulls in the regular stud owned by the New York Artificial Breeders' Cooperative, Inc. The percentage of motile sperm, and sperm concentration per milliliter of freshly collected semen were used to calculate the extension rate needed to give the desired concentration of sperm per milliliter of extended semen. Semen was added to either CUE or CU-16 extender and processed as described previously by Foote et al. (8). The catalase preparation used has been described in a previous report (7).

Extended semen for sperm survival studies was stored at 5 C in 2.5-ml plastic tubes. Periodically, these tubes were mixed, subsampled, and the percentage of motile sperm determined microscopically at 430 $\times$ in a stage incubator set at 37 C. The pH's of the extenders were determined with a Beckman Model G pH meter.

Field Trial 1. This trial consisted of a simple comparison of CUE versus CUE containing catalase added at the rate of 20 μg/ml of extender. Semen from 40 bulls, representing five dairy breeds plus Angus, was used during two 2-wk periods in August and September, 1959. All semen was extended to ten million motile sperm per milliliter of extender at the time of collection. Semen was not shipped into the field on the day of collection, excepting on Sundays. Therefore, most of the inseminations were performed with semen stored one to six days following collection.

During each 2-wk period semen from all bulls was extended with each extender. Within each week the order of treatment was randomized to prevent any possible bias due to time trends. All technicians had equal opportunities to use each treatment, but none knew the identification of each treatment.

Field Trial 2. This trial, conducted in January and February, 1960, consisted of CUE extender with and without 20 μg/ml of added catalase, and five million versus ten million motile sperm per milliliter of extended semen. The four treatments were arranged factorially in a 2 × 2 design. Ejaculates of semen were split to compare the sperm numbers' treatments, and during a 2-wk period semen from each bull was extended with both of the extenders. Within each week the order of treatment was randomized to prevent any possible bias due to time trends. The whole experiment was replicated during a second 2-wk period with 12 Holstein bulls.

Field Trial 3. A comparison of five million vs. ten million motile sperm per milliliter of extended semen was made in this trial, replicated over a period of 6 wk in May, June, and July, 1961. A total of 23 Holstein and Angus bulls completed the experiment. Again, the treatments were randomized to days within weeks to prevent any bias due to time trends.

Statistical analyses of all results were carried out as outlined by Henderson (10) and Steel and Torrie (18). Bulls were considered to represent a random sample of bulls, but all experimental treatments, age of semen, and other sources of variability were considered to be fixed effects.

RESULTS

The results of the first field trial are shown in Table 1. The small over-all difference of 0.5 percentage units did not approach statistical significance ($P > .1$). The extender with added catalase seemed to give better fertility results with older semen than the control, but the interaction of age of semen × extender was not statistically significant ($P > .1$).

It was observed that some of the semen samples with high extension rates showed excellent motility in the catalase-containing extender. The results of an experiment designed to compare survival of sperm extended to five and ten million motile sperm per milliliter with different extenders containing catalase is shown in Table 2. The effects of catalase, sperm numbers, and the interaction of catalase × sperm numbers were highly significant statistically ($P < .01$). The interaction is particularly important because it appears that catalase can partly eliminate the so-called dilution effect on sperm motility observed by all workers in this field. The lower motility recorded for the lower concentration of sperm may, in part, be due to a bias in the method of estimation, since this effect was observed from the outset of the storage period.

TABLE 1

Fertility of semen extended with CUE and CUE plus catalase

	Fertility based on 60- to 90-day nonreturns				
	CUE		CUE + catalase [a]		
Age of semen in days	No. of first services	% non-returns	No. of first services	% non-returns	Difference
1	1,162	73.4	1,221	72.5	− 0.9
2	3,630	71.9	4,022	71.4	− 0.5
3	1,891	69.1	2,078	71.3	+ 2.2
4–6	230	60.4	294	70.4	+10.0
Total or mean	6,913	71.0	7,615	71.5	+ 0.5

[a] Assays for catalase indicated that about 95% of the initial activity was maintained until the end of the storage period in the extender containing added catalase. The amount naturally present in egg yolk was negligible by comparison.

TABLE 2

Effect of catalase and extension rate on survival of sperm during storage at 5 C (Average of 20 ejaculates)

	CUE				CU-16			
	No catalase		Catalase, 20 μg/ml		No catalase		Catalase, 20 μg/ml	
Days stored at 5 C	5×10^6 sperm	10×10^6 sperm	5×10^6 sperm	10×10^6 sperm	5×10^6 sperm	10×10^6 sperm	5×10^6 sperm	10×10^6 sperm
				(% motile sperm)				
1	61	64	61	64	58	62	57	62
4	52	57	54	56	58	62	59	61
8	36	50	42	52	42	54	52	57
12	21	42	38	48	21	45	43	56
Mean	42	53	49	55	45	56	53	59
pH at 12 days	7.0	7.0	7.0	7.0	6.8	6.8	6.8	6.8

TABLE 3

Fertility of semen extended to five million and ten million motile sperm per milliliter of CUE and CUE plus catalase

	Fertility based on 60- to 90-day nonreturns				
	Five million sperm/ml		Ten million sperm/ml		
Extender	No. of first services	% non-returns	No. of first services	% non-returns	Over-all mean
CUE	5,133	75.4	5,176	76.9	76.1
CUE + catalase	5,480	73.9	5,471	75.4	74.6
Total or mean	10,613	74.6	10,647	76.1	75.4

The good survival of sperm observed at high extension rates when catalase was present suggested that this combination should be fertility-tested. The results of a second field trial are shown in Table 3. The differences between extenders and between sperm concentration (number of sperm inseminated) were not significant statistically ($P > .05$). However, there was nothing to indicate any beneficial effects of catalase, and this finding was consistent with the over-all result obtained in the first field trial.

TABLE 4

Fertility of semen extended to five million and ten million motile sperm per ml of CUE

		Fertility based on 60- to 90-day nonreturns			
		Five million sperm/ml		Ten million sperm/ml	
Breed	Age [a] of semen in days	No. of first services	% non-returns	No. of first services	% non-returns
Holstein	1	16,198	75.3	13,046	76.7
	2	6,557	74.0	4,034	73.6
Angus	1	1,120	78.8	1,046	78.4
	2	862	76.7	807	76.8
Both breeds	1	17,318	75.6	14,092	76.8
	2	7,419	74.3	4,841	74.1
Total or mean		24,737	75.2	18,933	76.1

[a] Semen used the day of collection (Day 0) was not tabulated because it was not possible to distinguish this from three- or four-day-old semen from bulls collected twice weekly.

A third field trial designed to obtain enough inseminations to detect differences in nonreturns of 1% at $P_I = .05$ and $P_{II} = .20$ (18) was undertaken. The results of this trial are shown in Table 4. The per cent nonreturns for each ejaculate were used for the analysis of variance, since the 138 ejaculates in the experiment averaged over 300 inseminations per ejaculate. The average nonreturn percentages on this basis was 76.0 for each treatment, indicating no difference between treatments. Fertility results obtained with nine other bulls (excluded from the analysis because they did not complete all 6 wk) also were equal for each treatment. In this trial, more inseminations were obtained with the lower sperm concentration, because it was possible to meet field needs each day with this treatment, but it was not always possible with the other treatment. On the average, the number of motile sperm per ejaculate was sufficient to breed more than 2,000 cows, using 5×10^6 motile sperm per milliliter of extender and per insemination.

DISCUSSION

The consistent beneficial effects of catalase on survival of sperm over a 12-day period at 5 C in yolk extenders previously observed was confirmed. The effect of catalase was particularly pronounced when high extension rates were used. This finding is in agreement with a report by Andersen and Rottensten (1), and a preliminary report from this laboratory (6). This beneficial effect on motility was usually not observable until after four or more days of storage. The lack of any beneficial effect of catalase on fertility of bull semen may be because most of the semen is used for breeding before it has been kept in vitro for four days. The nonreturn rates for catalase-treated semen tended to be higher with older semen, but this effect was not significant ($P > .1$). The lack of response is in agreement with earlier work by VanDemark et al. (19) and a recent report by Hafs (9).

The fertility results with 5×10^6 motile sperm per milliliter of CUE extender were essentially equal to those obtained with 10×10^6 motile sperm per milliliter. This is in contrast to earlier work with yolk-citrate (3), which indicated that a decline of several percentage units in fertility was to be expected when motile sperm numbers were reduced to 5×10^6 per milliliter or per insemination.

Extension rates resulting in 5×10^6 and 10×10^6 values were based on the initial percentage of motile sperm in the sample. The actual number of motile sperm inseminated probably was less than this, because some of the sperm die during handling and storage, some of the inseminators do not fill the catheter with 1 ml of extender, and not all of the extended semen is expelled at the time of insemination. Studies were made of these variables. From these studies it was estimated that during the first four days after semen was collected an average of about 7.6, 6.9, 6.4, and 5.8 million motile sperm were deposited in the cow, when the initial extension was to ten million motile sperm per milliliter.

The results of these experiments indicate that bull semen extended to five million motile sperm per milliliter of CUE and stored at 5 C, will give essentially as high fertility results as will the inclusion of twice as many motile sperm. This method can be used to increase substantially the number of breedings to outstanding sires, and it is currently being applied successfully to some of the AB-proven Holstein sires in New York State.

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REFERENCES

(1) Andersen, H., and Rottensten, K. The Effect of Glucose, Fructose, Catalase and Peroxidase in the Extender on the Viability of Bull Spermatozoa. (In Danish.) Ann. Rept. Ster. Research Inst., Roy. Vet. Agr. Coll., Copenhagen. 33. 1961.

(2) Branton, C., Kellgren, H. C., and Patrick, T. E. The Importance of Numbers of Spermatozoa in Relation to Semen Quality and Fertility of Dairy Bulls. J. Dairy Sci., 36: 1301. 1953.

(3) Bratton, R. W., Foote, R. H., and Henderson, C. R. The Relationship Between Fertility and the Number of Spermatozoa Inseminated. J. Dairy Sci., 37: 1353. 1954.

(4) Bratton, R. W., Foote, R. H., Henderson, C. R., Musgrave, S. D., Dunbar, R. S., Jr., Dunn, H. O., and Beardsley, P. J. The Relative Usefulness of Combinations of Laboratory Tests for Predicting the Fertility of Bovine Semen. J. Dairy Sci., 39: 1542. 1956.

(5) Cheng, P., Casida, L. E., and Barrett, G. R. Effects of Dilution on Motility of

Bull Spermatozoa and the Relation Between Motility in High Dilution and Fertility. J. Animal Sci., 8: 81. 1949.

(6) FOOTE, R. H. Effect of Catalase in Semen Extenders on Fertility and on Sperm Survival at High Extension Rates. J. Dairy Sci., 44: 1180. 1961.

(7) FOOTE, R. H. Survival of Bull Sperm in Milk and Yolk Extenders with Added Catalase. J. Dairy Sci., 45: 907. 1962.

(8) FOOTE, R. H., GRAY, L. C., YOUNG, D. C., AND DUNN, H. O. Fertility of Bull Semen Stored Up to Four Days at 5° C in 20% Egg Yolk Extenders. J. Dairy Sci., 43: 1330. 1960.

(9) HAFS, H. D. Fertility of Bull Semen with Added Catalase. J. Dairy Sci., 44: 1529. 1961.

(10) HENDERSON, C. R. Design and Analysis of Animal Husbandry Experiments. In Techniques and Procedures in Animal Production Research. A.S.A.P. Bethesda, Md. 1959.

(11) MELROSE, D. R. Semen Dilution Rates in Relation to Handling Technique. Vet. Record, 64: 124. 1952.

(12) MILK MARKETING BOARD. The Extreme Breeding Potential of A.I. Sires Without the Use of Frozen Semen. Milk Mkt. Board Rept., 6: 86. 1955/56.

(13) NEW ZEALAND DAIRY BOARD. Research into Semen Diluents and Dose Rates. New Zealand Dairy Board 37th Ann. Rept. 76. 1961.

(14) OLDS, D., SEATH, D. M., CARPENTER, M. C., AND LUCAS, H. L. Interrelationships Between Site of Deposition, Dosage, and Number of Spermatozoa in Diluted Semen and Fertility of Dairy Cows Inseminated Artificially. J. Dairy Sci., 36: 1031. 1953.

(15) ROTTENSTEN, K., AND ANDERSEN, H. The Effect of Dilution Rate on the Fertility of Bull Semen. (In Danish.) Beretn. Forsøgslab., 289: 1. 1956.

(16) SALISBURY, G. W. Fertility of Bull Semen Diluted at 1:100. J. Dairy Sci., 29: 695. 1946.

(17) SALISBURY, G. W., AND BRATTON, R. W. Fertility Level of Bull Semen Diluted at 1:400 with and Without Sulfanilamide. J. Dairy Sci., 31: 817. 1948.

(18) STEEL, R. G. D., AND TORRIE, J. H. Principles and Procedures of Statistics. McGraw-Hill Book Co., New York. 1960.

(19) VANDEMARK, N. L., BRATTON, R. W., AND FOOTE, R. H. The Fertility of Bovine Semen in Citrate-Yolk Extenders Containing Added Catalase. J. Dairy Sci., 33: 661. 1950.

(20) WILLETT, E. L. Fertility and Livability of Bull Semen Diluted at Various Levels to 1:300. J. Dairy Sci., 33: 43. 1950.

(21) WILLETT, E. L. Decline in Fertility of Bull Semen with Increase in Storage Time as Influenced by Dilution Rate. J. Dairy Sci., 36: 1182. 1953.

(22) WILLETT, E. L., AND LARSON, G. L. Fertility of Bull Semen as Influenced by Dilution Level, Antibiotics, Spermatozoan Numbers and the Interaction of These Factors. J. Dairy Sci., 35: 899. 1952.

MOTILITY AND FERTILITY DURING POST-THAWING STORAGE OF BOVINE SPERMATOZOA FROZEN CONCENTRATED, THAWED, AND RE-EXTENDED [1]

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SUMMARY

Twenty ejaculates of semen from 17 dairy bulls were extended to contain 200×10^6 and 20×10^6 sperm per milliliter in yolk citrate and whole milk extenders and frozen with 7% glycerol. The semen frozen with 200×10^6 sperm per milliliter was re-extended, after thawing, to 20×10^6 sperm per milliliter in yolk citrate, whole milk, skimmilk, and CUE, and the motility of these semen samples was estimated during 72 hr of storage at 5 C.

Freezing semen with 200×10^6 sperm per milliliter resulted in significantly improved motilities ($P<.01$), particularly as the storage interval increased ($P<.01$). The four re-extension extenders differed in their ability to maintain motility ($P<.01$), particularly as the storage interval increased ($P<.01$). In general, sperm survived post-thawing storage much better when re-extended in extenders other than those in which the sperm were frozen.

The average 60- to 90-day nonreturn percentages for semen frozen with 200×10^6 sperm per milliliter and re-extended after thawing to 15×10^6 motile sperm per milliliter in yolk-citrate and CUE was 51.8 and 63.0%, respectively, as measured with a total of 2,062 inseminations made between 24 and 60 hr after thawing. The 63.0% for CUE did not significantly differ from the average of 66.5% for control frozen semen thawed immediately before 675 cows were inseminated ($P > .05$), but both of these values were significantly higher than the 51.8% for the semen re-extended in yolk citrate ($P < .05$).

The present use of frozen semen depends upon storage at −79 C or lower until it is used to breed cows immediately after thawing (3, 7). The maintenance of these very low temperatures for storing frozen semen and transporting it to the cow appreciably increases the cost of a frozen semen program. As an alternative, it would be desirable, at least in certain circumstances, to maintain satisfactory fertility of thawed semen during storage at 5 C for periods as long as two or three days.

Bratton et al. (2) suggested the possibility of freezing bovine semen concentrated and re-extending it after thawing. These workers obtained uniformly lower motility and fertility with frozen semen frozen concentrated and thawed from −79 C and stored at 5 C, than with semen from the same ejaculate used unfrozen or immediately after thawing from −79 C. Recently, Sevinc (10) obtained a decided improvement in the maintenance of motility during post-thawing storage at 5 C with sperm frozen concentrated and re-extended after thawing in extenders other than that in which the sperm were frozen.

This investigation was initiated to compare the effects of post-thawing storage on the motility and fertility of sperm frozen concentrated, thawed, and re-extended in extenders other than those in which the sperm were frozen.

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EXPERIMENTAL PROCEDURES

Laboratory equipment. Twenty semen samples were collected from 17 bulls and were extended to 400×10^6 sperm per milliliter at 38 C in a nonglycerol 20% yolk-citrate (YC) extender described by Foote and Dunn (4), and in a nonglycerol whole milk (WM) extender described by Almquist et al. (1). After this partially extended semen was slowly cooled to 5 C over 90 min, 1 ml of each of the two samples of partially extended semen from each ejaculate was further diluted to 40×10^6 sperm per milliliter in its respective diluter. Six hours after collection, the samples were glycerolated

to obtain a final concentration of 7% glycerol by the method of Foote and Dunn (4). One-milliliter aliquots of the glycerolated semen were dispensed into glass ampules and the ampules were hermetically sealed. Eighteen hours after glycerolation, the ampuled semen was frozen at the rate of 0.8 C per minute from 5 to −15 C, and about 5 C per minute from −15 to −79 C, by adding Dry Ice to an alcohol freezing bath. The ampules of frozen semen were stored at −79 C for 24 to 48 hr, then thawed in a 5 C water bath with constant agitation to prevent the formation of an ice capsule around the ampule.

Immediately after thawing, the contents of three replicate ampules of semen frozen with 20×10^6 sperm per milliliter were placed in each of two 5-ml test tubes. Semen frozen with 200×10^6 sperm per milliliter was re-extended 1:9 in YC, WM, skimmilk (SM), described by Almquist et al. (1), and Cornell University Extender (CUE) described by Foote et al. (5). Duplicate test tubes were prepared for each re-extension extender, so that each tube contained 3 ml of extended semen with 20×10^6 sperm per milliliter. Duplicate motility estimations were made from each tube after 0, 24, 48, and 72 hr of storage at 5 C, by a person unaware of the identity of the sample being examined.

The analysis of variance of the motility data considered the following sources of variation: concentration of sperm at freezing, freezing extender, re-extension extender, storage time, bull, duplicate tube, duplicate motility, and 19 appropriate interactions among these seven main effects. The concentration of sperm at freezing, freezing extender, re-extension extender, and storage time were considered to be fixed and factorial. Bulls were considered to be random and factorial and tubes to be random and nested within concentration of sperm at freezing, freezing extender, re-extension extender, and bulls. Motilities were considered to be random and completely nested.

Fertility experiment. Two ejaculates of semen from each of eight Holstein bulls in routine service at Michigan Artificial Breeders Cooperative were frozen in glycerolated YC with 200×10^6 sperm per milliliter. After 2 to 3 wk of storage at −196 C, these ejaculates were thawed and one was re-extended to 15×10^6 motile sperm per milliliter in YC, whereas the other was similarly re-extended in CUE. In other words, these two ejaculates from each of the eight bulls were treated comparably until they were re-extended after thawing. Both were shipped to nearly all of the breeding technicians as unfrozen semen. To be certain that any fertility difference between these two treatments was not solely due to the two re-extension extenders, a third and fourth ejaculate were extended to 15×10^6 motile sperm per milliliter in CUE and YC, respectively, and these were shipped to nearly all of the breeding technicians without freezing. The order of application of these four treatments to the four ejaculates from each bull was at random.

Each of the four ejaculates from each of the eight bulls was initially extended to 25 ml with YC at 38 C and allowed to cool to 5 C over about 90 min. The two ejaculates that were frozen were glycerolated and equilibrated similarly to those in the laboratory experiment. One and one-tenth-milliliter quantities of the extended semen were dispensed into 1.2-ml glass ampules and the ampules were hermetically sealed. The ampuled semen was frozen on ampule racks in an automatic liquid nitrogen freezer.[2] The rate of temperature change during freezing and thawing was similar to that in the laboratory experiment. The operational routine of the Cooperative dictated that inseminations be performed between 24 and 60 hr after the unfrozen semen was extended or after the concentrated frozen semen was re-extended.

Analysis of variance of the nonreturn data considered the following sources of variation: extender, freezing, age of semen, bull, and 11 appropriate interactions among these four main effects. The sources of variation due to extender, freezing, and age of semen were considered to be fixed and factorial. Bulls were considered to be random and factorial.

A fifth ejaculate from each of the eight bulls, which served as a control, was extended in YC, glycerolated to 7% glycerol, and frozen with 22.5×10^6 motile sperm per milliliter. This concentration of sperm was chosen, anticipating a 40% loss of motile sperm during freezing so as to obtain an average of at least 12×10^6 motile sperm per milliliter after thawing. This control semen was stored at −196 C for at least 2 wk, then shipped to the breeding technicians who maintained the −196 C temperature until the semen was thawed in 5 C water immediately before a cow was to be inseminated. Six breeding technicians performed all of these control inseminations over a 3-months' period.

Although, ideally, it would have been preferable to have measured fertility of this control frozen semen during post-thawing storage,

[2] Model No. 3005, available from Frozen Semen Products, Inc., R. D. 1, Breinigsville, Pennsylvania.

TABLE 1

Average per cent of motile sperm during post-thawing storage at 5 C

Freezing extender	Millions of sperm per milliliter at freezing	Re-extension extender	Hours stored after thawing				
			0	24	48	72	Avg
YC	200	YC	37	27	26	20	28
	200	CUE	44	45	43	41	43
	200	WM	40	33	33	29	34
	200	SM	43	43	42	39	42
		Avg	41	37	36	32	37
	20	None	32	18	16	14	20
WM	200	YC	27	22	19	18	22
	200	CUE	38	34	34	34	35
	200	WM	28	28	28	23	27
	200	SM	33	31	30	27	30
		Avg	32	29	28	26	29
	20	None	33	23	22	19	24
Avg	200	YC	32	25	23	19	25
	200	CUE	41	40	39	38	39
	200	WM	34	30	31	26	30
	200	SM	38	37	36	33	36
		Avg	36	33	32	29	33
	20	None	33	21	19	17	22

as was done for the other four treatments, this could not be justified, because of the extremely low fertility to be expected from such a treatment (7). Because of this limitation, the average fertility of this control treatment was compared with the over-all means for the four other treatments in a Duncan's Multiple Range Test.

RESULTS

Laboratory experiment. The average motilities of the sperm in each of the ten extender treatments during 72 hr of post-thawing storage at 5 C are shown in Table 1, and the results of the analysis of variance of these data are presented in Table 2.

Freezing semen with 200×10^6 sperm per milliliter resulted in an average of 33% motility, a considerable improvement upon the average of 22% for the semen frozen with 20×10^6 sperm per milliliter ($P < .01$). It is apparent that, although freezing semen with 200×10^6 sperm per milliliter of WM resulted in a considerable improvement, the comparable improvement for the YC extender was much more pronounced ($P < .01$), and this statement holds for each storage time, since the CFS interaction was not significant ($P > .50$). The CUE and SM re-extension extenders resulted in averages of 39 and 36%, respectively, markedly higher than the 30 and 25% for WM and YC, respectively ($P < .01$).

TABLE 2

Summary of the important sources of variation in the analysis of variance of the motility of sperm during post-thawing storage

Source of variation	Degrees of freedom	Mean square	F ratio	Probability (P_1)
Concentration (C)	1	538.0	25.87	$P < .01$
Freezing extender (F)	1	268.0	17.70	$P < .01$
Re-extension extender (R)	3	266.0	24.86	$P < .01$
Storage time (S)	3	120.0	23.08	$P < .01$
Interaction (CF)	1	213.0	32.27	$P < .01$
(CS)	3	22.3	13.12	$P < .01$
(FR)	3	8.0	1.23	$P \cong .32$
(FS)	3	4.6	3.29	$P \cong .03$
(RS)	9	7.1	6.36	$P < .01$
(CFS)	3	1.3	0.50	$P > .50$
(FRS)	9	3.7	0.97	$P \cong .47$
Bulls (B)	19	71.8	82.53	$P < .01$

The YC freezing extender resulted in an average of 33% motility, significantly higher than the 27% for WM ($P < .01$), but the sperm frozen in WM declined eight percentage units during storage, significantly less than the 12 percentage units for YC ($P \cong .03$). As expected, post-thawing motilities declined appreciably during storage ($P < .01$). However, an unexpected interaction between concentration of sperm at freezing and storage times resulted. This interaction is illustrated by the fact that semen frozen with 200×10^6 sperm per milliliter declined only seven percentage units during post-thawing storage, much less than the decline of 16 percentage units for semen frozen with 20×10^6 sperm per milliliter ($P < .01$).

The re-extension extenders significantly affected post-thawing longevity ($P < .01$), with CUE, SM, WM, and YC resulting in average motility declines of three, five, eight, and 13 percentage units, respectively. This fact appears of prime importance in a consideration of improvements in post-thawing survival of sperm. Whether sperm were frozen in YC or in WM seemed to have little or no effect upon the manner in which the re-extension extenders affected motilities ($P \cong .32$), and this was true for each storage time ($P \cong .47$). Most of the interactions between bulls and treatments were significant, but since these sources of variation are of secondary importance in the interpretation of the above results, they are omitted from Table 2.

Field experiment. The total number of cows bred with sperm frozen concentrated, thawed, and re-extended in either CUE or YC, and the total number of cows bred with unfrozen semen in the same two extenders, along with their average 30- to 60-day and 60- to 90-day nonreturn percentages, are presented in Table 3. In this table, the first and second days of storage refer to cows bred with semen 24 to 36 hr and 48 to 60 hr, respectively, after the semen was collected or re-extended.

Cows bred with sperm extended in CUE had an average 60- to 90-day nonreturn rate of 69.9%, considerably higher than the 63.1% for cows bred with sperm in YC ($P \cong .17$). As expected, the fertility declined measurably during storage ($P < .01$). Although, on the average, the two extenders maintained fertility equally well during storage ($P > .50$), the 60- to 90-day nonreturn rate for semen frozen with 200×10^6 sperm per milliliter, thawed, and re-extended in CUE did not appreciably decline from the first to the second day of storage, whereas the comparable decline for YC was 10.7 percentage units ($P \cong .12$). The 60- to 90-day fertility of unfrozen sperm declined an average of 2.8 percentage units, considerably less than the 5.8 percentage unit decline for frozen sperm ($P \cong .17$). However, this interaction effect was largely restricted to the YC extender ($P \cong .12$).

The control semen frozen at 22.5×10^6 motile sperm per milliliter, and used to breed 675 cows immediately after thawing, resulted in a 66.5% 60- to 90-day nonreturn rate. The Duncan's Multiple Range Test showed no significant difference between this 66.5% average for the control frozen semen and the 63.0% over-all average for the concentrated frozen

TABLE 3

The average[a] nonreturn percentages for unfrozen semen and for sperm frozen concentrated, thawed, and re-extended

		CUE			YC		
	Day of storage	No. of services	% nonreturn 30-60 d.	% nonreturn 60-90 d.	No. of services	% nonreturn 30-60 d.	% nonreturn 60-90 d.
Unfrozen	1	1,073	83.0	78.7	905	81.7	75.4
	2	1,058	80.4	74.9	827	79.4	73.4
	Total	2,131			1,732		
	Avg		81.7	76.8		80.5	74.4
Frozen	1	498	68.5	63.5	498	66.2	57.2
	2	550	69.7	62.6	516	54.7	46.5
	Total	1,048			1,014		
	Avg		69.1	63.0		60.4	51.8
Totals or avg	1	1,571	75.8	71.1	1,403	74.0	66.3
	2	1,608	75.0	68.8	1,343	67.0	60.0
	Total	3,179			2,746		
	Avg		75.4	69.9		70.4	63.1

[a] Unweighted averages.

semen re-extended in CUE and used to breed cows up to 60 hr after thawing ($P > .05$). However, the 66.5% average for control frozen semen was significantly better than the over-all average of 51.8% for the concentrated frozen semen re-extended in YC and used to breed cows up to 60 hr after thawing ($P < .05$).

TABLE 4

Average decline from 30- to 60-day to 60- to 90-day nonreturn percentages for unfrozen sperm and sperm frozen concentrated

	Day of storage	Extender	
		CUE	YC
Unfrozen	1	4.3	6.3
	2	5.5	6.0
	Avg	4.9	6.2
Frozen concentrated	1	5.0	9.0
	2	7.1	8.2
	Avg	6.1	8.6

Average values for embryonic mortality, as measured by decline in fertility from 30- to 60-day to 60- to 90-day nonreturn rates (9), are tabulated in Table 4 for unfrozen semen and for semen frozen concentrated, thawed, and re-extended in YC and CUE, respectively. The average decline for frozen semen was 7.4 percentage units, somewhat higher than the average of 5.5 percentage units for unfrozen semen, suggesting the possibility that freezing sperm increases the embryonic mortality rate over and above that normally expected with unfrozen semen ($P \cong .20$).

DISCUSSION

Results of the experiments described above indicate that the semen frozen with 200×10^6 sperm per milliliter survived post-thawing storage much better when re-extended after thawing in extenders other than those in which the sperm were frozen. Although most commercial artificial insemination centers use a YC freezing extender (3), it is apparently inferior in terms of preserving motility and fertility during post-thawing storage at 5 C. CUE, on the other hand, is inferior from the standpoint of preserving sperm during the freezing process (Kirton and Hafs, unpublished data), whereas it is superior when used to extend unfrozen semen (5, 6). The combination of freezing sperm concentrated in YC, an extender which provides protection during the freezing process, and subsequently re-extending them in CUE, an extender which promotes survival during post-thawing storage, appears to prevent the expected drop in fertility (2, 7) normally observed during post-thawing storage of frozen semen.

Due to the small number of observations on sperm motility during storage for the fertility experiment, tests of significance were not performed for these data. Nevertheless, motility estimates were made for each extended semen sample used in the fertility experiment at storage intervals similar to those in the laboratory experiment. There were three comparable treatments in the two experiments. In the field experiment, the control frozen semen averaged 21% motility and the concentrated frozen semen re-extended in YC and CUE averaged 29 and 40% motility, respectively. The comparable values from the laboratory experiment were 20, 28, and 43%, respectively. Such repeatability permits additional confidence in the results of these experiments.

A report by Kinney and VanDemark (8) indicates that a greater percentage of spermatozoa survives freezing in the second than in the first ejaculate when they are collected a few minutes apart. Since the sperm concentration of second ejaculates is usually less than that of first ejaculates, the concentration of plasma constituents during freezing would be greater in second ejaculates. Semen frozen with 200×10^6 sperm per milliliter also has an elevated concentration of seminal plasma and, as shown in the experiments reported above, the motility of sperm treated in this manner is considerably higher than when the semen is frozen with 20×10^6 sperm per milliliter. These facts indicate that some factor(s) in seminal plasma may play an important role in protecting sperm during freezing.

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REFERENCES

(1) ALMQUIST, J. O., FLIPSE, R. J., AND THACKER, D. L. Diluters for Bovine Semen. IV. Fertility of Bovine Spermatozoa in Heated Homogenized Milk and Skimmilk. J. Dairy Sci., 37: 1303. 1954.

(2) BRATTON, R. W., FOOTE, R. H., AND CRUTHERS, J. C. Preliminary Fertility Results with Frozen Bovine Spermatozoa. J. Dairy Sci., 38: 40. 1955.

(3) DOMBROSKE, F. Report of Semen Preservation Committee, Proc. 11th Ann. Conv. Natl. Assoc. Artificial Breeders, p. 91. 1958.

(4) FOOTE, R. H., AND DUNN, H. O. Buffers, Extenders, and Methods for Freezing Semen. Routine Laboratory Procedure No. 11,

Lab. Animal Breeding, Dept. of Animal Husbandry, Cornell University. 1954.

(5) FOOTE, R. H., GRAY, L. C., YOUNG, D. C., AND DUNN, H. O. Fertility of Bull Semen Stored up to Four Days at 5° C. in 20% Egg Yolk Extenders. J. Dairy Sci., 43: 1330. 1960.

(6) HAFS, H. D. Fertility of Bull Sperm with Added Catalase. J. Dairy Sci., 44: 1529. 1961.

(7) HURST, V., AND KELLY, J. W. Results from Breeding Cows with Frozen Semen Thawed for Varying Lengths of Time. J. Dairy Sci., 41: 736. 1958.

(8) KINNEY, W. C., AND VANDEMARK, N. L. The Effect of Yolk and Citrate Levels and Stage of Maturity on the Survival of Bull Sperm at Subzero Storage Temperatures. J. Dairy Sci., 37: 650. 1954.

(9) SALISBURY, G. W., BRATTON, R. W., AND FOOTE, R. H. The Bull as One Cause of Delayed Returns to Service in Artificial Breeding. J. Dairy Sci., 35: 250. 1952.

(10) SEVINC, M. A. Preservation of Motility in Bull Sperm After Thawing from −79° C. Michigan Agr. Expt. Sta., Quart. Bull., 43: 576. 1961.

STUDY OF SUCCESSIVE EXPERIMENTAL LACTATIONS IN DAIRY COWS [1]

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SUMMARY

Regrowth of the mammary gland with daily injections of 200 mg progesterone and 200 μg estradiol benzoate for periods of 120 or 180 days followed by injections of 0.3 mg of estradiol benzoate per 100 lb body weight for 14 days to initiate lactation was attempted in 19 lactating cows.

In no case did the milk yield following the second attempt approach the level of the first experimental lactation. The mean maximum average daily milk yield of the 19 cows following the second treatment was only 22.7% of that obtained after the first hormone treatment.

The 14-day estrogen injection at the end of the 120- or 180-day estrogen-progesterone treatment stimulated a slight increase in milk yield in six of the 11 lactating cows ranging from 0.4 to 4.2 lb per day, whereas six of the eight cows that were dry the last 30 to 60 days produced from 0.3 to 8.5 lb per day, with a mean maximum daily milk yield of 3.2 lb per day for the group.

Daily injections of decreasing amounts of progesterone (150 to 50 mg) and increasing estradiol benzoate (1 to 3 mg) were administered to 11 dry and seven lactating cows for 63 days, which had failed to show increased production after the 120-day constant level P-EB treatment. Following the 14-day estrogen treatment to initiate milk secretion this group reached a mean maximum average daily milk yield of 17.8 lb per day, which was 79.5% of the mean maximum daily production of these animals in the first experimental lactation. The 11 cows which were dry during treatment were stimulated to produce a maximum average of 16.3 lb per day, ranging from 8.6 to 31.4 lb following the hormone administration. The lactating animals reached a maximum daily milk yield of 20.3, which represented an average increase of 15.9 lb, with a range of 0.0 to 29.4 lb per day.

The previous data presented on experimental growth of the mammary gland in cattle from the Missouri Station has been concerned primarily with nulliparous heifers (6, 8). In general, successive hormonal induced lactations have not been as successful as the first attempt to grow the udder experimentally. Hill (3) and Dzuik et al. (1) report their second attempt on a limited number of animals as unsuccessful.

In a previous study one lactating and three multiparous dry cows were injected daily with 100 mg progesterone and 100 μg estradiol for six months, followed by 6 mg of estradiol benzoate per day for ten days to initiate milk secretion. Only the lactating cow failed to respond to the hormone treatment, whereas the others produced a maximum daily milk yield of 19 to 29 lb per day (7).

Turner (6) reported on four cows in which lactation was induced after one or more previous lactations. Two failed to secrete appreciable amounts of milk, one produced about the same amount of milk at maximum as she did during her first experimental lactation, but much less than she had produced following a normal pregnancy, whereas the fourth animal produced about one-half as much as her previous normal lactation following parturition.

To provide further data concerning growth of the udder experimentally during lactation and the need of a dry period to grow the mammary gland for the next lactation, part of the animals were continued in milk throughout the hormone treatment while the remaining animals were turned dry the last 30 to 60 days of the treatment period.

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MATERIALS AND METHODS

A total of 19 animals were included in this study. Fourteen heifers which were induced to lactate experimentally were in their 180th to 188th day of lactation, when daily injections of 200 mg of progesterone and 200 μg of estradiol benzoate were initiated. After 120 days' treatment, 0.3 mg estradiol benzoate per 100 lb body weight was injected daily for 14 days in an attempt to initiate an increase in milk yield. Seven of these animals were turned dry the last 30 days of injection period. The remaining heifers were milked throughout the experiment without a dry period.

A Guernsey cow, no. 38, was administered progesterone and estrogen for 365 days starting with Day 78 of the induced lactation and was dry the last 60 days of the treatment period.

Two Guernseys, no. 41 (induced to lactate experimentally), and no. 440 (a cow which, after normal parturition, had continued to lactate over 8 yr), and two Jerseys, no. 348 (following a normal pregnancy) and no. 89 (lactating following abortion of a mummified fetus at approximately 200 days) were treated with 200 mg progesterone and 200 μg of estradiol benzoate for 180 days. Daily injections of 0.3 mg of estradiol benzoate per 100 lb body weight were then administered for 14 days in an attempt to increase milk yield.

RESULTS

The injection of levels of progesterone and estradiol benzoate (200 mg P. and 200 μg E.B.) followed by 0.3 mg E.B. per 100 lb body weight for 14 days, which were uniformly successful in heifers and dry cows in the first attempt, failed to stimulate a second experimental lactation in all cows lactating during the treatment. Animals in this experiment which were dry during the last 30 or 60 days of the treatment period either failed to milk or produced at a very low level following the second hormone treatment (Table 1). The mean maximum average daily milk yield of the 19 cows following second treatment was only 22.7% of that obtained in the first experimental lactation. Following the 14-day estrogen treatment, five of the 11 lactating cows continued to decline in production, whereas six showed an increase ranging from 0.4 to 4.2 lb per day. The mean increase in average daily milk yield above the production level at the time of estrogen stimulation was only 0.25 lb per day. The mean

TABLE 1

Comparative maximum daily yield stimulated in the first and second hormone treatment period

Breed and herd no.	Maximum avg daily milk yield—first experimental lactation	Avg daily milk yield at time of estrogen stimulation	Maximum avg daily milk yield—second experimental lactation	No. days treated with 200 mg progesterone 200 μg estradiol benzoate
	(lb)	*(lb)*	*(lb)*	
Group I—Cows lactating throughout second experimental period				
H 870	30.8	4.7	0.2	120
H 872	28.8	0.1	0.5	120
H 873	26.5	4.2	1.4	120
J 594	20.1	0.7	4.6	120
G 478	23.0	3.4	1.1	120
BS 25	32.0	7.2	0.5	120
BS 27	17.7	7.2	9.6	120
G 41	30.0	24.6	28.8	180
G 440	43.2	13.2	17.2	180
J 348	27.4	25.6	22.0	180
J 89	30.1	5.1	8.3	180
Group II—Cows dry at the end of second experimental period				
H 863	30.2	Dry 30 days	7.0	120
H 868	30.9	Dry 30 days	8.5	120
H 869	25.6	Dry 30 days	0.0	120
J 630	24.0	Dry 30 days	3.3	120
J 631	26.4	Dry 30 days	1.5	120
BS 24	26.8	Dry 30 days	0.0	120
BS 26	24.1	Dry 30 days	0.3	120
G 38	29.8	Dry 60 days	4.7	365

maximum daily milk yield for the group of eight cows that were turned dry at the end of the second experimental period reached a maximum of 3.2 lb per day, with a range of 0.0 to 8.5 lb per day following the estrogen treatment to initiate a second lactation.

EXPERIMENT II

The failure to stimulate milk secretion by the second course of ovarian hormones even equal to that stimulated by the first course suggested that the hormone required for lactation was not stimulated to increased secretion by the treatment or that the cells were not properly conditioned for response.

In late pregnancy it has been shown that estrogen secretion increases to a relatively high level in the cow (2), whereas progesterone shows a tendency to decline (4, 6). It was thought that this condition might be simulated by a more gradual transition from the udder growth stimulating phase to the lactation stimulating phase. It was decided to try this transition program during a 63-day period. In this treatment, the progesterone was gradually reduced and the estrogen gradually increased.

MATERIALS AND METHODS

The animals used in the present experiment were essentially the same as those used in the previous experiment. Part of the animals, Group I, were turned dry, whereas Group II were milked regularly, although they were producing at a low level.

Group I contained 11 animals, seven from the previous experiment plus a J × H crossbred heifer, no. 601, previously induced to lactate experimentally, a Jersey heifer, no. 635, two Holstein heifers, no. 263 and 265, which produced only a few pounds per day following a 60-day progesterone and estradiol benzoate treatment. Group II included seven animals from the previous experiment that were continued in milk during the treatment.

The hormone treatment consisted of 1 mg estradiol benzoate (E.B.) and 150 mg progesterone (P.) for 21 days, 2 mg E.B. and 100 mg P. for 21 days, and 3 mg E.B. and 50 mg P. for 21 days. The cows were then injected with 0.3 mg E.B./100 lb body weight for 14 days to initiate lactation. In the dry cows, milking was started when the final E.B. treatment was begun.

RESULTS

A steady increase in daily milk yield was obtained in each of the 11 dry cows of Group I following the injections of 0.3 mg E.B./100 lb body weight daily for 14 days. The maximum average daily milk yield of 16.2 lb per day for the group was reached in the seventh week (Figure 1), whereas individual maximum yield ranged from 8.6 to 31.4 lb per day which was reached in 5 to 12 wk (Table 2).

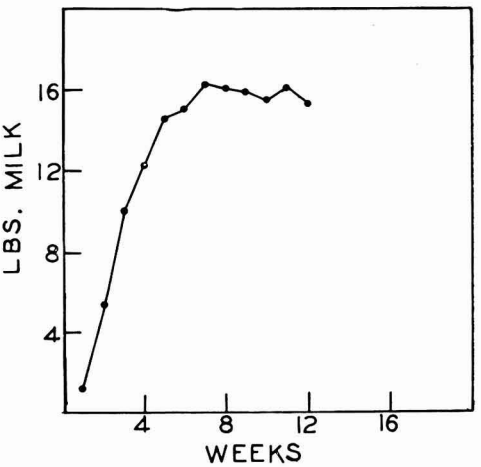

FIG. 1. The mean daily milk yield (based on a weekly average) for the 11 dry cows treated with decreasing progesterone and increasing levels of estradiol benzoate for 63 days.

The average daily milk yield of the animals of Group II ranged from 0.3 to 8.5 lb per day, with an average of 4.5 at the beginning of decreasing progesterone (150 to 50 mg) and increasing estradiol benzoate (1 to 3 mg) treatment. Six of the seven animals showed an increase in milk yield during the last week of the second treatment period (50 mg P. and 3 mg E.B.). The average daily milk yield for the group was 12.2 lb per day at the start of the 14-day E.B. injection. The lactating group showed an increase of 0.0 to 28.9 lb, with a mean average maximum yield of 15.9 lb per day within 6 wk after the daily injections were discontinued (Figure 2).

The mean maximum average daily milk yield of the 18 cows treated with decreasing levels of progesterone and increasing levels of estradiol benzoate following the 14-day estrogen treatment was 79.5% of the mean obtained in the first experimental lactation for these animals (Table 2).

DISCUSSION

Over 30 nulliparous dairy heifers have now been administered estrogen (estradiol benzoate and diethylstilbestrol) and progesterone for 180 and 120 days to stimulate the growth of the udder followed by estradiol benzoate to

stimulate the lactogenic hormone and milk secretion. The maximum milk yield attained in these animals has varied from 13.1 to 33.3, with a mean of 23.8 lb per day, but the best estimates of their productivity indicate that the experimental treatment produced 80 to 90% of that which could have been expected of these heifers after normal pregnancy.

It is believed that levels of estrogen (100 μg estradiol benzoate by injection or 10 mg diethylstilbestrol orally per day) plus 100 mg progesterone injected daily are as effective in stimulating extensive lobule-alveolar development as higher dosage levels of these hormones. Following the growth phase estradiol benzoate will stimulate the secretion of lactogenic hormone and milk secretion.

The failure of this treatment to stimulate a successive lactation comparable to that which might be expected to occur during a second pregnancy requires analysis.

In a normal pregnancy it has been shown that the progesterone levels of the blood (5), ovarian and placental tissue (4) reach a peak during the first trimester and remain near that level until 30 to 65 days before calving, then decline to a low level near parturition. However, estrogen secretion is low in early pregnancy, increasing throughout the latter half of gestation to a relatively high level at parturition (3).

Eleven dry and seven lactating cows were treated with decreasing levels of progesterone (150 to 50 mg) and increasing levels of estradiol benzoate (1 to 3 mg) for 63 days, followed by the 14-day estrogen treatment administered to initiate lactation. Seventeen of the 18 animals secreted greater yield than when treated with constant levels of hormones. The mean daily milk yield (based on a weekly average) of the group that was dry during treatment was 16.3 lb, which ranged from 8.6 to 31.4 lb in 6 to 7 wk, whereas the lactating group showed a maximum average daily increase of 15.9 lb in 6 wk, with a range of 0.0 to 28.9 lb per day. The mean maximum average daily milk yield

TABLE 2

Comparative maximum daily yield stimulated by constant and altered levels of hormones

Breed and herd no.	Maximum avg daily milk yield first experimental lactation	Maximum avg daily milk yield second experimental lactation	Maximum avg daily milk yield after increasing E.B. and decreasing P. treatment	Per cent of first experimental lactation obtained following the increasing E.B. and decreasing P. treatment
	(lb)	*(lb)*	*(lb)*	
Group I. Cows dry during increasing E.B. and decreasing P. treatment				
H 869	25.6	0.0	18.2	71.1
H 870	30.8	0.5	31.4	101.9
H 873	26.5	4.2	19.1	72.1
J 630	24.0	3.3	16.8	70.0
G 478	23.0	1.1	8.7	37.8
BS 24	26.8	0.0	14.0	52.2
BS 25	32.0	0.5	13.9	43.4
H 263	3.3		15.3	463.6
H 265	3.6		16.8	466.7
J 635	6.2		8.6	138.7
J × H 601	23.0		15.3	66.5
Group II. Cows lactating during increasing E.B. and decreasing P. treatment				
H 863	30.2	7.0	21.5	71.2
H 868	30.9	8.5	28.3	91.6
H 872	28.8	0.5	29.4	102.1
J 594	20.1	4.6	21.1	105.0
J 631	26.4	1.5	19.5	73.9
BS 26	24.1	0.3	14.0	58.1
BS 27	17.7	9.6	8.4	47.5
Mean =	22.4	3.0	17.8	79.5

E.B. = Estradiol benzoate.
P. = Progesterone.

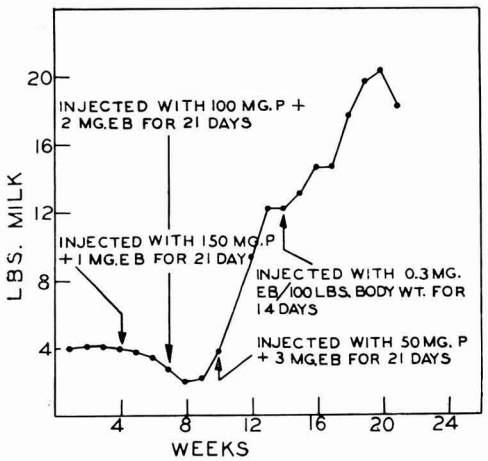

FIG. 2. The mean daily milk yield (based on a weekly average) for the seven lactating cows receiving altered levels of progesterone and estradiol benzoate.

obtained by this treatment was 79.5% of the mean observed in the first experimental lactation for these animals. The three heifers induced to lactate following the 60-day estrogen-progesterone administration showed the greatest response to the altered level of ovarian hormone injections, producing from 138.7 to 466.7% of their first maximum average daily milk yield, whereas the 15 animals treated for 120 or 180 days in the first trial produced 37.8 to 105.0% of their first maximum yield. While this improved response over the constant level of ovarian hormone treatment is encouraging, it should be noted that these heifers were approximately 1 yr older and should have been expected to produce 12 to 15% more milk at this time. Therefore, milk yields of 112 to 115% of the first experimental treatment would have been considered an equal response due to the hormone treatment. Since considerable success was obtained with the altered levels of the ovarian hormones in these 63-day trials, as compared with the failure in both dry and lactating cows with the constant level of hormones previously used, it is believed that the altered levels and ratios of estrogen and progesterone used in this experiment may be a more physiological method of stimulating successive experimental lactation.

REFERENCES

(1) DZIUK, P. J., KOSHI, J. H., AND PETERSEN, W. E. Alternate Administration of Progesterone and Diethylstilbestrol in Hormonal Induced Lactation. J. Animal Sci., 14: 1180. 1955.

(2) EL-ATTAR, T., AND TURNER, C. W. Spectrophotofluorometric Determination of Estrogens in the Urine and Feces of Cows During Different Stages of Pregnancy. Missouri Agr. Expt. Sta., Research Bull. 64. 1957.

(3) HILL, D. L. Experimental Development of the Mammary Gland of the Bovine. J. Dairy Sci., 35: 504. 1952.

(4) RAKES, J. M., HERN, W. R., AND MELAMPY, R. M. A Method of Determination of Progesterone in Tissue and Body Fluids. Iowa State College J. Sci., 33: 13. 1958.

(5) SHORT, R. V. Progesterone in Blood. II. Progesterone in the Peripheral Blood of Pregnant Cows. J. Endocrinology, 16: 426. 1958.

(6) TURNER, C. W. The Experimental Induction of Growth of the Cow's Udder and the Initiation of Milk Secretion. Missouri Agr. Expt. Sta., Research Bull. 697. 1959.

(7) WILLIAMS, R., CHILDS, O. A., SMITH, D., AND TURNER, C. W. The Effects of the Hormones Progesterone and Estrogen in Initiating Lactation in Dairy Cows. J. Dairy Sci., 38: 609. 1955.

(8) WILLIAMS, R., AND TURNER, C. W. Effects of Increased Levels of Ovarian Hormones and Duration of Treatment on the Experimental Induction of Growth of the Cow's Udder. J. Dairy Sci., 44: 524. 1961.

RATE OF ABSORPTION OF L-THYROXINE I^{131} FROM THE GASTROINTESTINAL TRACTS OF DAIRY CATTLE [1, 2]

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SUMMARY

Radiothyroxine was administered orally to seven dairy cattle and the rate of absorption was measured by following the radioactivity of plasma samples. These samples were taken at intervals up to 96 hr after administration. The half-time ($t\frac{1}{2}$) which can be defined as the time required for a substance to double in concentration or to decline one-half, was calculated from the radioactivity of the plasma. The absorption of thyroxine I^{131} from the gastrointestinal tract followed two phases, a rapid absorption phase with a $t\frac{1}{2}$ of 2.65 hr and a much slower phase with a $t\frac{1}{2}$ of 20.1 hr. After this period, a declining phase with a $t\frac{1}{2}$ of 160.1 hr was observed as metabolism and excretion predominated. The recycling of I^{131} from metabolized thyroxine I^{131} was blocked by daily administration of a potent goitrogen, tapazole.

Investigation of the rate of absorption of orally administered thyroidal materials in cattle appear to be limited to the study of Mixner and Lennon (2). These workers used the increase in PBI as an index of absorption. After thyroxine administration, the first blood samples were taken after 10 hr and an increase in PBI was observed. Maximum levels were found 30 to 40 hr after administration, followed by an exponential decline.

These observations, as well as the work of Premachandra and Turner (6) on the kinetics of subcutaneous absorption of thyroxine I^{131}, prompted an investigation of the rate of absorption, particularly during the early phase of orally administered L-thyroxine I^{131}. The present paper describes such observations.

EXPERIMENTAL PROCEDURE

Six nonlactating heifers of the Holstein, Jersey, and Guernsey breeds and one lactating Guernsey cow were used in these trials. All animals were confined in a dry lot and fed alfalfa hay and a ground dairy ration. Access to an iodized salt block was allowed at all times.

Three hundred microcuries of I^{131} tagged thyroxine were placed in gelatin capsules filled with filter paper and administered by a balling gun to each animal. Blood samples were taken from the jugular vein at intervals up to 96 hr after administration. The plasma was separated by centrifugation and its radioactivity was measured in a National Radiac Scintillation Well Counter (Model 5A-2D).

Four grams of tapazole per 1,000 lb body weight was given by gelatin capsule one day prior to the administration of L-thyroxine I^{131} and each day during blood sampling. Tapazole (5) prevents the recycling of iodine from metabolized thyroxine.

The per cent absorbed thyroxine I^{131} was calculated from the radioactivity of the plasma, using an estimated value of 4% of body weight as plasma [Turner and Herman, (7)].

The half-time for increase and subsequent decline in radioactivity ($t\frac{1}{2}$) in plasma was calculated by the equation $\frac{ln^2}{m}$ (1) when ln is the natural logarithm and m is the slope of the regression line (i.e., per cent dose in total plasma against time).

RESULTS

Radioactivity could be detected in the plasma 3 hr after administration of thyroxine I^{131}, when the mean per cent absorbed was 0.34. The maximum was reached after 44 to 45 hr, when the radioactivity of the plasma ranged from 5.6 to 14.9% of injected dose, with a mean of 10.8 ± 1.67.

The time course of absorption of thyroxine I^{131} into the blood followed two phases, a rapid initial phase and a second more prolonged phase

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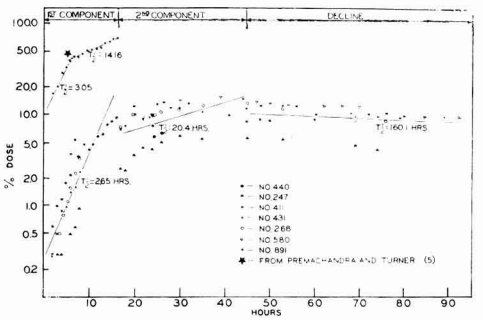

FIG. 1. The time course of absorption of thyroxine I^{131} from the gastrointestinal tract of dairy cattle.

(Figure 1). The initial phase of absorption had a half-time ($t_{1/2}$) of 2.65 hr and lasted approximately 16 hr. This was followed by a second phase with a $t_{1/2}$ of 20.4 hr and lasted until 45 hr after administration. At this time, the radioactivity of the blood began to decline with a $t_{1/2}$ of 160.1 hr. Measurement of thyroidal I^{131} by the method of Pipes and Turner (3) confirmed that I^{131} was not taken up by the thyroid and that the goitrogen blockage was complete.

DISCUSSION

Absorption of orally administered thyroxine I^{131} was slightly more rapid during the initial phase but more prolonged during the second phase than that found by Premachandra and Turner (6) following subcutaneous injection (Figure 1). However, the amount absorbed into the blood was far less than that observed from subcutaneous injection (Figure 1). Since the $t_{1/2}$ of intravenous injected thyroxine [Pipes et al. (4)] is of the order of 59 hr, daily oral administration would result in a much higher blood level of thyroxine than that observed.

The slow apparent rate of absorption during the second phase was not complicated by recycling of I^{131} from metabolized thyroxine I^{131}, since tapazole was administered in sufficient amounts to prevent the uptake of I^{131} by the thyroid. The second slower phase of absorption was undoubtedly influenced by the simultaneous absorption and metabolism of thyroxine I^{131}, as well as subsequent excretion of I^{131} in the urine. During the declining phase (Figure 1), metabolism and excretion overshadowed absorption, but the $t_{1/2}$ of 160.1 hr was far greater than the $t_{1/2}$ of 59.3 hr found by Pipes et al. (4), after intravenous injection of thyroxine I^{131} into the dairy cow. This indicates that absorption was still taking place.

Preliminary fractionation studies indicate the excretion of thyroxine I^{131} in large amounts in the feces during the second and third phase.

The possibility exists that the radioactivity found in the blood after thyroxine I^{131} administration was largely inorganic iodine, but the radioactivity was not rapidly eliminated by the kidney, as it would have been if such had been the case.

On the other hand, the blood from the rumen and intestines must pass first through the portal vein to the liver, where a certain degree of deiodination may take place before it reaches the general circulation. Chemical fractionation of the plasma indicates that 80% of the radioactivity of plasma behaves as thyroxine I^{131} (i.e., is protein-bound).

In view of the rapid absorption of orally administered thyroxine I^{131}, it is strongly suggested that a certain amount of absorption takes place in the rumen. While amino acids are not generally believed to be absorbed from the rumen, thyroxine may prove to be unique in this respect. To support this view, limited data were obtained in fistulated, rumen-ligatured sheep. Significant amounts of radioactivity in the blood were observed under these conditions.

The mean absorption at the peak (Figure 1) of 10.8% confirms the studies of Mixner and Lennon (2), who found a mean absorption efficiency of 11.6% for orally administered thyroxine.

It should be considered that this study was made in the dynamic state, since absorption, metabolism, and excretion were occurring. True values for total absorption, therefore, are probably higher.

At any rate, the comparison of oral and parenterally administered thyroxine (Figure 1) show that thyroxine is poorly absorbed from the gastrointestinal tract of the bovine and tends to explain the low potency of orally administered thyroidal materials.

Further confirmation is the finding of large amounts of thyroxine-like substances in the feces.

REFERENCES

(1) BROWNELL, G. L. Analysis of Techniques for Determination of Thyroid Function with Radioiodine. J. Clin. Endocrinol., 11: 1095. 1951.

(2) MIXNER, J. P., AND LENNON, H. D., JR. Efficiency of Absorption of Thyroxine in Various Forms from the Gastrointestinal Tracts of Lactating Cows. J. Dairy Sci., 43: 1480. 1960.

(3) PIPES, G. W., AND TURNER, C. W. The Effect of Thyroxine on Thyroid Function. Missouri Agr. Expt. Sta., Research Bull. 617. 1956.

(4) PIPES, G. W., PREMACHANDRA, B. N., AND TURNER, C. W. The Biological Half-Life of L-Thyroxine and L-Triiodothyronine in the Blood of the Dairy Cow. J. Dairy Sci., 42: 606. 1959.

(5) PREMACHANDRA, B. N., PIPES, G. W., AND TURNER, C. W. Comparative Goitrogenic Activity of Tapazole and Carbimazole in Cattle. J. Animal Sci., 19: 553. 1960.

(6) PREMACHANDRA, B. N., AND TURNER, C. W. Kinetics of Subcutaneous Absorption of L-Thyroxine I^{131} as Measured by Increase of I^{131} in Blood of Cattle. J. Dairy Sci., 44: 1544. 1961.

(7) TURNER, C. W., AND HERMAN, H. A. A Determination of the Blood and Plasma Volume of Dairy Cattle. Missouri Agr. Expt. Sta., Research Bull. 159. 1931.

BIOLOGICAL ASSAY OF THYROPROTEIN [1]

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SUMMARY

A new method for the biological assay of thyroidal substances based upon the determination of the thyroxine secretion rate (TSR) of rats is suggested. The mean TSR of a group of rats is first determined by thyroxine replacement. Thyroprotein is then injected subcutaneously in amount sufficient to block thyroidal-I^{131} release in the individual rats. Biological estimation of thyroidal activity in five samples of thyroprotein indicated that samples contained 42, 27, 45, 31, and 53% more activity than chemical estimation of 1% thyroxine. It was suggested that the increased biological activity might be due to slower absorption of thyroxine from the injection site or to the presence of significant amounts of triiodothyronine in thyroprotein.

A number of methods have been suggested for the biological assay of thyroidal substances. These methods have been reviewed by Turner and Premachandra (7). The object of the present report is to suggest a new method for the biological assay of thyroidal substances based upon the method of determining the thyroxine secretion rate of experimental animals such as rats (3) or mice (8).

Materials and methods. Male rats of Sprague-Dawley-Rolfsmeyer strain were maintained on laboratory pellet feed at a constant temperature of 78 ± 1 F. The thyroxine secretion rate (TSR) of each of 63 animals was determined by the method described (3), except that thyroxine (T_4) was administered in increasing increments of 0.25 μg and tapazole at the rate of 400 μg/100 g body weight was injected daily to prevent recycling of I^{131}. The T_4 used was supplied by Travenol Lab., Inc., Morton Grove, Illinois, and was guaranteed to contain not less than 97% of L-thyroxine.

Thyroidal substances in the form of synthetic thyroprotein (thyroactive iodinated casein) with commercial name of Protamone [3] were assayed. These preparations were shown by Reineke (6) on the basis of isotope dilution technique to contain 1.04% thyroxine. Five commercial samples which were standardized by the manufacturer to contain 1% thyroxine by a countercurrent distribution fractionation system and a differential spectrophotometric method were used. On assumption that thyroprotein contained 1% T_4, 100 μg thyroprotein would contain 1 μg T_4. Thyroprotein was homogenized in solution of 50% propylene glycol and 50% saline. Both T_4 and thyroprotein were injected subcutaneously. The L-thyroxine secretion rate of each rat in the group was determined. The TSR of surviving rats was compared in each successive sample with amount of thyroprotein required to block thyroidal-I^{131} release and thus indicate the thyroxine equivalent effect.

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[3] Supplied by Agri-Tech., Inc., Kansas City, Missouri.

RESULTS

Mean TSR of 63 rats was 1.42 μg T_4/100 g body weight. Sample I thyroprotein required only a mean of 100 μg/100 g body weight to block thyroidal-I^{131} release rate; thus, this amount of thyroprotein produced a biological effect equal to 1.42 μg T_4 or was 42% above estimate of 100 μg thyroprotein, being equal to 1 μg T_4.

Sample II required a mean of 113 μg to equal 1.43 μg T_4/100 g body weight, indicating an increase of 27% above estimated potency of thyroprotein. Samples III, IV, and V were calculated to be 45, 31, and 53% more potent than T_4 on the basis of thyroprotein containing 1% T_4. It will be noted that successive bioassays were based on reduced numbers of comparisons due to mortality during the time span required to conduct successive assays. The mean TSR of surviving group was compared in each case.

DISCUSSION

The proposed method of bioassay of thyroidal material is based upon the method of determining the TSR of rats. Comparison is made of the amount of thyroidal material re-

TABLE 1

Biological assay of thyroprotein in comparison with thyroxine by thyroxine secretion rate equivalent method

No. of animals	Mean TSR μg/100 g B.W./of surviving animals	Thyroprotein equivalent dosage (100 μg = 1 μg T_4)				
		Sample I	Sample II	Sample III	Sample IV	Sample V
		(μg)				
		100.0				
63	1.42		113.0			
62	1.43			99.0		
50	1.44				109.0	
38	1.43					94.0
16	1.44					
Potency [a]		+42%	+27%	+45%	+31%	+53%

[a] Potency determined by comparison of mean TSR of group with thyroprotein equivalent. Thus, in Group I, mean TSR = 1.42 μg T_4 per 100 g body weight. 100 μg thyroprotein equals 1.42 μg T_4. Thyroprotein 42% more potent than chemical estimate of 1% thyroxine.

quired to equal the T_4 effect. Because of the variation in TSR of individual rats and even groups of rats, it is important that comparisons be made using the same animals.

Thyroprotein samples were from batches of commercially manufactured material standardized by chemical analysis to contain 1% T_4. Present bioassays indicate that these samples contained from 27 to 53% more biological activity than chemical analysis indicated.

Several possible explanations may be advanced. In comparison with T_4, thyroprotein was injected in complete protein combination. Present study suggests that the thyroprotein is hydrolyzed at injection site and T_4 is released. It is possible that a slower rate of absorption of T_4 from hydrolyzed thyroprotein may increase the effectiveness of T_4 contained, thus increasing the apparent effectiveness. In studies with dairy cattle, the rate of absorption of thyroxine-I^{131} from a subcutaneous site was shown to be exponential with a faster component lasting 6 to 10 hr, followed by a slower component for the remainder of the 24-hr period (5). If absorption of T_4 were slower from thyroprotein, it is possible that the apparent biological effectiveness might be increased.

A second and more probable explanation may be the presence of significant amounts of triiodothyronine in thyroprotein. Friedberg and Reineke (2) reported that hydrolyzed thyroprotein contained at least ten iodinated compounds including mono- and diiodotyrosine, diiodo- and triiodothyronine, as well as thyroxine. Since triiodothyronine has been shown to be three or more times as biologically active in the rat as thyroxine (7), the presence of this compound in thyroprotein could increase its apparent biological activity as shown in the present study.

In dairy cattle, Premachandra et al. (4) reported that triiodothyronine was slightly over twice as effective as thyroxine when injected in inhibiting pituitary thyroprotein secretion. However, Bartlett et al. (1) reported that triiodothyronine was much less effective orally than thyroxine, as measured by their influence on milk yield. Since the response of thyroxine on milk yield in dairy cattle is quite variable, the results may not indicate a significant difference.

The significance of the present study of the biological asay of successive commercial samples of thyroprotein standardized to 1% thyroxine by a chemical method is the indication that such a method is satisfactory, since all samples indicate biological activity above the guaranteed minimum of 1%, in standardizing this product. That the biological method indicates potency from 27 to 53% above the chemical method may be due to the difference in rate of subcutaneous absorption of thyroprotein or to the presence of triiodothyronine. If the latter is true and the oral biological value of triiodothyronine is less than thyroxine, the chemical and biological methods of assay of thyroprotein would indicate similar oral activity.

REFERENCES

(1) Bartlett, S., Burt, A. W. A., Folley, S. J., and Rowland, S. J. Relative Galactopoietic Effects of 3:5:3′-Triiodo-L-Thyronine and L-Thyroxine in Lactating Cows. J. Endocrinology, 10: 193. 1954.

(2) FRIEDBERG, W., AND REINEKE, E. P. Thyroxine-like Compounds in Iodinated Protein and Thyroid Gland. Federation Proc., 11: 50. 1952.

(3) GROSVENOR, C. E., AND TURNER, C. W. Effect of Lactation upon Thyroid Secretion Rate in the Rat. Proc. Soc. Exptl. Biol. Med., 99: 517. 1958.

(4) PREMACHANDRA, B. N., PIPES, G. W., AND TURNER, C. W. Comparative Abilities of Thyroxine and Triiodothyronine as Inhibitors of Pituitary Thyrotropin Secretion in Cattle. Am. J. Physiol., 201: 77. 1961.

(5) PREMACHANDRA, B. N., AND TURNER, C. W. Kinetics of Subcutaneous Absorption of L-Thyroxine-I^{131} as Measured by Increase of I^{131} in Blood of Cattle. J. Dairy Sci., 44: 1544. 1961.

(6) REINEKE, E. P. The Thyroxine Content of Thyroactive Iodinated Proteins as Determined by a Radioactive Isotope Dilution Technique. J. Dairy Sci., 37: 1227. 1954.

(7) TURNER, C. W., AND PREMACHANDRA, B. N. Methods in Hormone Research, edited by R. I. Dorfman. Chap. 10, Thyroidal Substances, p. 385, Publ., Academic Press, New York. 1962.

(8) WADA, H., v. BERSWORDT-WALLRABE, R., AND TURNER, C. W. Thyroxine Secretion Rate of Mice. Proc. Soc. Exptl. Biol. Med., 102: 608. 1959.

TECHNICAL NOTES

SIMPLE DEVICE FOR OBTAINING SAMPLES OF HEADSPACE GAS DIRECTLY FROM SEALED CONTAINERS FOR ANALYSIS BY GAS CHROMATOGRAPHY [1]

The determination of oxygen in the headspace gas of sealed containers of food products has been a commonly used quality control measure for many years. The Orsat method which is often employed generally requires large volumes of gas and is time-consuming. Instruments are available for detecting a gas by measuring its paramagnetic susceptibility, but such devices cannot yield a complete analysis of mixtures of gases. Several methods (3, 4) have been described for the removal of gas mixtures from the headspace of sealed containers and procedures have been developed (1, 2) for the separation and identification of microquantities of gases by gas chromatography.

A simple, inexpensive device (Figure 1) has

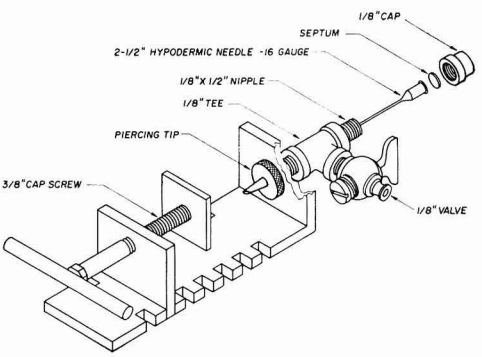

FIG. 1. Detail of gas sampling device.

been developed in this laboratory to permit the removal of headspace gas from sealed cans without contamination of the sample from an outside atmosphere. The materials used in construction of the device were:

1. Base Plate—2″ × 14″ × 3⁄16″ mild steel with a 90° bend 2″ from end
2. End Plate—2″ × 2″ × 3⁄16″ mild steel
3. Screw—3⁄8″ × 4″ hex head cap screw
4. Screw Plate—1″ × 1″ × 3⁄16″ mild steel
5. Piercing tip with rubber stopper
6. 2—1⁄8″ × 1⁄2″ pipe nipples
7. 1⁄8″ × 1⁄8″ × 1⁄8″ pipe tee
8. 1⁄8″ pipe cap with a 1⁄8″ drilled hole
9. 1⁄8″ valve
10. Hypodermic needle, 16 ga., 4″
11. Rubber injection gasket, 3⁄8″ dia.

Adjustments made by moving the end plate of this device adapt the base to any size container. The can is first held loosely in the apparatus and the screw is tightened until a seal is accomplished between the rubber stopper and the container. The system can then be purged by attaching a cylinder of inert gas and flushing or, alternatively, the contaminating atmospheric gases in the unit may be removed with a vacuum pump. The screw is tightened further, compressing the rubber stopper (not shown in the diagram) until the can is punctured by the piercing tip. A gas-tight syringe with a 4-inch hypodermic needle (23 to 27 ga.) is inserted through the rubber septum. An internally contained hypodermic needle (16 ga.) provides a passageway to guide the inserted needle through the can piercing tip and into the can. Without this internally contained hypodermic needle, probing is necessary to find the hole in the piercing tip. Consequently, time is wasted and the needle is dulled after only a few uses. Two rinsings of the syringe with the headspace gas are necessary to obtain reproducible results when the gas sample is injected into a gas chromatograph. The hypodermic needle on the air-tight syringe may be withdrawn and inserted many times without danger of contaminating the internally contained gases.

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[2] Published with the approval of the Directors of the Michigan Agricultural Experiment Station as Journal Article No. 3018 (n.s.).

REFERENCES

(1) BRENNER, N., AND CIEPLINSKI, E. Gas Chromatographic Analysis of Mixtures Containing Oxygen, Nitrogen, and Carbon Dioxide. Ann. N. Y. Acad. Sci., 72: 705. 1959.
(2) MURAKAMI, Y. Rapid Gas Chromatographic Analysis of a Mixture of Oxygen, Nitrogen, Methane, Carbon Monoxide, and Carbon Dioxide. Bull. Chem. Soc. Japan, 32: 316. 1959.
(3) STAHL, W. H., VOELKER, W. A., AND SULLIVAN, J. H. A Gas Chromatographic Method for Determining Gases in the Headspace of Cans and Flexible Packages. Food Technol., 14: 14. 1960.
(4) VOSTI, D. C., HERNANDEZ, H. H., AND STRAND, J. B. Analysis of Headspace Gases in Canned Foods by Gas Chromatography. Food Technol., 15: 29. 1961.

COMPARISON OF 305- AND 365-DAY PRODUCTION RECORDS UNDER STRATIFIED LEVELS OF HERD PRODUCTION[1, 2]

The relative merit of lactation records of varying duration has been studied by numerous research workers. Thoele (5), in a review of the literature dealing with 305- and 365-day records, generally concluded that for purposes of selection the 305-day record is at least as useful as the 365-day record. The increased influence of calving interval, age, and season of calving on records longer than ten months is apparent (2-5). This, plus the knowledge that variation in feeding and management have greater influence on later than on early stage of lactation, has stimulated recent research dealing with the usefulness of records of shorter duration than ten months.

The issue of 305- vs. 365-day records has been renewed, by dairymen, as a result of the rapid growth of Dairy Herd Improvement Registry (DHIR) through which 305-day and completed lactation records but not 365-day records are provided as a result of routine Dairy Herd Improvement Association (DHIA) procedures. The question raised by dairymen is whether 365-day records are more useful for selection purposes in high-producing herds under Advanced Registry (AR), Herd Improvement Registry (HIR), and DHIR conditions. This study was made in an attempt to appraise the relative merits of 305- and 365-day records for selection purposes in stratified levels of AR and HIR herd production.

[1] Supported in part by Wisconsin's AI Cooperatives.

[2] Approved for publication by the Director of the Experiment Station, University of Wisconsin.

Production data were provided by the Holstein-Friesian Association of America and consisted of 81,226 records made by 15,892 cows in 76 AR and HIR herds. The herds used in this study were selected on the basis of having been on test for 15 or more consecutive years and for variation in level of herd fat production (low = 400-449 lb; medium = 450-499 lb; high = 500-550 lb). The data included actual lactations of 306 to 365 days and both M.E. and actual 305-day or less records completed through 1959. A total of 15,430 records represented 3× milkings in 17 herds.

The general description of the data with respect to five length-of-lactation groups is as follows:

Lactation days	Herd groups: Low 2× (%)	Medium 2× (%)	High 2× (%)	3× (%)
<305	21.9	23.5	17.8	19.1
305	39.7	38.7	41.5	41.0
306–335	13.4	14.8	14.3	11.4
336–364	9.3	8.5	9.8	8.4
365	15.8	14.5	16.7	20.1

There appeared to be no important trends in the distribution of length of lactation groups over the three levels of 2× herds. However, in 3× herds a somewhat greater percentage of 365-day records was noted. The average age at calving, length of lactation, and number of records per cow were very similar and in most cases identical over the four herd groups. The

TABLE 1

Repeatability estimates of first and second available 305- and 365-day actual and M.E. production

Herd group	No. herds	No. cows	1st 305-day fat $\bar{x}$	1st 305-day fat s	Repeatability estimates: 1st vs. 2nd 305 Milk	1st vs. 2nd 305 Fat	1st vs. 2nd 305 % Fat	1st vs. 2nd 365 Milk	1st vs. 2nd 365 Fat	1st vs. 2nd 365 % Fat
2×, M.E.										
Low	24	767	471	84	.421	.375	.658	.417	.376	.690
Medium	22	602	516	79	.417	.348	.732	.437	.390	.763
High	27	1,050	582	89	.496	.494	.748	.496	.501	.770
Over-all	73	2,419	530	85	.453	.429	.719	.458	.442	.746
2×, Actual										
Low	24	767	402	80	.420	.383		.428	.390	
Medium	22	602	440	80	.408	.363		.427	.381	
High	27	1,050	501	94	.456	.474		.476	.501	
Over-all	73	2,419	454	87	.443	.424		.451	.444	
3× on M.E., 2× basis	17	426	502	70	.514	.535	.750	.506	.530	.781
3× actual	17	426	507	81	.504	.510		.503	.512	

distribution of cows having one or more 365-day records was 49.3, 45.0, 50.6, and 57.6% in the low, medium, high, and 3× herds, respectively.

Repeatability estimates of 305- and 365-day records were calculated after selecting from the data those cows having two or more 365-day records. Mature equivalent yield was determined, using the age adjustment factor corresponding to the same lactation in 305 days. The repeatability estimates were based on intra-herd simple correlation coefficients, and represented the first and second available records of 2,419 cows milked at least 365 days under 2× conditions, and 426 cows milked at least 365 days under 3× conditions. The results are summarized in Table 1.

It is apparent from Table 1 that no significant differences exist in the estimates of repeatability of 305- vs. 365-day records. The trend toward slightly greater values for the 365-day records is possibly attributed to the more deliberate effort on the part of these dairymen to delay breeding the cows involved in Table 1 for 12-month lactations. It was not considered feasible, however, to study the influence of calving interval in these data.

There is no obvious explanation for the significantly higher repeatability estimates involving fat yield in the high 2× herds. When herd averages were computed over all years from all available 305-day lactations, variation in herd averages was greatest in high herds and smallest in medium herds. However, coefficients of variation were 5.8, 4.7, and 6.1 for high, medium, and low groups, respectively. The average interval between the first and second available record was 25.5, 26.7, 27.5, and 26.8 months in the 3×, high, medium, and low herds, respectively. There was no indication that short-time environmental influences were a contributing factor. The percentage of cows from the original data and represented in the analysis involving low, medium, and high herds was 13.5, 13.6, and 18.3, respectively. Consequently, it is possible that the greater selection of cows in the low and medium herds could have been contributing factors to the lower repeatability estimates in these groups.

There was little difference in the repeatability estimates involving actual and 2×, M.E. lactations. The average age at calving for the first and next available record was 41 and 67 months, respectively. The DHIA-M.E. factors used here were effective in reducing within-herd variation in milk and fat yield as measured by the coefficient of variation.

Simple correlations between concurrent 305- and 365-day lactations were in the order of 0.99 for milk, fat, and percentage of fat and did not vary with herd level group. Among the 2,419 first available records, 89.0 and 88.3% of the milk and fat, respectively, in 365 days was made in the concurrent 305 days. The corresponding values for second available records were 90.5 and 90.0%, respectively.

There were no indications from this study that 365-day records are more useful than 305-day records for selection purposes under AR and HIR conditions. In high-producing seed stock herds, their value is largely for promotion and advertising purposes. For selection purposes, they add little to the usefulness of 305-day records and, indeed, appear to be more confusing than useful in sire provings, pedigree evaluation, and record appraisal in general. If there is a need for lifetime production, completed lactations should be used and can be provided by machine processing centers.

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REFERENCES

(1) Dickerson, G. E. Evaluation of Different Measures of Inherited Producing Ability in Dairy Cattle. J. Dairy Sci., 20: 437. 1937.
(2) Dickerson, G. E. Estimates of Producing Ability in Dairy Cattle. J. Agr. Research, 61: 561. 1940.
(3) Gaines, W. L. Persistency of Lactation in Dairy Cattle. Illinois Agr. Expt. Sta., Bull. 288. 1927.
(4) Johansson, I., and Hansson, A. Causes of Variation in Milk and Butterfat Yield in Dairy Cows. Kgl. Lantbrukasakad. Tidskr., Nr 6 1/2. 1940.
(5) Thoele, H. W. Factors Influencing Lactation Records of Dairy Cows. (Mimeo). Holstein-Friesian Assoc. of America, Brattleboro, Vermont. 1960.

SYMPOSIUM ON LACTIC STARTER CULTURES[1]

CONTENTS

[1] This symposium was held at the 57th Annual Meeting of the American Dairy Science Association, College Park, Maryland, on June 21, 1962, under the sponsorship of the Manufacturing Section, with Dr. M. L. Speck as convenor.

I. CULTURE IDENTITY AND SELECTION

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The first starters used in manufacturing butter and cheese were natural cultures. The first recorded instructions appear to have been those of C. A. Flint, of Boston (8), who in 1866 wrote: "After the milk has been brought from the yard or stable, strain immediately into the pans, in which has been put a little sour milk from which the cream has been removed, the quantity varying from a tablespoon to half a teacupful according to the state of the weather."

In 1873, Lister (17) isolated *Streptococcus lactis* (called *Bacterium lactis*) from sour milk, and studied it. Interest increased in using natural starters and in isolating organisms for use in manufacturing dairy products. Papers on the subject were published in 1889 by Conn of Connecticut (6), and in 1890 by Storch of Denmark (24) and Weigman of Germany (27). Commercial cultures for manufacturing butter became available in Denmark shortly after Storch's report. By 1897, 802 of the 866 creameries in Denmark were reported to have been using commercial cultures (14). At about the same time commercial cultures appeared to have become available in America.

In manufacturing dairy products, cultures are added for one or both of two purposes: To produce lactic acid, or to produce desired aroma. With these purposes in mind, my discussion will be divided into two parts: identity and selection of the bacteria responsible for producing aroma; and identity and selection of the lactic streptococci responsible for producing acid. I have in mind primarily the identity and selection of organisms normally used in the manufacture of such products as Cheddar cheese, Cottage cheese, and buttermilk—not products such as Swiss cheese and yogurt, which require different organisms, though some of the thoughts expressed might be applicable to them.

Culture selection will not be discussed from the viewpoint of buying several commercial mixed-strain cultures and testing them in the dairy plant to determine which is best for manufacturing a particular product. This approach should not be necessary. One culture is not enough. For manufacturing cheese, one needs about four different cultures that can be depended on to produce acid. For manufacturing buttermilk, one needs at least two or three different cultures that can be depended on to produce acid and the desired aroma.

Cultures should be composed—not developed. The selection of organisms for use in cultures, and the preparation of cultures for specific purposes, are the responsibilities of the composer—not the dairy-products manufacturer. When a culture is composed of properly selected organisms, it can and should be expected to do the specific job for which it was prepared. Even so, the manufacturer has the responsibility of proper care and correct use of cultures.

IDENTITY AND SELECTION OF THE BACTERIA RESPONSIBLE FOR PRODUCING AROMA

Beginning in 1890, there was confusion as to whether the desired aroma imparted to butter by starters was the result of a single organism or a mixture of organisms. Conn (7) reported in 1899 that desired aroma was produced by a single organism in some European cultures, and by a mixture in others. By 1919 interest was centered on certain low-acid-producing bacteria that produced good butter aroma only when grown together with lactic streptococci (2, 13, 25). Hammer (12) named these associate organisms *Streptococcus citrovorus* and *Streptococcus paracitrovorus*. In 1929, Knudsen and Sørensen (16) isolated some organisms presumably the same as Hammer's *S. citrovorus*, and proposed the name *Betacoccus cremoris*. Hucker and Pederson (15) studied some of the associate organisms in 1930, and concluded that the names *Leuconostoc citrovorum* and *Leuconostoc dextranicum* are more appropriate than *S. citrovorus* and *S. paracitrovorus*. In 1960 Garvie (11) reported a study of the genus *Leuconostoc*. She feels that *L. citrovorum* should be called *L. cremoris*, after Knudsen and Sørensen's *B. cremoris*, on the basis that the organisms named *S. citrovorus* by Hammer might have been *Pediococcus cerevisiae*. These various names should not be confusing if one keeps in mind that all of the following names in the literature are intended to refer to a single species: *S. citrovorus, B. cremoris, L. cremoris,* and *L. citrovorum*. Since the name *L. citrovorum* is the one now used in Bergey's Manual (3), it is the one I use.

For many years following the 1890's, little attention was given to the fact that some single-strain cultures had been found able to produce good butter aroma in the absence of *S. lactis* or *S. cremoris* or both. More recently, several investigators have isolated and defined strains of citric-acid-fermenting streptococci. In 1936, Matuszewski et al. (20) studied such streptococci and gave them the name *Streptococcus diacetilactis*. In 1951, Swartling (26) made an extensive study of citric-acid-fermenting streptococci and concluded that the name *S. diacetilactis*, rather than other proposed names, should be retained. Hesitation about giving species distinction to these organisms, expressed by Swartling and later by Sherman (23), stemmed from the fact that cultures of *S. dia-*

cetilactis sometimes lose their ability to form aroma compounds, and in this state appear indistinguishable from *S. lactis*. Recent results in our laboratory show that such mutants differ from *S. lactis* by containing citritase (5).

Unfortunately, the specific identity of the aroma-producing organisms in most commercial cultures has not been known. In most instances emphasis has been on securing cultures that produced good aroma, with little consideration given to identity other than assuming that they were some of those I have mentioned.

Garvie (11) suggested that some of the low-acid-producing cultures studied by Hammer and by Orla-Jensen might have been *P. cerevisiae*, and that this species might be useful in dairy cultures. But I know of no evidence indicating that pediococci are useful in this way. Galesloot (9) of the Netherlands found no pediococci in any cultures. We recently accumulated nine strains of *P. cerevisiae*, and found none of them to produce desired aroma.

Garvie (11), in accumulating and studying organisms of the genus *Leuconostoc*, found *L. dextranicum* (Hammer's *S. paracitrovorus*) to be a rare species, which suggests that this organism is not often present in cultures.

Swartling of Sweden (26) mentioned in 1951 that *S. diacetilactis* is of considerable importance in butter cultures. All starter cultures presently used in Russia contain this organism, according to a recent report (18). In 1959, Sandine et al. (22), in determining the identity of organisms responsible for floating Cottage cheese curd, isolated several strains of *S. diacetilactis* from cultures, emphasizing the fact that many of our commercial cultures contain this organism. But many of our commercial cultures must not contain this species, since many of them do not produce sufficient gas to cause floating curd. Galesloot and coworkers (1, 10), in the Netherlands, began about 1950 to find some starters containing *S. diacetilactis* as aroma bacteria and some starters containing both *S. diacetilactis* and *L. citrovorum*. Before 1950, they had found *L. citrovorum* to be the organism producing aroma in starters used in the Netherlands.

In summary, available information indicates that aroma production by cultures primarily involves two species: *L. citrovorum* or *S. diacetilactis* or both. In view of recent findings of Badings and Galesloot (1) that yoghurt flavor in butter is due to acetaldehyde produced by *S. diacetilactis*, in view of the problem of floating curd produced by this species (22), and in view of the difficulties we have had in attempts to make uniformly good buttermilk with cultures containing this species (4), it is perhaps unfortunate that *S. diacetilactis* is present in many commercial cultures.

Simple procedures have been used to separate *L. citrovorum* from *S. diacetilactis*. Sandine et al. (22) used incubation in litmus milk at 30 C. *L. citrovorum* produces little or no change. *S. diacetilactis* produces sufficient acid to coagulate milk in 48 hr or less. Galesloot and Hassing (10) found it possible to detect *S. diacetilactis* in mixed-strain cultures without strain isolation and separation. A positive creatine test for a culture after one propagation at 35 C indicated the presence of *S. diacetilactis*.

Though *L. citrovorum* appears to be the organism of choice, it is important to recognize that some strains of this species do not produce the desired aroma of diacetyl. Results not yet published show that organisms of this species mutate to citrate-permease-negative, the same as we reported for *S. diacetilactis* (5). Without citrate permease an organism cannot produce the desired buttermilk aroma from citrate. The composer of cultures should select the proper strains in composing cultures to be used in products in which a diacetyl aroma is desired. For this selection we have found useful a method similar to that of Mather and Babel (19). A culture is inoculated heavily (about 10%) into sterile skimmilk and incubated 24 hr at 22 C. Sterile citric acid is added to reduce pH to 4.3, or a few drops of *S. cremoris* is added. After an additional incubation of 18 hr the culture is tested for odor and for reaction to the creatine test.

IDENTITY AND SELECTION OF THE LACTIC STREPTOCOCCI RESPONSIBLE FOR PRODUCING LACTIC ACID

Let us now consider the organisms responsible for producing lactic acid in cultures. In 1873, Lister (17) isolated *Streptococcus lactis* (called *Bacterium lactis*) from sour milk, and studied it. In 1919, Orla-Jensen (21) found in sour milk a lactic streptococcus that was slightly different from *S. lactis*. This organism, which he named *Streptococcus cremoris*, differs by forming longer chains, by not growing at 40 C, by not producing ammonia from peptone or arginine, by not fermenting maltose or dextrin, and by being somewhat less tolerant to a variety of conditions. The primary species for producing acid in lactic cultures is *S. cremoris*, though strains of *S. lactis* may be found occasionally. Differences between the species are of little concern in composing cultures. More important are the differences among the strains of either species. Three important characteristics in which strains differ are: rate of acid production, production of nisin-like antibiotics, and bacteriophage sensitivity. For a more detailed discussion of these characteristics, I cite the published account (4) of a recent seminar. Here, time permits me only to mention a few pertinent facts.

Many strains of these lactic streptococci are too slow in acid production to be used satisfactorily in making cultured dairy products. Some strains produce antibiotics active against other strains of lactic streptococci; others do

not. With regard to bacteriophage sensitivity there is strain specificity, though not nearly as much as we would like. There are different degrees of sensitivity to bacteriophage action that must be understood. I refer to the nascent effect, the lysin reported by Naylor and Czulak, and host-controlled variations. These have been discussed more fully previously (4).

The number of strains of lactic streptococci suitable for use in composing cultures is quite limited, selecting merely on the basis of the three mentioned characteristics. These and other characteristics must be determined by the composer if the resulting cultures are to serve satisfactorily in producing lactic acid. Experience as to the characteristics and capabilities of individual strains is important. There is only a general correlation between culture activity in a laboratory and activity in a dairy plant. Some strains serve well in one product and not so well in another. Strains selected for Cheddar cheese cultures should be sufficiently heat-resistant to survive the cheese cooking temperatures without damage.

Strains selected as being sensitive to different types of bacteriophages, found not to produce antibiotics, and known to be suitable for use in making the intended product, cannot be mixed at will. Of particular importance is the matter of strain domination (4). Some strains of lactic streptococci dominate others in mixtures, soon becoming responsible for most of the acid produced. Domination among strains actually seems to be the rule rather than the exception, even among strains that do not produce antibiotics. When strain domination has occurred, a single bacteriophage active against the dominant strain can cause failure in the production of lactic acid. Except with strains that produce antibiotic, the only known method of determining compatibility or lack of it is to grow strains together in the laboratory and check for domination.

Beyond an attempt to indicate the present situation regarding culture identity and selection, I have attempted to define those which I believe present knowledge indicates to be some of the responsibilities of the culture composer. He is the one who can and should determine culture composition. His flexibility in composing cultures is limited only by the number of good strains he has available, by his knowledge and experience with those strains, and by his awareness of the manufacturer's needs. There should be close cooperation between composer and user with regard to the results the user is getting with the cultures supplied, particularly with regard to the control of bacteriophages.

I have not attempted to discuss bacteriophages, because time does not permit adequate coverage. I once saw bacteriophages cause a dairy plant to lose in one day enough skimmilk to have made ten tons of Cottage cheese. Seeing such losses makes one aware of the importance of bacteriophages. The subject has been discussed in an earlier report (4). Those of you who have read that paper know I am convinced that the key steps in controlling bacteriophages are tests for bacteriophages, cultures composed of strains selected to resist the bacteriophages found, and close cooperation between culture composer and culture user. These steps only supplement—they do not replace—good sanitation and correct procedures in the dairy plant.

There are no cast iron cultures. A dairy products manufacturer who has propagated and used one culture for a long period is fortunate. But he should not feel that he has by lucky chance found the solution to culture problems. Rather, he should recognize that he has given opportunity for several possibilities to influence culture composition. Strain domination has been mentioned. Another possibility is piecemeal elimination of strains, or culture types, by the action of bacteriophages that are permitted to contaminate the mother culture. His culture, though it might be giving excellent results, might now be, for all practical purposes, a single-strain culture. That is, the one or more strains remaining might be of the same phage type. If this is the case, the manufacturer is gambling that a particular active bacteriophage will not get into the plant and cause difficulty.

REFERENCES

(1) Badings, H. T., and Galesloot, T. E. Studies on the Flavor of Different Types of Butter Starters with Reference to the Defect "Yoghurt Flavour" in Butter. Proc. 16th Intern. Dairy Congr., Copenhagen, Sec. 111-2. 1962.

(2) Beokhout, F. W. J., and Ott de Vries, J. J. Aromabildner bei der Rahmsauerung. Centr. Bakteriol. 11 Abt., 49: 373. 1919.

(3) Breed, R. S., Murray, E. G. D., and Smith, N. R. Bergey's Manual of Determinative Bacteriology. 7th ed. Williams and Wilkins Co., Baltimore. 1957.

(4) Collins, E. B. Behavior and Use of Lactic Streptococci and Their Bacteriophages. J. Dairy Sci., 45: 552. 1962.

(5) Collins, E. B., and Harvey, R. J. Failure in the Production of Citrate Permease by *Streptococcus diacetilactis*. J. Dairy Sci., 45: 32. 1962.

(6) Conn, H. W. Bacteria in Milk and Its Products. Storrs Agr. Expt. Sta., Bull. 4. 1889.

(7) Conn, H. W. Some Practical Applications of Bacteriology in European Dairying. Storrs Agr. Expt. Sta., Ann. Rept., 1898. p. 67. 1899.

(8) Flint, C. F. Milch Cows and Dairy Farming. p. 231. Crosley and Ainsworth, Boston. 1866.

(9) Galesloot, T. E. Personal communication. 1962.

(10) Galesloot, T. E., and Hassing, R. Some Differences in Behavior Between Starters Containing as Aroma Bacterium Either *Streptococcus diacetilactis* or *Betacoccus*

cremoris. Netherlands Milk Dairy J., 15: 242. 1961.

(11) GARVIE, E. I. The Genus *Leuconostoc* and Its Nomenclature. J. Dairy Research, 27: 283. 1960.

(12) HAMMER, B. W. Volatile Acid Production of *S. lacticus* and the Organisms Associated with It in Starters. Iowa Agr. Expt. Sta., Research Bull. 63. 1920.

(13) HAMMER, B. W., AND BAILEY, D. E. The Volatile Acid Production of Starters and of Organisms Isolated from Them. Iowa Agr. Expt. Sta., Research Bull. 55. 1919.

(14) HANSEN, S. Versuche mit Bakterienkulturen fur die Rahmsäuering. Milch-Z., 26: 590. 1897.

(15) HUCKER, G. J., AND PEDERSON, C. S. Studies on the Coccaceae. XVII. The Genus *Leuconostoc*. New York State Agr. Expt. Sta., Tech. Bull. 156. 1930.

(16) KNUDSEN, S., AND SØRENSEN, A. Bidrag til Syrevaekkernes Bacteriologi. p. 64. Kgl. Veterinaer-og Landbohøjskole Aarsskrift. 1929.

(17) LISTER, J. A Further Contribution to the Natural History of Bacteria and the Germ Theory of Fermentation Changes. Quart. J. Microbiol. Sci., 13: 380. 1873.

(18) LUKOVNIKOVA, L. Selection of Lactic Streptococci for the Preparation of Cultures. Molochnaya Prom., 21: 25. 1960.

(19) MATHER, D. W., AND BABEL, F. J. A Method for Standardizing the Biacetyl Content of Creamed Cottage Cheese. J. Dairy Sci., 42: 1045. 1959.

(20) MATUSZEWSKI, T., PIJANOWSKI, E., AND SUPINSKA, J. *Streptococcus dicetilactis* n. sp. i jego zastosowanie przy wyrobie masla. Polish Agr. Forest Ann., 36: 1 (in English, 25). 1936.

(21) ORLA-JENSEN, S. The Lactic Acid Bacteria. And. Fred. Host. & Søn, Copenhagen. 1919.

(22) SANDINE, W. E., ELLIKER, P. R., AND ANDERSON, A. W. Taxonomic Study of High Carbon Dioxide–Producing Lactic Acid Streptococci Isolated from Mixed Strain Starter Cultures. J. Dairy Sci., 42: 799. 1959.

(23) SHERMAN, J. M. *Streptococcus lactis* and the Streptococci of the So-called Lactic Group. J. Dairy Sci., 38: 1184. 1955.

(24) STORCH, V. Nogle Undersøgelser over Flødens Syrning. 18de Beretning fra Forsøgslaboratoriet. Kgl. Veterinaer-og Landbohøjskoles Laboratoruim for Landøkonomiske Forsøg. København. 1890.

(25) STORCH, V. Forstatte Undersøgelser over Fremstillingen of Syrevaekkere. 102de Beretning fra Forsøgslaboratoriet. Kgl. Veterinaer-og Landbohøjskoles Laboratorium for Landøkonomiske Forsøg. København. 1919.

(26) SWARTLING, P. F. Biochemical and Serological Properties of Some Citric Acid Fermenting Streptococci from Milk and Dairy Products. J. Dairy Research, 18: 265. 1951.

(27) WEIGMAN, H. Neue Mitteilungen uber Rahmsauerung mittelst Reinkulturen von Saurebakterien. Milch-Z. p. 944. 1890.

II. GENETIC EXCHANGE AND VARIABILITY IN LACTIC STREPTOCOCCUS STARTER ORGANISMS[1]

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I would like to begin with a brief description of culture variation, in order to emphasize the nature and magnitude of this problem in the dairy industry. For many years dairy manufacturers have been plagued by lack of uniformity in flavor and acid production by mixed-strain lactic streptococcus starter cultures. Some of this variation has been found due to specific environmental factors such as bacteriophages (30), antibiotics (15), quaternary ammonium compounds (18), and changes in milk composition (2). More recently, strain dominance (8) and strain compatibility (9, 12, 19) have been shown to play roles in culture variability. However, other factors undoubtedly also are involved, since variation in mixed-strain starter cultures frequently is observed in the apparent absence of any of these aforementioned influences. For example, a number of cells in a mixed-strain starter culture from time to time will gain or lose certain characteristics, independent of any obvious environmental effects. This gain or loss is a permanent change, for all the progeny or descendents of the cells involved are like the parents from which they originated. Good examples of this may be seen from some of the familiar variety designations for *Streptococcus lactis*, which identify the characteristic present or absent, as the case may be. The terms maltigenes for malty-flavored strains, hollandicus for ropy strains, anoxyphilus for slow litmus-reducing strains, tardus for slow acid-producing strains, and aromaticus for diacetyl-producing strains have all been used in this regard (14).

These observations suggest that mutation and selection occur in mixed-strain starter cultures. Moreover, since different strains of lactic streptococcus and *Leuconostoc* organisms are grown in mixtures to provide starter cultures, the probability that exchange of genetic material may cause culture variation is even more likely. We now know that the genetic material of bacteria (3) and most bacteriophages (10,

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20) is deoxyribonucleic acid (DNA). We know further that there are at least three mechanisms by which DNA from one bacterium may become associated with DNA from another bacterium, in order that recombination can occur to give rise to new cells that differ from each of their ancestors. These well-known processes may be listed as follows:

TABLE 1

Mechanisms of bacterial recombination

Method	Process inhibited by	
	DNAse	Phage Antiserum
Transformation	+	−
Transduction	−	+
Conjugation	−	−

Transformation involves the use of DNA isolated from donor bacteria and added to suitable recipient bacteria; naked DNA carries the genetic information. Transduction involves the use of bacteriophages propagated on donor bacteria. The phages, which carry the DNA, are used to infect suitable recipient bacteria. Conjugation is a sexual process requiring physical contact, during which there is a one-way transfer of genetic material from a fertile cell (F^+) to a non-fertile cell (F^-).

In each of these three cases, the actual exchange or recombination of genetic material occurs between the intact DNA of the recipient cell and a portion of the DNA of a donor cell or donor phage. One of the popular theories concerning how this recombination occurs is called copy choice (16). A simplification of this process is presented in the following figure, where it is shown how DNA having markers ABC might recombine with DNA having markers 123 to yield DNA which would be capable of expressing the characters 1B3 and A2C in individual, segregated cells.

At the top of the figure is the double-stranded Watson-Crick DNA, which has been redrawn below using straight lines to represent one of the strands; on the left is the ABC DNA, on the right the 123 DNA. Now the copy choice theory proposes that a new strand of DNA is formed using one of the parental strands as a replication model. Occasionally, however, a strand will switch over and copy from the second strand, which will require the replicating piece on the other strand also to switch over. This can be seen from the middle portion of the figure, the two halves of which would have to be superimposed to see the complete picture. The hypothetical recombinant characters 1B3 and A2C would then show up in some of the progeny after segregation had occurred.

Conjugation has only been demonstrated among gram-negative bacteria of the *Escherichia, Shigella, Salmonella, Vibrio,* and *Pseudomonas* genera. I know of no attempts to demonstrate this phenomenon among organisms used in the dairy industry. For the past year, however, we have been studying transformation, lysogeny, and transduction in lactic streptococci as part of a program to determine whether variation in mixed-strain lactic streptococcus starter cultures might arise by genetic exchange. I would like to present some of our preliminary findings at this time.

Organisms of the *Streptococcus* genus have been transformed by other investigators (4, 22, 23), but no original articles on this phenomenon in lactic streptococci are found in the literature; Reiter and Oram (25), however, cite a personal communication from Moller-Madsen in which he indicated transformation of citrate utilization from *Streptococcus diacetilactis* to *S. lactis.*

The first difficulty we encountered in our transformation work was the lack of a suitable, gentle lysing system for lactic acid streptococci by which we could extract highly polymerized DNA; lysozyme, sodium lauryl sulfate, and sodium desoxycholate were relatively ineffective under a variety of conditions. Other workers (23) had used an enzyme preparation from *Streptomyces albus* to lyse streptococci, but this material was not generally available. Then one of us (W. C. B.) observed that old cultures (four to seven days) of *S. diacetilactis* strains grown in citrate broth (28) became quite viscous. This suggested a possible role for chelating agents in altering streptococcal cell walls and reminded us of a report by Repaske (26) concerning the lysis-accelerating effect of ethylenediaminetetraacetate (EDTA) on lysozyme-lysis of gram-negative bacteria. When the lysozyme-EDTA procedure described by Repaske was applied to *S. lactis,* significant lysis resulted (6). Dr. R. Pakula of the Department

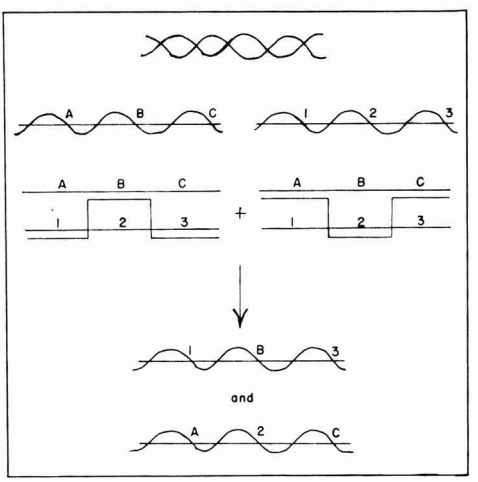

FIG. 1

of Microbiology and Hygiene, School of Medicine, Warsaw, Poland, graciously provided two strains of transformable *Streptococcus* organisms (SBE and challis), in order that we could test the activity of DNA prepared using this lysis technique; the DNA was active and typical data from one of our experiments for transformation of streptomycin resistance follow:

TABLE 2

Number of streptomycin resistant transformants per milliliter [a]

Modification of transforming system [b]	Treatment of DNA [c]	
	Deproteinized	Undeproteinized
None	480	160
DNAse added	0	0
DNA omitted	0	0
DNA boiled	0	0

[a] Total cell count was 3×10^7 cells per milliliter. Organism was *Streptococcus* strain challis.
[b] The method and transforming system of Pakula et al. (22) were used.
[c] 2,000 μg per milliliter.

Transformation of the challis strain also has been accomplished in nonfat milk, but at a very low frequency. DNA extracted from over 40 strains of lactic streptococcus organisms using this lysis procedure has been used in an effort to transform streptomycin resistance and tryptophan independence (5). No transformation has as yet been observed, suggesting a paucity of competent or transformable strains among these bacteria. However, DNA prepared from a streptomycin resistant mutant of *S. lactis* C10, has been found to confer antibiotic resistance upon the streptomycin-sensitive, transformable *Streptococcus* strain challis.

I would like now to comment briefly regarding our studies on lysogeny in lactic streptococci, but let me first of all define lysogeny and phage conversion. Lysogenic bacteria are those that carry bacteriophages as prophage in association with their genetic material; they appear perfectly normal but from time to time some of the cells in the population will lyse and release free phage particles. The remaining lysogenic bacteria are immune to infection by these liberated phages, but other unrelated strains of cells, as would be present in a mixed-strain starter culture, may be infected. Phage conversion is similar to transduction, except that the recipient cells gain a new character only as a result of being lysogenized. It is well known that conversion by bacteriophages also is a factor in bacterial variation (13). For example, Freeman (11) reported the conversion of a nontoxin-producing *Corynebacterium diphtheriae* organism into a toxin-producer by lysogenizing the bacterium. Other examples of phage conversion also have been shown (13).

In 1949, Reiter (24) reported the occurrence of lysogenic lactic streptococcus organisms in bulk mixed-strain starter cultures and we were interested in isolating strains exhibiting this phenomenon for use in variation studies. We were interested in determining whether there was any change, for example, in flavor production or rate of acid production, when lactic streptococci were lysogenized. Several strains from our stock culture collection expressed possible lysogeny in cross streak tests; cross streaks of *Streptococcus cremoris* W and *S. cremoris* C3, for example, provided the results shown in the following figure:

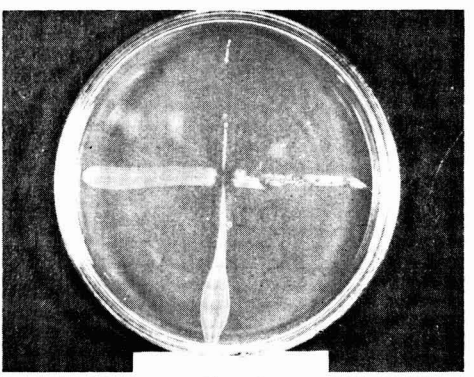

FIG. 2

This suggested that one or both of these two *S. cremoris* strains carried phages which, when liberated, would lyse the other strain. When these two strains were grown together, a filtrate spotted on a lawn of *S. cremoris* C3 resulted in the lytic spot which may be seen as follows:

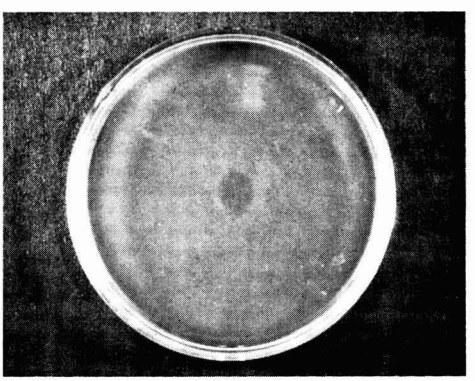

FIG. 3

Phages which repropagate on *S. cremoris* C3 have been isolated from the lysis area. We have not yet had an opportunity to pursue this matter further, but subsequent studies will con-

cern an examination of the role lysogenization or phage conversion may play in variation among lactic streptococci.

Transduction among organisms of the *Streptococcus* genus has not been reported. In fact, the only gram-positive bacteria reported to undergo transduction are *Bacillus subtilis* (29) and *Staphylococcus aureus* (21, 27). Recently, however, transduction of tryptophan independence in *Streptococcus diacetilactis* 18-16 and streptomycin resistance in *S. lactis* C2 were accomplished in our laboratory (1). Unlike most other successful transductions, these were brought about using a virulent rather than a temperate phage. Results of typical qualitative experiments for tryptophan independence may be seen in the following table:

TABLE 3

Transduction of tryptophan independence in *Streptococcus diacetilactis* 18-16

PFU per milliliter	MOI [a]	Survivors per milliliter	Transductants per milliliter [c]	Frequency
1×10^8	1	3×10^5	0	0
..... [b]	.....	5×10^8	0	0
1×10^{10}	100	5.2×10^6	>1 [d]	$>1/5.2 \times 10^6$
..... [b]	.....	6×10^7	0	0

[a] Multiplicity of infection: The ratio of bacteriophages to bacterial cells.
[b] Nonphage control.
[c] The method of Allen (1) was used to carry out the transduction.
[d] Detected by observing growth in synthetic broth medium lacking tryptophan.

The frequency of transduction is shown as greater than one per so many surviving cells per milliliter, because the recipient survivors were screened in broth medium lacking tryptophan. It may be seen that a high multiplicity of infection (100) was required. The next table shows the same type of data for streptomycin resistance:

TABLE 4

Transduction of streptomycin resistance in *Streptococcus lactis* C2

PFU per milliliter	MOI	Survivors per milliliter	Transductants per milliliter	Frequency
5×10^9	1	1×10^3	0	0
..... [a]	.....	1×10^9	0	0
1×10^9	100	1×10^3	>1 [b]	>1/1,000
..... [a]	.....	1×10^8	0	0
1×10^9	100	4×10^4	>1 [b]	>1/40,000
..... [a]	.....	4×10^8	0	0

[a] Nonphage controls.
[b] Detected by observing growth in medium containing 1,000 units per milliliter of streptomycin.

Information regarding the frequency with which this transduction was occurring in streptomycin resistance was obtained using the replica plating procedure of the Lederbergs (17). Results are shown in the next table:

The streptomycin-sensitive recipient used in these experiments has been found to exhibit no significant mutation to streptomycin resistance; in fact, the antibiotic resistant mutant donor used in the transduction was obtained only after several attempts. It can be seen that a frequency of about one in 27 cells was found with the first experiment. Phages in the second experiment were irradiated; this increased the number of survivors but reduced the frequency of transduction.

On the basis of our findings, I would like to speculate a little regarding the role that genetic exchange may play as a contributor to culture variation in the Dairy Industry. Transformation, while it undoubtedly occurs in starter cultures, may take place less frequently than other exchange methods. Presumably, autolysis of cells to release DNA would occur only at pH 5.0 or below, where the activity of transforming DNA is much reduced (3). External lysing agents, however, such as bacteriophages and antibiotics, could provide the release of DNA under conditions that would favor transformation. The consequences of the ensuing recombinations may not seem undesirable if all the organisms in the mixed-strain starter culture possess desirable characteristics. That is, recombinants of desirable parents might be expected to yield desirable progeny. This, however, is not necessarily true, for it is well known that biosynthetic recombinants can

TABLE 5

Frequency of transduction of streptomycin resistance in *Streptococcus lactis* C2 as determined by replica plating

PFU per milliliter	Survivors per milliliter	Avg no. colonies per master plate	Avg no. transductants per rep. plate	Frequency
1×10^9	8×10^2	186	6	1/27
.....[b]	1×10^8	TNTC	0	0
1×10^{9}[c]	4×10^3	TNTC	5.3	1/150
.....[b]	2×10^8	TNTC	0	0

[a] MOI was 100.
[b] Nonphage controls.
[c] Irradiated phage preparation.

synthesize compounds which cannot be produced by the parents (7).

It appears that transduction may be more significant in starter culture variation, especially since this process would allow for the introduction of new characteristics as a result of bacteriophage infection. While the role of phage conversion in this variation is yet to be assayed, it also may be important, especially since lysogenic lactic streptococci appear to be common. All this emphasizes further the pressing need for continued research to find suitable milk additives which will prevent bacteriophage adsorption. Furthermore, the frequent rotation of starter cultures originating from a lyophilized powder becomes even more necessary in view of pending genetic exchange.

REFERENCES

(1) ALLEN, LOIS K. Studies on Transduction in Lactic Streptococcus Organisms. M.S. thesis, Oregon State University. 1962.

(2) ANDERSON, A. W., PARKER, R. W., AND ELLIKER, P. R. The Nutritional Requirements of Lactic Streptococci Isolated from Starter Cultures. III. Variations in Growth-Promoting Properties of Fresh Whole Milk. J. Dairy Sci., 38: 1083. 1955.

(3) AVERY, O. T., MACLEOD, C. M., AND MCCARTY, M. Studies on the Chemical Nature of the Substance Inducing Transformation of Pneumococcal Types. J. Exptl. Med., 79: 137. 1944.

(4) BRACCO, R. M., KRAUSS, R. M., ROE, A. S., AND MACLEOD, C. M. Transformation Reactions Between Pneumococcus and Three Strains of Streptococci. J. Exptl. Med., 106: 247. 1957.

(5) BROWN, W. C. Studies on Transformation in Streptococci. M.S. thesis, Oregon State University. 1962.

(6) BROWN, W. C., SANDINE, W. E., AND ELLIKER, P. R. Lysis of Lactic Acid Bacteria by Lysozyme and Ethylenediaminetetraacetic Acid. J. Bacteriol., 83: 697. 1962.

(7) CATCHESIDE, O. G. Relation of Genotype to Enzyme Content. Microbial Genetics, 10th Sympos. Soc. for Gen. Microbiol. p. 181. 1960.

(8) COLLINS, E. B. Domination Among Strains of Lactic Streptococci with Attention to Antibiotic Production. Appl. Microbiol., 9: 200. 1961.

(9) DAHIYA, R. S., AND SPECK, M. L. Symbiosis Among Lactic Streptococci. J. Dairy Sci., 45: 607. 1962.

(10) DOI, R. H., AND SPIEGELMAN, S. Properties and Intracellular Localization of the RNA Injected by an RNA Bacteriophage. Bacteriol. Proc., p. 153. 1962.

(11) FREEMAN, V. J. Studies on the Virulence of Bacteriophage-Infected Strains of *Corynebacterium diphtheriae*. J. Bacteriol., 61: 675. 1951.

(12) GREEN, V. W. Interaction Among Pure Strains of Lactic Streptococci Isolated from Multiple Strain Cheese Starters. Paper M-50, 54th Ann. Meet., Am. Dairy Sci. Assoc. June, 1959.

(13) GROMAN, N. B. Conversion by Bacteriophage—A Factor in Bacterial Variation. Microbial Genetics. Biol. Colloq., Oregon State University, 41: 1960.

(14) HAMMER, B. W., AND BABEL, F. J. Dairy Bacteriology. 4th ed. John Wiley and Sons, New York. 614 pp. 1957.

(15) KOSIKOWSKI, F. V., AND MOCQUOT, G. Antibiotic Residues in Milk for Cheese. Advances in Cheese Technology. FAO Agr. Studies No. 38. Rome, 1958.

(16) LEDERBERG, J. Recombination Mechanisms in Bacteria. J. Cellular Comp. Physiol., 45: Suppl. 2: 75. 1955.

(17) LEDERBERG, J., AND LEDERBERG, ESTHER M. Replica Plating and Indirect Selection of Bacterial Mutants. J. Bacteriol., 63: 399. 1952.

(18) LUNDSTEDT, ERIK. Don't Let Quats Ruin that Flavor. Food Ind., 22: 2056. 1950.

(19) LUNDSTEDT, ERIK. The Symbiosis Between Lactic Acid Bacteria and the Aroma Bacteria in Starters. The Milk Dealer, Part I, March: 47, and Part II, April: 42. 1959.

(20) MEYER, F., MACKAL, R. P., MABEL, T., AND EVANS, E. A., JR. Infectious Deoxyribonucleic Acid from Bacteriophage. J. Biol. Chem., 236: 1141. 1961.

(21) MORSE, M. L. Transduction by Staphylococcal Bacteriophage. Proc. Natl. Acad. Sci., 45: 722. 1959.

(22) PAKULA, R., HULANICKA, E., AND WALCZAK, W. Transformation Between Streptococci, Pneumococci, and Staphylococci. Bulletin de l'Academie Polonaise des Sciences, Classe II, 6: 435. 1958.

(23) PERRY, D., AND SLADE, H. D. Transformation of Streptococci to Streptomycin Resistance. J. Bacteriol., 83: 443. 1962.
(24) REITER, B. Lysogenic Strains of Lactic Streptococci. Nature, 164: 667. 1949.
(25) REITER, B., AND ORAM, J. O. Nutritional Studies on Cheese Starters. I. Vitamin and Amino Acid Requirements of Single Strain Starters. J. Dairy Research, 29: 63. 1962.
(26) REPASKE, ROY. Lysis of Gram Negative Organisms and the Role of Versene. Biochim. et Biophys. Acta, 39: 225. 1958.
(27) RITZ, H. L., AND BALDWIN, J. N. Induction of Penicillinase Production in Staphylococci by Bacteriophage. Bacteriol. Proc., p. 40. 1958.
(28) SANDINE, W. E., ELLIKER, P. R., AND HAYS, HELEN. Cultural Studies on *Streptococcus diacetilactis* and Other Members of the Lactic Streptococcus Group. Canadian J. Microbiol., 8: 161. 1962.
(29) THORNE, C. B. Transduction in *Bacillus subtilis*. J. Bacteriol., 83: 106. 1962.
(30) WHITEHEAD, H. R., AND COX, G. A. The Occurrence of Bacteriophage in Cultures in Lactic Streptococci. New Zealand J. Sci. Technol., 16: 319. 1935.

III. CERTAIN ASPECTS OF STARTER CULTURE METABOLISM

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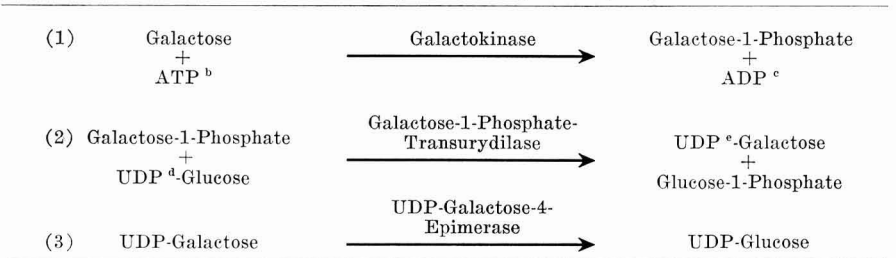
The metabolism of starter cultures is complex, in that it encompasses not only production of lactic acid and flavor or aroma substances but includes production of other compounds from carbohydrates, utilization of nitrogenous compounds, vitamins, salts, and fats as well as development of antibiotic-like substances. This paper will attempt to summarize some of the available information, first, on metabolism of homofermentative lactic starter streptococci, and, secondly, on heterofermentative starter organisms. The discussion on homofermentative bacteria will be restricted to *Streptococcus lactis, Streptococcus cremoris,* and *Streptococcus thermophilus,* whereas the consideration of heterofermentative organisms will deal primarily with *Streptococcus diacetilactis* and *Leuconostoc citrovorum.*

HOMOFERMENTATIVE LACTIC STREPTOCOCCI

Utilization of carbohydrates. Lactose is the carbohydrate of greatest concern in a consideration of dairy starter cultures. The organisms split lactose into its components, namely, glucose and galactose. Glucose then is converted to lactic acid via the well known Embden-Meyerhof-Parnes (EMP) metabolic pathway. The final reaction of this scheme involves reduction of pyruvate to lactic acid with the aid of lactic acid–dehydrogenase. This homofermentative reaction, theoretically, yields two molecules of lactic acid for each molecule of glucose utilized (54).

Before galactose can be utilized by the organism it must be converted to a glucose-form which can enter into the EMP metabolic scheme. This conversion of galactose to glucose-1-phosphate occurs according to the equations illustrated in Figure 1. First, galactose is converted to galactose-1-phosphate with the aid of galactokinase. The galactose-1-phosphate then reacts with uridinediphosphoglucose to form glucose-1-phosphate and uridinediphosphogalactose. The enzyme galactose-1-phosphate transurydilase catalyzes the reaction. Finally, the UDP-glucose utilized in the previous reaction is formed by conversion of UDP-galactose through the action of UDP-galactose-4-epimer-

FIG. 1. Conversion of galactose to glucose-1-phosphate.[a]

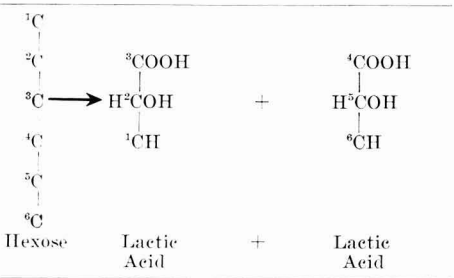


[a] From Kandler, Milchwissenschaft, 16: 523. 1961.
[b] Adenosinetriphosphate.
[c] Adenisinediphosphate.
[d] Uridinediphosphoglucose.
[e] Uridinediphosphogalactose.

ase (54). The glucose-1-phosphate formed in the second reaction now is ready to enter the EMP scheme at the same point as glucose. Both glucose-1-phosphate and glucose are initially converted to glucose-6-phosphate in the EMP scheme, although different enzymes serve as catalysts (54).

Studies with radioactive glucose labeled in different positions have shown how hexose carbon atoms are distributed in lactic acid molecules. This is illustrated in Figure 2.

FIG. 2. Location of hexose carbon atoms in product of homofermentative lactic acid bacteria.[a]



[a] From Kandler, Milchwissenschaft, 16: 523. 1961.

Carbon atoms one, two, and three are located in one molecule of lactic acid and the remaining three serve as the carbon component of the other lactic acid molecule (54).

Production of acid by both *S. lactis* and *S. cremoris* is greatest at 94 F and decreases considerably at 100 and 105 F (127). Acid production in milk by *S. thermophilus*: (a) was higher in autoclaved and lower in raw and pasteurized milks, (b) varied from day to day, (c) varied according to the cow which produced test milks, (d) was not affected by season of year, and (e), in one instance, was partially inhibited by rennet (28).

Cells of *S. cremoris*, when grown in a glucose medium without pH control, produced acetic and formic acids, carbon dioxide, and ethanol in addition to lactic acid. *S. lactis* did not produce formic acid but, instead, developed some acetoin. *S. thermophilus* produced all of the products mentioned for the other two (97). Control of pH at 7.0 increased the number of products formed by *S. cremoris* and altered the kinds produced by *S. lactis* and *S. thermophilus*. When *S. lactis* was grown in a glucose medium controlled at pH 7.0 and sparged with nitrogen; lactic, acetic, and formic acids, carbon dioxide, ethanol, diacetyl, acetoin, and 2,3-butanediol were formed (99). Other products developed by both *S. lactis* and *S. cremoris* include acetaldehyde and acetone (40).

Although heterofermentative starter bacteria will be considered in greater detail later in this paper, it is appropriate to mention now that they differ from homofermentative bacteria, in that they do not possess an aldolase and hence are unable to degrade hexoses by means of the EMP scheme (54). Homofermentative bacteria, however, do possess the enzymes necessary for direct oxidation and decarboxylation of glucose-6-phosphate to ribulose-5-phosphate and, hence, could also carry out heterofermentative reactions (54). This is mentioned as a possible explanation for formation of products other than lactic acid by homofermentative bacteria.

S. lactis contains dehydrogenase enzymes and hence the organism activated carbohydrates but lacked the ability to dehydrogenate noncarbohydrate substances. A few could be oxidized, however, and they appeared to be glycerol, lactate and pyruvate, butyrate, and some of the alcohols (115). Endogenous respiration of *S. lactis* in a glucose medium was found to occur and was affected by: (a) glucose and pH levels, (b) age of cells (9 hr appeared best), and (c) presence of sodium fluoride, which was stimulatory (112). It was postulated that *S. lactis* may have two respiratory systems (one involved in glucose and ascorbic oxidation and the other in oxidation of noncarbohydrate substances), neither is able to function during glycolysis (115).

When grown in milk for 24 hr, *S. lactis* and *S. thermophilus* bring about a fivefold increase in pyruvic acid and a 1.5-fold increase in alpha-ketoglutaric acid (39). An increase in frequency of transfer was accompanied by an increase in levels of these acids.

Cells of *S. lactis* adapted most readily to new sugars if they were just emerging from the lag phase and were in the period of physiological growth. During the logarithmic phase, adaptability decreased rapidly and continuously (43).

Production of acid by *S. thermophilus* is related to the surface-to-depth ratio of the medium in which the organism was previously grown. A surface-to-depth ratio of the old culture which was less than that of the new medium resulted in slower acid production than if both ratios were similar (38).

Effect of carbon dioxide. Laboratory experiments using an artificial medium indicated that both *S. lactis* and *S. cremoris* required carbon dioxide to initiate growth (31, 103). When the organisms were incubated in an atmosphere free from carbon dioxide, growth failed to occur. The addition of 10% carbon dioxide to the atmosphere permitted good growth and acid production, but an excessive amount of the gas proved to be inhibitory (31).

The initial growth rate of *S. lactis* and *S. cremoris* in skimmilk also depends on the proportion of carbon dioxide present in solution (123). When carbon dioxide was constantly swept away from a milk medium all strains showed a very long lag period. The organisms

eventually emerged from this lag period and then grew well enough to coagulate milk in 24 hr. To obtain optimal initial growth, lactic streptococci require the presence, in solution, in milk of 0.2 to 2.3% carbon dioxide. The exact requirement varied with the strain (123).

To fully understand the significance of carbon dioxide in the metabolism of lactic acid bacteria, it is necessary to consider what can happen to pyruvate formed through degradation of carbohydrates (54).

First, pyruvate can be reduced to lactate. This is the primary reaction of lactic streptococci. Second, a reductive amination can take place in which pyruvate is converted to alanine (one of the more important amino acids in the build-up of protein). Third, the malic enzymes can catalyze the carboxylation and reduction necessary to produce malic acid. Carbon dioxide is utilized in this reaction. Malic acid may then be oxidized to oxalacetate. This compound is an important precursor of asparagine, which can be converted to threonine and leucine. Fourth, pyruvate can be converted to phosphoenolpyruvic acid with the aid of pyruvatekinase. The phosphoenolpyruvic acid can be carboxylated to form phosphoenoloxalacetate, then oxalacetate. Carbon dioxide is also utilized in this reaction. This conversion is the predominant one in lactic acid bacteria and is necessary for maintenance of life, since these organisms cannot form oxalacetate by means of the citric acid cycle, as do other bacteria. Fifth, formation of acetaldehyde, acetolactate, and acetoin, which will be considered in greater detail later in this paper.

Utilization of fats. Information on degradation of fat and utilization of fatty acids by homofermentative lactic acid bacteria is quite limited. It has been noted that milk which has undergone sufficient lipolysis so its surface tension is reduced to about 10 dynes per centimeter is definitely inhibitory toward *S. lactis*. The inhibitory action previously was not ascribed to reduction in surface tension nor to the slight accompanying reduction in pH (19), but recent studies have shown that reduction in surface tension may be a factor (82). The presence in milk of certain free fatty acids is accompanied by an inhibition of acid production by *S. lactis*. Lauric, capric, and caprylic acids, when present at the 0.1% level, were inhibitory, whereas oleic, butyric, linoleic, linolenic, arachidic, or palmitic at the same level showed no effect. Caproic and stearic acids were not inhibitory, but 0.5% capric almost completely stopped acid production by *S. lactis* (18). Some strains of *S. lactis* have been found to grow in a chemically defined medium only if it was supplemented with acetate (58) or acetate and oleate radicals (15). The presence of fat in milk has been shown to be inhibitory to some strains of *S. cremoris* (34). It is believed that cells of susceptible strains rise with the cream and then are in an area of insufficient nutrients.

Cells of *S. lactis* treated with lysozyme yielded 5% of their dry weight as lipid (73). All lipids obtained contained the following five major fatty acids: myristic, palmitic, palmitoleic, lactobacillic, and an 18 carbon acid which possessed one double bond and may be oleic acid or one or several of its isomers (73).

Recently, it was reported that ethylendiaminetetraacetic acid accelerated lysing of *S. lactis* by lysozyme (7). Whether the use of this technique would change the lipid picture is not known.

Effects of vitamins, salts, and metals. The homofermentative lactic streptococci require a number of vitamins for growth. Various published reports indicate that *S. lactis* requires: riboflavin (2, 91, 110), pantothenic acid (2, 91, 104, 110), pyridoxine (2, 91, 110), thiamine (2, 91, 110), biotin (2, 91, 104, 110), nicotinic acid (91, 110), and niacin (2, 104). Some variations in vitamin requirements exist among different strains of *S. lactis*. Vitamin requirements for *S. cremoris* appear to be similar to those described for *S. lactis*. For good growth, *S. thermophilus* requires: pantothenic acid, riboflavin, thiamin, nicotinamide (or nicotinic acid), biotin, and pyridoxine or its derivatives.

The growth of *S. lactis* in milk was not affected by addition of pantothenic acid, niacin, B_{12}, biotin, or folic acid, whereas slight inhibition was noted when thiamin, xanthine, or pyridoxamine was added (3). Folic acid or B_{12} additions to a synthetic medium either in the presence or in the absence of added methionine had no effect on growth of *S. lactis* (3).

In spring, changes in milk composition (lowered protein nitrogen and vitamin content) may bring about disturbances in lactic acid production by some strains of *S. lactis*, whereas others are not affected (118). The unaffected strains appear to require less riboflavin and more biotin than others. They are also more proteolytic and can synthesize nicotinic acid (118). Another strain has been found able to utilize phenylalanine in the synthesis of folinic acid (93). *S. lactis* also produces folic acid (89).

Vitamin K, when added to milk in concentrations as low as 3.0 ppm, reduced the rate of acid production by lactic dairy starters (125). Levels of 100 ppm or more were required to arrest acid production for 24 hr at an incubation temperature of 30 C (125).

Acid production by *S. lactis* and *S. cremoris* was reduced in the absence of iron (101). Cobalt or zinc could replace iron (101). Sodium chloride, when added to milk at the 0.5% level, served to stimulate acid production by *S. lactis* (112). Higher levels caused a decline in acid development (112).

When added to milk, potassium nitrate or chlorate had little effect on the growth rate of *S. cremoris*, whereas potassium persulfate

showed slight inhibition and potassium bromate and iodate were strongly inhibitory (45).

S. thermophilus was inhibited slightly by the presence of 2 and 4 ppm copper in the medium. Higher levels, up to 16 ppm, may have retarded growth somewhat more, but the effect was not pronounced (87).

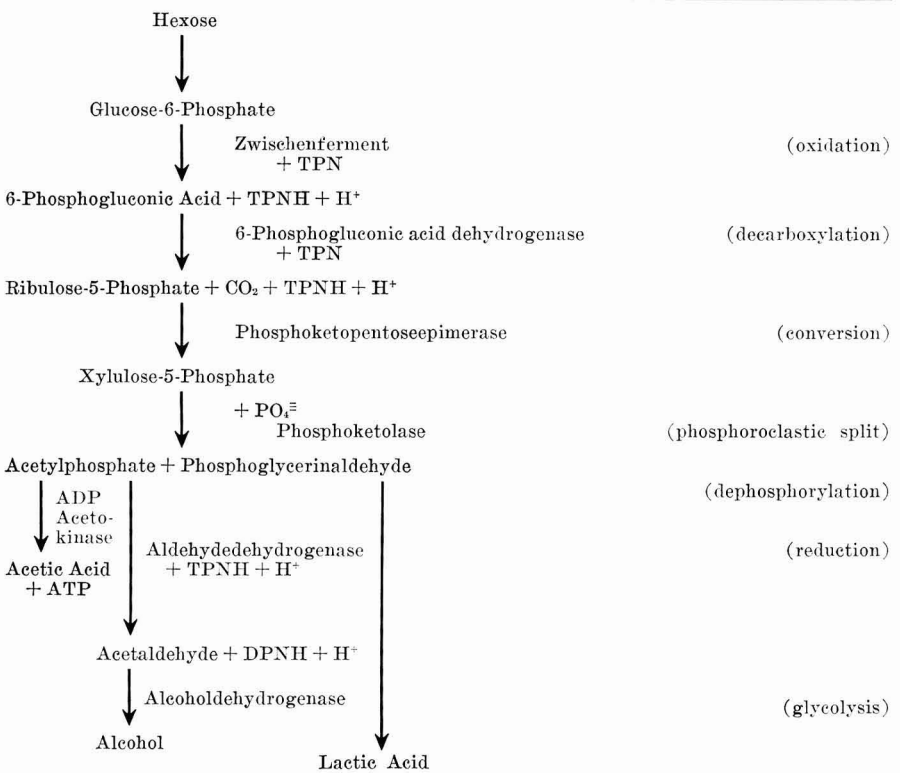
Metabolic variations of S. lactis *var.* maltigenes. The malty variant of *S. lactis* has been the object of considerable interest. At one time it was believed that this organism produced the malty flavor through liberation of acetaldehyde (125). This was later disproved when it was found that nonmalty strains of *S. lactis* produced as much or more acetaldehyde than did the malty variant (51). Subsequent studies showed the aroma resulted from 3-methylbutanal and a small amount of 2-methylbutanal. Both of these compounds probably were derived from free leucine in milk (51). The mechanism by which this occurred appeared to be as follows: (a) the organism possesses a transaminase system which effects a transfer of the amino group of leucine to alpha-ketoglutaric acid and this results in formation of alpha-ketoisocaproic and glutamic acids, (b) an alpha-ketoisocaproic acid decarboxylase, in the presence of magnesium ions and thiaminpyrophosphate, decarboxylates the keto acid to form 3-methylbutanal plus carbon dioxide (70).

S. lactis var. *maltigenes* was found to require leucine, isoleucine, and valine for multiplication. The leucine requirement could be satisfied with alpha-ketoisocaproic acid (69).

HETEROFERMENTATIVE STARTERS

Utilization of carbohydrates. The mechanism by which heterofermentative starter bacteria utilize carbohydrates and produce a variety of products is indicated in Figure 3. In this instance, glucose-6-phosphate is oxidized to 6-phosphogluconic acid through activity of the Zwischenferment first described by Warburg. The 6-phosphogluconic acid is decarboxylated with formation of ribulose-5-phosphate and carbon dioxide. Ribulose-5-phosphate is converted to xylulose-5-phosphate, which then undergoes phosphoroclastic splitting and yields acetylphosphate and phosphoglycerinaldehyde.

FIG. 3. Carbohydrate metabolism of heterofermentative lactic acid bacteria.[a]

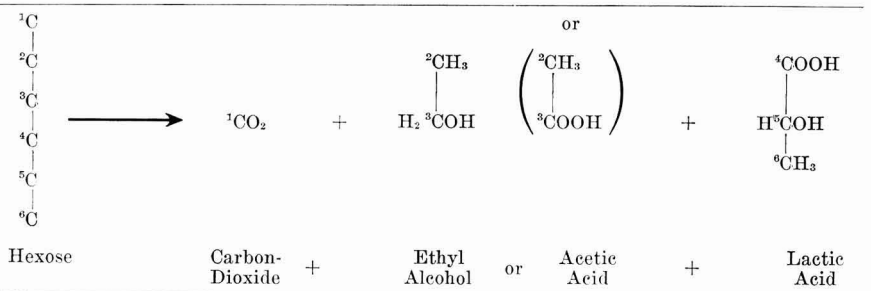


[a] From Kandler, Milchwissenschaft, 16: 523. 1961.

The phosphoglycerinaldehyde is transformed into lactic acid by means of the EMP glycolytic pathway. Acetylphosphate is dephosphorylated and forms acetic acid, or is reduced to produce alcohol. Under anaerobic conditions, generally, complete reduction of acetylphosphate occurs. If oxygen is present, some of the acetylphosphate in converted to acetic acid and some to alcohol. Theoretically, heterofermentative organisms convert one molecule of glucose into one molecule each of carbon dioxide, lactic acid, and alcohol or acetic acid (54).

Studies with labeled glucose have provided information about distribution of hexose carbon atoms in products of heterofermentative bacteria. These data are outlined in Figure 4.

FIG. 4. Location of hexose carbon atoms in products of heterofermentative lactic acid bacteria.[a]



[a] From Kandler, Milchwissenschaft, 16: 523. 1961.

The carbon atom numbered one is present in carbon dioxide, whereas the No. 2 and 3 carbon atoms are found in alcohol or acetic acid. Carbon atoms 4, 5, and 6 become a part of the lactic acid molecule (54).

L. citrovorum, in a series of tests, was able to convert glucose to the products described above under both aerobic and anaerobic conditions (5).

When *S. diacetilactis* was grown in milk for 4 hr at 30 C, 94 to 128 μl of oxygen were utilized and 455 to 591 μl of carbon dioxide were produced (105). Gas production is greatest at elevated incubation temperatures (49). Development of gas by *L. citrovorum* has caused slit-openness in Cheddar cheese at ripening temperatures of 50 and 58 F (96). The decarboxylase system of *S. diacetilactis* has been shown to be very heat stable (100% activity after heating to 80 C for 60 min) and to be most active when the pCO_2 was low (94, 95).

Aroma and flavor formation. The production of acetoin (and diacetyl) by heterofermentative bacteria has been the subject of many investigations. These studies have led to differences in opinion on the nature of the reaction. Briefly, some of the ideas previously held are as follows:

(a) Diacetyl results from sugar dissimilation by *Leuconostoc* and citrate serves only as a hydrogen acceptor (13).

(b) Lactose is the main source of C-4 compounds formed and citrate is only indirectly involved (17).

(c) Neither glucose nor citrate alone supports production of acetoin and barium or calcium salts are necessary to initiate the reaction (116).

(d) Aroma substances are produced by *L. citrovorum* when lactose and citric acid are present. Other carbohydrates could replace lactose but citric acid could not be replaced by other acids (63).

(e) Diacetyl and acetoin result from fermentation of citrate only (13).

(f) Diacetyl is produced from sodium pyruvate and citrate but not from glucose (13).

(g) Pyruvate occurs as an intermediate in fermentation of citrate to acetoin. Pyruvate is not formed from citrate either by a reversal of the condensing-enzyme reaction and subsequent decarboxylation of oxalacetate thus formed or by the tricarboxylic acid cycle (13).

(h) Formation of diacetyl appears to be an oxidation-reduction process in which methyl glyoxal and diacetyl are derived from pyruvate (25, 27). Adding oxidizing agents to the medium does not alter the O/R potential enough to permit diacetyl formation (26).

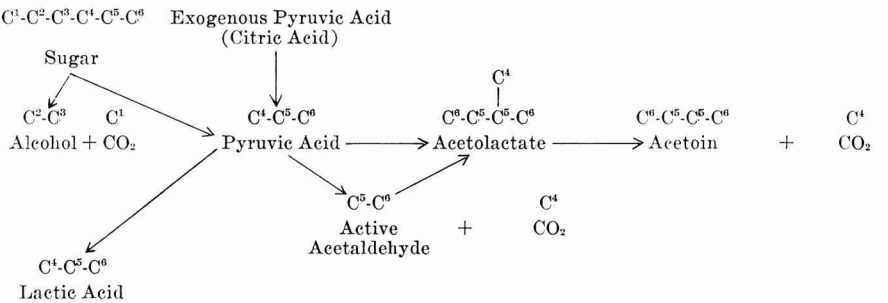
(i) Pyruvate is formed from citrate by cleavage of citrate into acetate and oxalacetate by citridesmolase. This is followed by a decarboxylation of oxalacetate to acetate (13).

The variability of these findings has been explained as follows: (a) Pyruvic acid is the key intermediate in the fermentation of lactose and citrate to acetoin and diacetyl, (b) in the fermentation of lactose to pyruvate by the

EMP scheme, sufficient DPN is produced to reduce pyruvate to lactic acid, (c) fermentation of citrate results in production of pyruvate without a simultaneous supply of reduced DPN and, hence, products other than lactic acid are formed. This difference may account for some of the controversial findings of different investigators.

The viewpoint on acetoin formation held by many investigators today, and based on studies with labeled hexoses and citrate, encompasses the following steps shown in Figure 5: (a) citrate is converted to acetate and oxalacetate, (b) oxalacetate is converted to pyruvate, (c) hexoses are converted to pyruvate, (d) pyruvate from the two sources is mixed, (e) pyruvate is converted to acetolactate directly or via active acetaldehyde, (f) acetolactate is converted to acetoin, which then is oxidized to form diacetyl (8, 13, 84, 85, 117). This figure also shows distribution of original carbon atoms in fermentation products.

FIG. 5. Scheme for formation of acetoin by *Leuconostoc citrovorum*.[a]



[a] From Kandler, Milchwissenschaft, 16: 523. 1961.

Addition of the following substances to culture media resulted in increased acetoin production by *L. citrovorum*: lactose (76), citric acid (44, 84), pyruvic acid (44, 84), sodium citrate (74), and oxalacetic acid (84). Most metabolic inhibitors stopped acid production by *L. citrovorum* more readily than acetoin formation (84). A reduction in pH to 5.0 (or below) accelerated acetoin production (84).

Apparently, *S. diacetilactis* produces acetoin in a fashion similar to that described for *L. citrovorum*. Experimental evidence indicates that the organism possesses a citritase enzyme able to convert citrate into acetate and oxalacetate (41, 105, 106), and an oxalacetic acid dehydrogenase able to catalyze production of pyruvate (106). Pyruvate is then converted to acetolactate and subsequently to acetoin (106). The citritase enzyme requires magnesium and manganese ions together with thiamine pyrophosphate for maximum activity (41).

Recently, a citrate transport system, somewhat similar to the beta-galactoside permease of *Escherichia coli*, was observed in *S. diacetilactis* (42). The transport system could be induced and permitted greatest entry of citrate into cells at pH values below 6.0. Lactose enhanced citrate uptake, whereas 2,4-dinitrophenol reduced it.

Conditions necessary for optimum acetoin (and diacetyl) production by *S. diacetilactis* include: (a) presence of citrate in medium (107), (b) pH near 5.0 (65, 107), (c) heat treatment of milk equivalent to 12 min at 121 C (107), and (d) incubation at temperatures of 18 C or above (65). The fermentation by *S. diacetilactis* proceeds in two stages; first, formation of volatile acids and cell mass, and secondly, formation of lactic acid, acetoin, and diacetyl (92).

S. diacetilactis fails to produce acetoin from lactose in the absence of citrate. It has been suggested that this occurs since all available pyruvate is used for the glycolytic reactions and none remains for synthesis of acetoin. If, however, citrate is also present, the amount of pyruvate exceeds that required for glycolysis and permits synthesis of acetoin (41).

When cultured products are held for longer periods of time, diacetyl aromas tend to disappear. Several explanations have been offered for this occurrence and are as follows: (a) acetoin (and diacetyl) can be reduced to 2,3-butanediol (54), (b) *S. diacetilactis* and organisms of the genus *Leuconostoc* possess an enzyme, diacetyl reductase, which irreversibly reduces diacetyl to acetoin (108), (c) spoilage bacteria (e.g., coliforms, pseudomonads) possess the same enzyme and hence carry out the same reaction (108), (d) contamination with bacteria able to produce strongly reducing conditions may cause a decrease in aroma and flavor substances (47), and (e) conversion of acetoin to acetic acid by micrococci that may be contaminants (54).

Effect of vitamins and salts. Cultures of

L. citrovorum require the following vitamins for growth: pantothenic acid (88), pyridoxine (88), nicotinic acid (88), biotin (88), and thymidine (57). Thymine desoxyriboside was found able to substantially increase the growth of this organism (111). The addition to the medium of pyridoxamine phosphate, para aminobenzoic acid, or B_{12} failed to improve growth. *S. diacetilactis* did not require folic or folinic acids, thiamine, and B_{12}.

The presence of 1 to 2% salt in skimmilk proved to be stimulatory for acid production by *S. diacetilactis* and *L. dextranicum* (112). The addition of more salt reduced acid production (112). Salt tolerance of these bacteria was enhanced by the addition to milk of phosphates and dried milk and reduced by raising or lowering the pH from the optimum (112).

PRODUCTION OF ANTIBIOTIC-LIKE SUBSTANCES AND EFFECT OF THOSE PRODUCED BY OTHER LACTIC STARTER BACTERIA

Studies in our laboratory (81) indicated that *L. citrovorum* produced an antibiotic-like substance(s) in skimmilk which inhibited a variety of gram-negative bacteria. The substance(s) also displayed considerable activity against *Staphylococcus aureus*. Activity appeared greatest at a pH near 4.5 and diminished as the pH was increased. Production of the inhibitory material appeared to vary from strain to strain, but all four strains tested elaborated detectable levels.

Antibiotic materials produced by *S. lactis* inhibited *L. citrovorum* (61) and *S. diacetilactis* (12). The production of antibiotics by some strains may serve to explain why mixed-strain lactic cultures change after a few transfers (12, 33).

OTHER ASPECTS OF HOMO- AND HETEROFERMENTATIVE STARTER METABOLISM

Space limitations prevent a complete consideration of starter culture metabolism. These aspects and appropriate references are indicated below. First, the utilization of nitrogenous compounds and proteolytic activity is discussed, in part, in other papers of this series and in the following references: 2, 3, 4, 9, 14, 16, 21, 22, 24, 37, 48, 50, 60, 62, 64, 68, 69, 71, 72, 75, 83, 86, 90, 98, 100, 102, 104, 119, 120, 121, and 126. Second, the effect of stimulants added to milk or to an artificial culture medium is also discussed in another paper of this series and in the following references: 3, 10, 11, 32, 35, 36, 53, 55, 56, 58, and 113. Third, the production by and effects of antibiotic-like substances on homofermentative lactic acid bacteria is considered in this series of papers and in the following references: 6, 18, 20, 23, 29, 30, 33, 34, 61, 67, and 124. Fourth, the effect of milk-borne antibiotics is discussed in the following references: 1, 66, 77, 78, 79, 80, and 109.

REFERENCES

(1) Albright, J. L., Tuckey, S. L., and Woods, G. T. Antibiotics in Milk—A Review. J. Dairy Sci., 44: 779. 1961.

(2) Anderson, A. W., and Elliker, P. R. The Nutritional Requirements of Lactic Streptococci Isolated from Starter Cultures. I. Growth in a Synthetic Medium. J. Dairy Sci., 36: 161. 1953.

(3) Anderson, A. W., and Elliker, P. R. The Nutritional Requirements of Lactic Streptococci Isolated from Starter Cultures. II. A Stimulatory Factor Required for Rapid Growth of Some Strains in Reconstituted Nonfat Milk Solids. J. Dairy Sci., 36: 608. 1953.

(4) Anderson, A. W., Parker, R. B., and Elliker, P. R. The Nutritional Requirements of Lactic Streptococci Isolated from Starter Cultures. III. Variation in Growth Promoting Properties of Fresh Whole Milks. J. Dairy Sci., 38: 1083. 1955.

(5) Bang, F. Om Omsältning av Glykos Under Aeroba Betingelser Genom Aromabakterier *Streptococcus citrovorus*. Svenska Mejeritidn., 35: 366. 1943.

(6) Baribo, L. E., and Foster, E. M. Some Characteristics of the Growth Inhibitor Produced by a Lactic Streptococcus. J. Dairy Sci., 34: 1128. 1951.

(7) Brown, W. C., Sandine, W. E., and Elliker, P. R. Lysis of Lactic Acid Bacteria by Lysozyme and Ethylenediaminetetraacetic Acid. J. Bacteriol., 83: 697. 1962.

(8) Busse, M., and Kandler, O. Biosynthesis of Acetoin by *Leuconostoc citrovorum*. Nature, 189: 774. 1961.

(9) Christophersen, J., and Thiele, H. Über den Einfluss von Substrat und Temperatur auf die proteolytische Aktivität einiger Käsebakterien. Kiel. milchwirtsch. Forschungsber., 4: 683. 1952.

(10) Claydon, T. J., and Koburger, J. A. Stimulation of Lactic Starter Cultures by Filtrates from Milk Cultures of *Pseudomonas fluorescens*. Appl. Microbiol., 9: 117. 1961.

(11) Colio, L. G., and Babb, Virginia. Study of a New Stimulatory Growth Factor. J. Biol. Chem., 174: 405. 1948.

(12) Collins, E. B. Domination Among Strains of Lactic Streptococci with Attention to Antibiotic Production. Appl. Microbiol., 9: 200. 1961.

(13) Collins, E. B., Jennings, W. G., and Anderson, V. B. Formation of Diacetyl and Acetoin by Certain Starter Organisms. Milchwissenschaft, 14: 429; 16: 151. 1959, 1961.

(14) Collins, E. B., and Nelson, F. E. The Effect of *Streptococcus lactis* and Coliform Organisms on the Soluble Nitrogen of Milk. J. Dairy Sci., 32: 652. 1949.

(15) Collins, E. B., Nelson, F. E., and Parmelee, C. E. Acetate and Oleate Requirements of the Lactic Group of Streptococci. J. Bacteriol., 59: 69. 1950.

(16) Conway, T. W., Ball, E., Ravel, Joanne M., Reger, J. L., and Shive, W. Some Functions of Glutamic Acid in Lactobacilli. Federation Proc., 14: 196. 1955.

(17) Coppens, R. Quelques Observations sur la

Biochimie des Ferments d'Arome. Netherlands Milk Dairy J., 8: 14. 1954.
(18) COSTILOW, R. N., AND SPECK, M. L. Inhibition of *Streptococcus lactis* in Milk by Fatty Acids. J. Dairy Sci., 34: 1104. 1951.
(19) COSTILOW, R. N., AND SPECK, M. L. Inhibitory Effect of Rancid Milk on Certain Bacteria. J. Dairy Sci., 34: 1119. 1951.
(20) CSISZAR, J., AND PULAY, G. Untersuchungen mit, gegen Clostridien wirkenden, Antibiotica bildenden *Str. lactis* Stämmen. I. Das Wirkungsspektrum der Antibioticabildenden Stämme und die Steigerung ihrer Antibiotica-Production. Proc. 14th Intern. Dairy Congr., Rome, 2: 423. 1956.
(21) CZULAK, J., AND SHIMMIN, P. D. Further Notes on Bitter Flavor in Cheese. Australian J. Dairy Technol., 16: 96. 1961.
(22) DIEN, L. T. H., RAVEL, JOANNE M., AND SHIVE, W. Some Inhibitory Intererlationships Among Leucine, Isoleucine and Valine. Arch. Biochem. Biophys., 49: 283. 1954.
(23) DUBINSKII, R. A. Zavisimost' Mezdhu Antimikrobnoi Aktivnost' yu i Kislotnost' yu Kislomolochnykh Produktov. Voprosy Pitaniya, 17: 68. 1958.
(24) EIDUSON, S., CAMIEN, M. U., AND DUNN, M. S. Response of Lactic Acid Bacteria to Amino Acid Derivatives. I. Optically Active and Racemic Forms of Phenylalamine and Phenyllactic Acid. Arch. Biochem., 29: 302. 1950.
(25) EVENHUIS, N. Diacetyl Formation in Starters. Netherlands Milk Dairy J., 6: 137. 1952.
(26) EVENHUIS, N. Diacetyl Formation in Starters. II. Netherlands Milk Dairy J., 6: 331. 1952.
(27) EVENHUIS, N. Quelques Remarques sur la Biochimie des Ferments d'Arome. Netherlands Milk Dairy J., 8: 19. 1954.
(28) FEAGAN, J. T. Inhibition of Thermoduric Starter Cultures Used in High Temperature Cheddar Cheese Manufacture. I. Seasonal Distribution and Strain Susceptibility. Australian J. Dairy Technol., 14: 110. 1959.
(29) GALESLOOT, T. E. Invloed van Nisine op die Bacterien Welke Betrokhen zijn of Kennen sizjn bij Bacteriologische Processen in Kaas en Smeltkaas. Netherlands Milk Dairy J., 11: 58. 1957.
(30) GALESLOOT, T. E. Produktie van Nisine door Antibiotische Zuursels. Netherlands Milk Dairy J., 11: 74. 1957.
(31) GARVIE, ELLEN I. Some Observations on Slow and Fast Acid-Producing Variants of Strains of *Streptococcus cremoris* and *Str. lactis* used as Cheese Starters. J. Dairy Research, 26: 227. 1959.
(32) GARVIE, ELLEN I., AND MABBITT, L. A. Acid Production in Milk by Starter Cultures—The Effect of Peptone and Other Stimulatory Substances. J. Dairy Research, 23: 305. 1956.
(33) GIBSHMAN, M., AND BELOUSOVA, N. Osobennosti Podbora Smeshannŷkh Mnogoshtammovŷkh Zakvasok. Molochnaya Prom., 19: 19. 1958.
(34) GILLES, A. J. Role of the Fat Globules in the Inhibition of Certain Strains of *Streptococcus cremoris* in Pasteurized Milk. Queensland J. Agr. Sci., 18: 123. 1961.
(35) GREENE, V. W., AND JEZESKI, J. J. Studies on Starter Metabolism. III. Studies on Cysteine-Induced Stimulation and Inhibition of Starter Cultures in Milk. J. Dairy Sci., 40: 1062. 1957.
(36) GUSS, M. L., AND DELWICHE, E. A. *Streptococcus thermophilus*. J. Bacteriol., 67: 714. 1954.
(37) HAMDY, M. K., HARPER, W. J., AND WEISER, H. H. Acidic-free Amino Compounds Formed in Various Lactic Acid Starter Cultures as Measured by Ion Exchange Chromatography. Appl. Microbiol., 3: 221. 1955.
(38) HARPER, W. J., AND HUBER, ROSE M. Acidity Development in Italian Cheese Starter Cultures. Milk Prod. J., 48(8): 16. 1957.
(39) HARPER, W. J., AND HUBER, ROSE M. Formation of Acidic Carbonyl Compounds by Some Homofermentative Lactic Acid Bacteria. Appl. Microbiol., 9: 184. 1961.
(40) HARVEY, R. J. Production of Acetone and Acetaldehyde by Lactic Streptococci. J. Dairy Research, 27: 41. 1960.
(41) HARVEY, R. J., AND COLLINS, E. B. Role of Citratase in Acetoin Formation by *Streptococcus diacetilactis* and *Leuconostoc citrovorum*. J. Bacteriol., 82: 954. 1961.
(42) HARVEY, R. J., AND COLLINS, E. B. Citrate Transport System of *Streptococcus diacetilactis*. J. Bacteriol., 83: 1005. 1962.
(43) HEGARTY, C. P. Physiological Youth as an Important Factor in Enzyme Formation. J. Bacteriol., 37: 145. 1939.
(44) HIBSHMAN, M. R. Vliyanie Khimicheskikh Veshchesto na Obrazovanie Letuchikh Kislot, Atsetoina i Diatsetila pri Molochnokisolom Brozhenii. Mikrobiologiya, 8: 432. 1949.
(45) HIETARANTA, M. Oxiderande Salters Inverkan På Mylksyrabakteriernas Tillväxt, Syraproduktion och Proteolytiska Egenskaper. Svenska Mejeritidn., 41: 63. 1949.
(46) HIETARANTA, M. Über die Bildung von Kohlendioxyd bei Säureweckern. Proc. 15th Intern. Dairy Congr., London, 2: 993. 1959.
(47) HIETARANTA, M., AND GYLLENBERG, H. Undersökningar över Bakterieväxtens och Redox-Potentialens Roll Vid Uppkonsten av Diacetyl och Acetylmethylkarbinal i mjölk. Svenska Mejeritidn., 42: 217. 1950.
(48) HUSAIN, I., AND MCDONALD, I. J. Amino Acids and Utilization of Sodium Caseinate by Lactic Streptococci. Canadian J. Microbiol., 3: 487. 1957.
(49) IRVINE, O. R. Gas Production by Lactic Starters. Canadian Dairy Ice Cream J., 38 (4): 44. 1959.
(50) IVERSON, C. A., BIRD, E. W., AND NELSON, F. E. Proteolytic and Lipolytic Degradation of Milk and Cream and the Effect of Such Degradation upon Dry Nonfat Milk Solids Obtained from These Products. Rept., Iowa Agr. Expt. Sta., 1947-1948, Part 1, 202. 1948.
(51) JACKSON, H. W., AND MORGAN, M. E. Identity and Origin of the Malty Aroma Substance from Milk Cultures of *Streptococcus lactis* var. *maltigenes*. J. Dairy Sci., 37: 1316. 1954.
(52) JAGO, G. R., AND SWINBOURNE, M. F. Factors Influencing the Lactic Acid–Producing Properties of Streptococci Used in the Manufacture of Cheddar Cheese. II. Observations Relating Susceptibility with In-

susceptibility. J. Dairy Research, 25: 123. 1959.
(53) JANDL, J. H. Failure of Cortisone and Hydrocortisone to Replace Pteroylglutamic Acid and Citrovorum Factor in Microbial Growth. Proc. Soc. Exptl. Biol. Med., 85: 166. 1954.
(54) KANDLER, O. Stoffwechsel der Säurewecker-Organismen. Milchwissenschaft, 16: 523. 1961.
(55) KENNEDY, H. E., AND SPECK, M. L. Studies on Corn Steep Liquor in the Nutrition of Certain Lactic Acid Bacteria. J. Dairy Sci., 38: 208. 1955.
(56) KENNEDY, H. E., SPECK, M. L., AND AURAND, L. W. Studies on Corn Steep Liquor in the Nutrition of Lactic Acid Bacteria. J. Dairy Sci., 37: 635. 1954.
(57) KITAY, ESTELLE, AND SNELL, E. E. Some Additional Nutritional Requirements of Certain Lactic Acid Bacteria. J. Bacteriol., 60: 49. 1950.
(58) KIZER, D. E., HANKIN, L., SPECK, M. L., AND AURAND, L. W. Stimulation of Lactic Acid Bacteria by Substances Concomitant to Certain Proteolytic Enzymes. J. Dairy Sci., 37: 634. 1954.
(59) KIZER, D. E., AND SPECK, M. L. Observations on the Acetate and Citrate Metabolism of *Streptococcus lactis* and *Streptococcus cremoris*. J. Dairy Sci., 38: 96. 1955.
(60) KIZER, D. E., SPECK, M. L., AND AURAND, L. W. The Effect of Methionine and Methionine Precursors on the Growth of *Streptococcus lactis*. J. Bacteriol., 69: 16. 1955.
(61) KOOY, J. S., AND PETTE, J. W. Remming van de Groei van Lactaatvergistende Boterzuurbacterien door Antibiotica van Milkzuur streptococcen. Netherlands Milk Dairy J., 6: 302. 1952.
(62) KORZENOVSKY, M., AND WERKMAN, C. H. Stoichiometry of the Citrulline Phosphorylase Reaction of *Streptococcus lactis*. Bio-Chem. J., 57: 343. 1954.
(63) KRENN, J., AND VALIK, DORIS. Untersuchungen über die Art und Weise der Bildung der Aromastoffe der Butter. Proc. 12th Intern. Dairy Congr., Stockholm, 2: 516. 1949.
(64) KUNKEE, R. E. Sparing Effect on the Folic Acid Requirement of *Streptococcus lactis* by 5-Amino-2,4-bis (Substituted-Amino) Pyrimidines. J. Bacteriol., 79: 55. 1960.
(65) LIGHTBODY, LORNA G. Diacetyl Production in Cream by *Streptococcus diacetilactis*. Australian J. Dairy Technol., 17: 36. 1962.
(66) LISKA, B. J. Effects of Penicillin on the Morphology of *Streptococcus lactis*, *Streptococcus thermophilus*, and *Leuconostoc dextranicum*. J. Dairy Sci., 42: 1391. 1959.
(67) LUKOVNIKOVA, L. Antibiotic Properties of Lactic Streptococci. Molochanaya Prom., 22: 39. 1961.
(68) MAC LEOD, PATRICIA, AND GORDON, D. F. Peptides as Sources of Essential Amino Acids for Lactic Streptococci. J. Dairy Sci., 44: 237. 1961.
(69) MAC LEOD, PATRICIA, AND MORGAN, M. E. Leucine Metabolism of *Streptococcus lactis* var. *maltigenes*. I. Conversion of Alpha-Ketoisicaproic Acid to Leucine and 3-Methylbutanal. J. Dairy Sci., 38: 1208. 1955.
(70) MAC LEOD, PATRICIA, AND MORGAN, M. E. Leucine Metabolism of *Streptococcus lactis* var. *maltigenes*. II. Transaminase and Decarboxylase Activity of Acetone Powders. J. Dairy Sci., 39: 1125. 1956.
(71) MAC LEOD, PATRICIA, AND MORGAN, M. E. Differences in the Ability of Lactic Streptococci to Form Aldehydes from Certain Amino Acids. J. Dairy Sci., 41: 908. 1958.
(72) MACLEOD, PATRICIA, GORDON, D. F., AND MORGAN, M. E. The Conversion of Alpha-Ketoisovaleric Acid to Valine by Lactic Streptococci. J. Dairy Sci., 43: 1147. 1960.
(73) MACLEOD, PATRICIA, JENSEN, R. G., GANDER, G. W., AND SAMPUGNA, J. Quantity and Fatty Acid Composition of Lipid Extracted from Cells of *Streptococcus lactis*. J. Bacteriol., 83: 806. 1962.
(74) MADSEN, F. Biacetyl Production by *Leuconostoc citrovorum* in Simplified Media. Arch. Esc. Vet. Minas Gerais, 6: 101. 1953.
(75) MALIN, RUTH B., CAMIEN, M. N., AND DUNN, M. S. Response of Lactic Acid Bacteria to Amino Acid Derivatives. II. Glycine. Arch. Biochem. Biophys., 32: 106. 1951.
(76) MARSHALL, R. D., STINE, C. M., AND BRUNNER, J. R. Effect of Lactose Fortification on Body, Flavor, and Biacetyl and Acetoin Content of Cultured Skimmilk. J. Dairy Sci., 44: 1159. 1961.
(77) MARTH, E. H. Antibiotics in Milk—A Review. I. Recent Developments. J. Milk and Food Technol., 24: 35. 1961.
(78) MARTH, E. H. Antibiotics in Milk—A Review. II. Methods for Detection of Antibiotics in Milk. J. Milk and Food Technol., 24: 70. 1961.
(79) MARTH, E. H., AND ELLICKSON, B. E. Antibiotic Residues in Milk and Milk Products—A Review. J. Milk and Food Technol., 22: 241. 1959.
(80) MARTH, E. H., AND ELLICKSON, B. E. Problems Created by the Presence of Antibiotics in Milk—A Review. J. Milk and Food Technol., 22: 266. 1959.
(81) MARTH, E. H., AND HUSSONG, R. V. Effect of Skimmilks Cultured with Different Strains of *Leuconostic citrovorum* on Growth of Some Microorganisms Associated with Cottage Cheese Spoilage. J. Dairy Sci., 45: 652. 1962.
(82) MAXCY, R. B., AND CHANDAN, R. C. Inhibitory Effects of Fatty Acids and Other Surface-Active Compounds on *Streptococcus lactis*. J. Dairy Sci., 45: 654. 1962.
(83) MCDONALD, I. J. Utilization of Sodium Caseinate by Lactic Streptococci and Enterococci. Canadian J. Microbiol., 2: 602. 1956.
(84) MIZUNO, W. G., AND JEZESKI, J. J. Studies on Starter Metabolism. IV. Effect of Various Substrates on the Formation of Acetoin by a Mixed-Strain Starter Culture. J. Dairy Sci., 42: 251. 1959.
(85) MIZUNO, W. G., AND JEZESKI, J. J. Starter Metabolism. V. The Mechanism of Acetoin Formation as Determined with C^{14}-Labeled Substrates. J. Dairy Sci., 44: 579. 1961.
(86) MORGAN, M. E., AND NELSON, F. E. The Distribution of Certain Amino Acids in Soluble Fractions of Milk Cultures of *Streptococcus lactis*. J. Dairy Sci., 34: 446. 1951.
(87) MUELLER, H. F., NELSON, W. O., AND WOOD,

W. A. The Effect of Copper upon the Activity of Bacteria Associated with Swiss Cheese Manufacture. J. Dairy Sci., 35: 929. 1952.

(88) NAMBUDRIPAD, V. K. N., GONSALVES, A., LAXMINARAYANA, H., AND IYA, K. K. Studies on the Nutrition of Lactic Acid Bacteria. Part II. Vitamin Requirements. Indian J. Dairy Sci., 10: 157. 1957.

(89) NAMBRUDRIPAD, V. K. N., LAXMINARAYANA, H., AND IYA, K. K. Synthesis of Growth Factors by Lactic Acid Bacteria in Dahi (Indian Fermented Milk). Proc. 14th Intern. Dairy Congr., Rome, 1: 388. 1956.

(90) NISHIKAWA, I. Studies on the Proteolytic Decomposition in Cheese. Part I. Proteolytic Properties of Starter Cultures. Rept. Research Lab., Snow Brand Milk Prod., Japan, 33: 7 pp. 1957.

(91) NIVEN, C. F., JR. Nutrition of *Streptococcus lactis*. J. Bacteriol., 47: 343. 1944.

(92) NOVIKOVA, G. A., PETROVA, E. A., USHAKOVA, V. I., AND FEOFILOVA, E. P. Production of Diacetyl and Acetoin in Lactic Streptococci. Trudy Inst. Mikrobiol. Akad. Nauk S.S.S.R., 1959: 87. 1959.

(93) NURMIKKO, V. Phenylalanine as a Precursor in the Biosynthesis of Folinic Acid (Citrovorum Factor) in Lactic Acid Bacteria. Suomen Kemistilehti, 28B: 62. 1955.

(94) OLIVER, W. H. The Stability of Some Bacterial Enzymes Toward Heat and Chemical Bactericides. J. Gen. Microbiol., 7: 329. 1952.

(95) OLIVER, W. H. The Effect of Carbon Dioxide Pressure on a Bacterial Decarboxylase System. J. Gen. Microbiol., 8: 38. 1953.

(96) OVERCAST, W. W., AND ALBRECHT, T. W. Gas Production in Cheddar Cheese Caused by *Leuconostoc citrovorum*. J. Dairy Sci., 35: 554. 1952.

(97) OXFORD, A. E. Diplococcin, an Antibacterial Protein Elaborated by Certain Milk Streptococci. Biochem. J., 38: 178. 1944.

(98) PETTE, J. W., AND LOLKEMA, H. Yoghurt. II. Groeifactoren voor *Sc. thermophilus*. Netherlands Milk Dairy J., 4: 209. 1950.

(99) PLATT, T. B., AND FOSTER, E. M. Products of Glucose Metabolism by Homofermentative Streptococci Under Anaerobic Conditions. J. Bacteriol., 75: 453. 1958.

(100) PROUTY, C. C. Amino Acid Requirements of *Leuconostoc citrovorum* and *Leuconostoc dextranicum* Isolated from Dairy Starters and Cultured Milk Products. J. Dairy Sci., 44: 2100. 1961.

(101) PULAY, G., TOTH, S. N., AND BAKOS, A. R. Die Bedeutung des Eisens im Stoffwechsel einiger in der Milchindustrie vorkommenden Mikroorganismen. Proc. 15th Intern. Dairy Congr., London, 2: 775. 1959.

(102) RAVEL, JOANNE M., AND SHIVE, W. Arginine Inhibition of Glutamate Utilization. Biochem. et Biophys. Acta, 18: 535. 1955.

(103) REITER, B., AND ORAM, J. D. A Note on the Carbon Dioxide Requirements of *Streptococcus lactis* Strain ML$_3$. J. Dairy Research, 28: 175. 1961.

(104) REITER, B., AND ORAM, J. D. Nutritional Studies on Cheese Starters. I. Vitamin and Amino Acid Requirements of Single Strain Starters. J. Dairy Research, 29: 63. 1962.

(105) SANDINE, W. E., ELLIKER, P. R., AND HAYS, HELEN. Cultural Studies on *Streptococcus diacetilactis* and Other Members of the Lactic Streptococcus Group. Canadian J. Microbiol., 8: 161. 1962.

(106) SANDINE, W. E., SEITZ, E. W., AND ELLIKER, P. R. Studies on the Mechanism of Diacetyl Synthesis by *Streptococcus diacetilactis*. J. Dairy Sci., 44: 1158. 1961.

(107) SEITZ, E. W., SANDINE, W. E., DAY, E. A., AND ELLIKER, P. R. Studies on Factors Affecting Diacetyl Production by *Streptococcus diacetilactis*. J. Dairy Sci., 44: 1159. 1961.

(108) SEITZ, E. W., SANDINE, W. E., ELLIKER, P. R., AND DAY, E. A. Enzymatic Destruction of Diacetyl by Bacteria. Bacteriol. Proc., 1962: 23. 1962.

(109) SHAHANI, K. M. Comparison of Nucleic Acids and Other Phosphorus Compounds of Terramycin-Sensitive and -Resistant Strains of *Streptococcus lactis*. J. Dairy Sci., 42: 1659. 1959.

(110) SNELL, E. E. Nutritional Requirements of Lactic Acid Bacteria. Wallerstein Lab. Commun., 11: 81. 1948.

(111) SNELL, E. E., KITAY, ESTELLE, AND MCNUTT, W. S. Thymine Desoxyriboside as an Essential Growth Factor for Lactic Acid Bacteria. J. Biol. Chem., 175: 473. 1948.

(112) SOKOL'SKAYA, A. Vliyanie Povarennoi Soli na Razvitie Molochnokislўkh i Gazoobrazuyushchikh Bakterii. Molochanaya Prom., 16: 19. 1955.

(113) SPECK, M. L., MCANELLEY, J. K., AND WILBUR, JEANNE D. Variability in Response of Lactic Streptococci to Stimulants in Extracts of Pancreas, Liver, and Yeast. J. Dairy Sci., 41: 502. 1958.

(114) SPECK, M. L., AND WILLIAMSON, W. T. The Influence of Stimulatory Peptides on Proteolysis of Milk by Lactic Streptococci. J. Dairy Sci., 41: 705. 1958.

(115) SPENDLOVE, R., WEISER, H. H., AND HARPER, W. J. Factors Affecting the Initiation of Respiration of *Streptococcus lactis*. Appl. Microbiol., 5: 281. 1957.

(116) STORGARDS, T. Tutkimuksia voin Aromiaineiden Muodostumisesta. Meijeritieteellinen Aikakauskiya, 2: 22. 1940.

(117) TÄUFEL, K., AND BEHNKE, V. Untersuchungen zur Bildung von Acetoin und Diacetyl durch Rahmsäuerungskulturen. Nahrung, 4: 197. 1960.

(118) TEVILEVICH, M. B. Vitamin Requirements and Synthesis of Some Group B Vitamins by *Str. lactis* Strains. Trudy Inst. Mikrobiol. Akad. Nauk S.S.S.R., 1959: 80. 1959.

(119) VANDERZANT, W. C., AND NELSON, F. E. Characteristics of an Endocellular Proteolytic Enzyme System of *Streptococcus lactis*. J. Dairy Sci., 36: 1212. 1953.

(120) VANDERZANT, W. C., AND NELSON, F. E. Proteolysis by *Streptococcus lactis* Grown in Milk with and Without Controlled pH. J. Dairy Sci., 36: 1104. 1953.

(121) VANDERZANT, W. C., AND NELSON, F. E. Amino Acids and Peptides in the Protein-Free Fraction of Milk Before and After Incubation with *Streptococcus lactis*. J. Dairy Sci., 37: 790. 1954.

(122) VANDERZANT, W. C., AND NELSON, F. E. Characteristics of Some Endocellular Pep-

tidases of *Streptococcus lactis*. J. Dairy Sci., 37: 795. 1954.

(123) WHITEHEAD, H. R., JONES, P. A., AND ROBERTSON, P. S. The Influence of Carbon Dioxide on the Growth of Lactic Streptococci. J. Dairy Research, 25: 24. 1958.

(124) WILHARM, G., AND CIBLIS, E. Die Bedeutung der Hemmungserscheinungen zwischen *Streptococcus lactis* und *Streptococcus cremoris* für die Neuzüchtung von Säureweckern. Milchwissenschaft, 6: 51. 1951.

(125) WILKOWSKE, H. H., KRIENKE, W. A., ARRINGTON, L. R., AND FOUTS, E. L. Influence of Certain Vitamin K Compounds on Lactic Acid Development in Milk. J. Dairy Sci., 38: 1077. 1955.

(126) WILLIAMSON, W. T., TOVE, S. B., AND SPECK, M. L. Purification and Properties of Extracellular Proteinase Produced by *Streptococcus lactis*. Bacteriol. Proc., 1962: 26. 1962.

(127) WOLK, J., AND TITTSLER, R. P. Effect of Temperature on Acid Production by *Streptococcus lactis* and *Streptococcus cremoris*. J. Dairy Sci., 41: 705. 1958.

(128) ZURAW, E., AND MORGAN, M. E. Acetaldehyde Production by *Streptococcus lactis* and *Streptococcus lactis* var. *maltigenes*. J. Dairy Sci., 35: 483. 1952.

IV. STARTER CULTURE GROWTH AND ACTION IN MILK[1]

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Milk is the substrate most widely used for growth of the lactic streptococci, as evidenced by the volume of cultured milks and cheeses produced. Cultures of these bacteria normally are propagated in milk. In spite of these facts, milk is not the natural habitat of the lactic streptococci; rather, they appear to be of plant origin. Evidently, plants furnish the proper nutrients by environmental conditions present on and within them; also, plants are subjected to contamination from various biological sources (i.e., soil, insects, other microorganisms, etc.). When the lactic streptococci encounter milk as an environment, certain adjustments would appear to be required in their metabolic habits. First of all, the lactenins usually present in milk are more or less inhibitory to many bacteria. However, the nutritive aspects of milk probably are more important in allowing starters to initiate growth. The complement of nutrients in milk is essentially adequate for the lactic streptococci, except that required nitrogen does not exist in forms that are particularly readily available. Milk as it is secreted contains very little nonprotein nitrogen and, thus, nitrogen for the needs of the culture must be obtained primarily by hydrolysis of the proteins. Not all streptococci are endowed with equal proteolytic activity, and this may limit the ability of cultures to obtain their nitrogen in amounts, or forms, necessary for maximum growth rates.

During the production and processing of milk, it may become contaminated with many bacteria capable of rapid growth which, in turn, can be very deleterious to the growth of starters and to the cultured product being manufactured. To prevent this undesirable situation, milk can be heated to destroy the undesirable bacteria. This heating, however, may have marked effects on the nutritive qualities of the milk for the lactic streptococci.

The purpose of this presentation is to collate certain of the information available which might serve as a guide for better utilization of lactic starter cultures in the manufacture of cultured milk products. Owing to limitations of time, only information more directly pertaining to the foregoing introductory comments will be discussed.

HEAT TREATMENT OF MILK AND STARTER GROWTH

To reduce competition from other microorganisms and attenuate lactenin, milk is nearly always heat-treated before being inoculated with starter cultures. The heating also usually results in the milk being more beneficial to starter growth, although this effect does not exist for all magnitudes of heat treatment.

Foster (11) reported that autoclaved (115 C-15 min) milk was markedly superior to milk heated at 80 C for 10 min for growth of lactic streptococci. Extension of autoclaving time beyond 15-20 min resulted in decreased growth-supporting ability of the milk. He concluded that milk normally is deficient in readily available nitrogen sources and that the improved growth in autoclaved milk was the result of partial hydrolysis of the casein.

Greene and Jezeski (13-15) have shown that progressing severity of heat treatments resulted in milk first showing stimulatory properties for cultures; this was followed by the milk being inhibitory on further heating; increased exposure produced a second zone of stimulation which was followed by a second zone of inhibition. The heating exposures and resulting character of the milk were:

62 C for 30 min to 72 C for 40 min—stimulation

72 C for 45 min to 82 C for 10-45 min, or 90 C for 1-45 min—inhibition

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90 C for 60-180 min to 120 C for 15-30 min—stimulation
120 C for longer than 30 min—inhibition

The first zone of stimulation was considered to be caused by a variety of factors acting independently and together. These were: (1) lowering of Eh due to expulsion of oxygen; (2) destruction of heat-labile inhibitors normally present in milk; (3) partial hydrolysis of milk proteins; and (4) the denaturing of serum protein. The first zone of inhibition was due to an excessive concentration of cysteine with the concomitant increase in toxic volatile sulfides. The second zone of stimulation was coincident with a heat-induced diminution of the toxic sulfides.

The association of free sulfhydryl groups with the nutritional qualities of milk was approached from a different viewpoint by Henningson and Kosikowski (21). *Streptococcus pyogenes,* which quickly dies in fresh raw milk or whey, was used as the test culture. These workers observed that the germicidal property of fresh raw whey was lost as the whey was heated, and that the loss of germicidal property was accompanied by the presence of free sulfhydryl groups in increasing concentration. As heating was continued and free sulfhydryls decreased, growth of *S. pyogenes* also decreased.

Evidence, therefore, indicates that heating of milk at certain levels denatures proteins and that such changes, accompanied by the presence of free sulfhydryl groups, are beneficial to bacterial growth. The actual compounds produced which directly cause this increased nutritional quality are still not completely identified. The inhibition, however, caused in milk by various heat exposures appears to reside in the volatile sulfides produced.

Heating of milk has been shown by Auclair and Portmann (3) to result in the formation of formic acid from lactose degradation. The formic acid appeared to be stimulatory for *Lactobacillus lactis.* However, a mixture of milk heated at 90 C and autoclaved milk was better than either alone. Autoclaving was considered to have resulted in diminution of free amino acids by their reaction with lactose and thus result in a decreased ability of the milk to support culture growth.

There is general agreement that certain heat treatments of milk benefit starter culture growth, and that others result in developing inhibitory properties in the milk. There still remains some question concerning the actual causative factors involved in these two opposing properties.

The practical importance of heat effects on milk are quite obvious. For example, dry skimmilk that may be used for preparation of various cultured products, particularly Cottage cheese and buttermilk, should be manufactured without heating to such an extent that its ability to support culture growth would be impaired. The increased use of UHT treatments of milk also may be expected to result in some alteration of the ability of the milk to support culture growth. This type of heat processing possesses many desirable features from the standpoint of plant operations, and it is reasonable to expect that it will be adopted for milk to be made into cultured products. Preliminary data in our laboratories have indicated that care must be exercised that inhibitory properties for starters are not developed in UHT treated milk.

MILK CONSTITUENTS AND STARTER GROWTH

Milk that appears normal in all recognizable properties and constituents often seems to vary in its ability to support growth of starters. Certain investigations have been directed toward obtaining an explanation for this apparent anomaly. Anderson et al. (2) have indicated that normal milk may vary in a nonprotein nitrogen component (designated as a peptide fraction) and that growth rates of many lactic streptococci varied directly with the content of this fraction in milk. There was, however, a variation in the amount of response by different cultures, indicating that nutritional requirements of the cultures were diverse.

Claydon and Fryer (5) studied variations in milk composition, including differences in the proteose-peptone content, due to pasture and dry feeding. The content of the proteose-peptone fraction was not correlated with culture activity. Differences in starter growth in milk from cows on pasture or dry feeding appeared to be dependent on characteristics of the cultures. In any event, the differences were of questionable practical significance.

Stadhouders (32) has noted a stimulatory effect resulting from the H.T.S.T. and steam heating of autumn and winter milk, but considered that precursors of the stimulatory substances were not naturally present in milk. Some inhibitory properties of milk which had been only H.T.S.T. pasteurized were found to correlate with seasonal variations of peroxidase concentrations.

Tevilevich (33) reported that strains of *S. lactis* often are unable to produce normal fermentation in milk secreted during the spring season. Strains that were able to grow properly in such milk, however, were found to require less riboflavin, but more biotin, were able to synthesize nicotinic acid, and had greater proteolytic activity.

The free amino acids present in fresh milk have been reported by Deutsch and Samuelsson (9) and by von Hetzel (22); those reported by Niven (26) to be needed for effective growth of *S. lactis* were found to be present with the exception of phenylalanine and, possibly, cystine. Glutamine, which is stimulatory, also has not been observed to be present in milk. Peptides and phosphopeptides were not detected

in freshly drawn milk that was nearly free from bacteria (9).

The present status of our knowledge does not enable us to predict with accuracy the preferential qualities of various lots of milk with respect to their support of starter growth. However, pooled herd milk that is free from unnatural inhibitors and products of microbiological growth appears to be relatively constant in nutritive qualities for lactic streptococci. Inherent differences in starter cultures result in much greater variability of growth than that caused by different lots of normal milk.

SUPPLEMENTATION OF MILK WITH NUTRIENTS FOR STARTERS

Although free amino acids have been reported to be present in milk, there has been some question regarding their presence in sufficient quantities for optimum culture growth. Bradshaw (4) noted that the addition of amino acids to milk have produced variable results, certain amino acids being somewhat beneficial to some cultures and other amino acids showing marked inhibition. The beneficial results obtained were of relatively low magnitude and of insufficient value to justify the cost of adding the amino acids. Although the inhibitory actions of various amino acids were quite marked, their action was overcome by others (i.e., serine antagonism was overcome by alanine; methionine was overcome by norleucine or threonine). This probably accounts for the apparent absence of inhibition by certain amino acids when milk is supplemented by mixtures of amino acids present in protein hydrolysates and other nitrogenous materials.

Improved growth of starter cultures can be obtained by supplementation of milk with various extracts of plant and animal tissues, as well as with hydrolysates of proteins (1, 12, 18, 24, 31). Cultures vary in their response to supplementation of milk by such adjuncts, slow cultures showing a much greater response than fast ones (1, 12, 31). Actually, the slow cultures grow nearly as well as fast ones when milk is fortified with such extracts. The attributes of such supplements apparently are associated with the more readily available forms of certain nitrogenous compounds. There has been some evidence that peptides contribute much of the growth-stimulatory qualities of certain adjuncts (1, 25, 28).

The supplementation of milk with certain extracts to obtain more active culture growth can be used to advantage in various ways. Speck and Ledford (30) have reported that ripening and cooking times in Cottage cheese manufacture can be reduced markedly by acceleration of starter growth through the addition of pancreas extract to the cheese milk. Similarly, Cheddar cheese manufacture through the hooping operation can also be accelerated; furthermore, the accelerated culture activity usually resulted in greater yields of curd (approximately 10%).

The reduced rate of growth and acid production by antibiotic-resistant strains of *S. lactis* was found by Kennedy (23) to be restored to a rate comparable to their antibiotic-sensitive counterparts by addition of 0.5% pancreas extract to the milk. Antibiotic-resistant cultures could, therefore, be used for manufacturing purposes; otherwise, their growth might be too slow for commercial fermentations.

The activation of lyophilized starter cultures was observed by Speck and Koburger (29) to be greatly accelerated by fortification of milk with 0.2% pancreas extract. Subsequent subcultures in plain milk possessed similar activity, whether the dry cultures were activated at 32 or 22 C, and whether or not in the presence of the pancreas extract. In addition to allowing a reduction in time for preparing active cultures, the fortification of milk was proposed as a means for preparing bulk starters from a dry culture within a 24-hr period. Thus, a dry culture could be grown up in fortified (0.2% pancreas extract) milk in about 8 hr at 32 C. Then this culture could be inoculated into milk for the bulk culture and incubated at 22 C for about 16 hr.

Certainly, many useful applications can be made of accelerated culture growth, and the foregoing are cited only as possible examples of such applications.

PROTEOLYSIS BY STARTERS IN MILK

Proteolytic activities of the lactic streptococci are subtle and manifestations of their proteolysis are relatively obscure. Consequently, the importance of these activities is often not fully appreciated. Presumably, the proteinase system of these microorganisms is needed for them to obtain certain nitrogenous constituents from milk proteins. The ability of lactic streptococci to grow properly in milk may, therefore, be directly related to their proteinase activity.

Harriman and Hammer (19) reported that cultures of *S. lactis* which coagulated milk rapidly caused a greater concomitant increase in soluble nitrogen than did slow-coagulating cultures. This relationship between rate of acid production and proteolysis was observed when cultures were incubated at room temperature, but not when incubated at 30 or 37 C (17). Studies by Williamson and Speck (36) have confirmed that rate of acid production at 32 C by lactic streptococci in milk is not directly related to the amount of proteolysis effected. Qualitative differences in proteinase activity may be related to these observations.

Proteolysis in milk by cultures of lactic streptococci is detectable within the first 4 hr during incubation at 32 C. The properties of the endocellular proteinase and peptidase sys-

tems of the lactic streptococci have been extensively studied by Van der Zant and Nelson (34, 35). An extracellular proteinase was isolated by Williamson et al. (37) from a casein medium after *S. lactis* developed through the logarithmic phase of growth. The enzyme was inducible and was at a maximum concentration only when casein was present in the medium.

Although the relation of proteolysis to nutrition of the lactic streptococci is not clear, there is increasing evidence that proteolysis by these cultures is very important in the manufacture of cultured milk products. Heinemann (20) found that various starters produced quite different curd tension by the time the cultures attained a whey titratable acidity of 0.55%. He attributed these differences to variations in proteolytic activity of the various cultures. Williamson and Speck (36) studied the curd tension produced by cultures in milk without addition of any extraneous coagulator. They observed that different cultures varied markedly in the strength of the curd produced, and that this was not related directly to the proteolytic activity of the cultures. Actually, there appeared to be a rennet-like activity exhibited by the most proteolytic strain. When milk was supplemented with pancreas extract all cultures showed an increased rate of acid production and sharp reductions in proteolysis, and in these cultures the curd tension of the coagulated milk was greatly increased. These activities may be related to the reduced cooking required and increased yield of Cottage cheese curd made with accelerated culture growth. Thus, decreased proteolysis might be expected to permit more rapid coagulation of the less altered protein in the acidified milk. Furthermore, the stronger curd shoulld result in increased yield of curd, due to less loss by shattering.

Differences in proteolytic activities of starters have also been observed to be associated with certain flavors that develop in Cheddar cheese. Emmons et al. (10) found that free amino nitrogen in Cheddar cheese correlated negatively with the degree of bitterness, and was a characteristic of the culture. They postulated that bitterness was due to a deficiency of proteolytic enzymes capable of hydrolyzing bitter primary breakdown products of the cheese protein. Czulak and Shimmin (7) confirmed these observations and also suggested that the bitter flavor resulted from the formation and accumulation of certain polypeptides.

Obviously, our knowledge of the proteolytic enzymes of lactic streptococci is very limited. Much more needs to be learned about this aspect of starter cultures before they can be used most judiciously in the manufacture of cultured products.

BACTERIAL INTERACTIONS AND STARTER GROWTH

The growth of lactic streptococci in milk is affected by many factors which are not inherent properties of milk. The effects of various inhibitors, such as antibiotics resulting from therapeutic treatment of the cow, improper use of sanitizers, rancidity resulting from milk lipase action, and products from growth of antagonistic bacteria are well known for their deleterious effects on culture growth and will not be discussed here. On the other hand, the beneficial attributes of certain bacteria on the growth of starters in milk has not been as well recognized. Improved activity of starters has been reported to result from the growth of *Bacillus subtilis* (27), *Escherichia coli* (16), and *Pseudomonas fluorescens* (6). Since all of these bacteria are commonly found in raw milk, their effects on subsequent growth of lactic starters must be considered when evaluating the nutritional qualities in milk. It also is possible for streptococci to exert very beneficial effects on one another during growth in milk, and this is an important reason for the use of a mixture of strains in the manufacture of cultured dairy products. There is little basis on which symbiotic strains can be selected, other than actual testing of various isolates. Beneficial interactions cannot be predicted solely on the basis of high acid production. Dahiya and Speck (8) found a low-acid-producing lactic streptococcus to be particularly stimulatory to a faster-growing one; the faster one was only slightly beneficial to the slower one. The biologically active material was liberated from the cells during growth, since cell-free medium from the single strain slow culture promoted growth equal to that when the cultures grew as a mixture. More information is needed on the mechanism of symbiosis between strains of lactic streptococci, and ways in which this type of interaction can be used to develop hardy cultures that will allow their most advantageous utilization in commercial fermentations.

The development of cultures of lactic streptococci for use in manufacturing cultured milk products obviously must be dependent on various factors. Such cultures must be selected for their ability to effect certain desirable changes in milk. Limitations in accomplishing these objectives have resulted from our lack of knowledge on certain of the actual changes desired of the cultures. In addition, the ability of cultures to effect certain changes, particularly in nitrogenous constituents, has not been fully understood. There is reason to believe that progress is being made in securing information that will allow more prudent use of lactic starters in the manufacture of cultured milk products.

REFERENCES

(1) Anderson, A. W., and Elliker, P. R. The Nutritional Requirements of Lactic Streptococci Isolated from Starter Cultures. II. A Stimulatory Factor Required for Rapid Growth of Some Strains in Reconstituted

Nonfat Milk Solids. J. Dairy Sci., 36: 608. 1953.
(2) ANDERSON, A. W., PARKER, R. B., AND ELLIKER, P. R. The Nutritional Requirements of Lactic Streptococci Isolated from Starter Cultures. III. Variation in Growth-Promoting Properties of Fresh Whole Milks. J. Dairy Sci., 38: 1083. 1955.
(3) AUCLAIR, J. E., AND PORTMANN, A. Influence du Chauffage du Lait sur le Developpement des Bacteries. Effet Stimulant du Lait Autoclave sur la Croissance de *Lactobacillus lactis* (Role de l'Acide Formique). Revue le Lait, 39: 496. 1959.
(4) BRADSHAW, J. G. The Growth of Lactic Streptococci in Milk as Affected by Added Amino Acids. M.S. thesis, North Carolina State College, Raleigh. 1961.
(5) CLAYDON, T. J., AND FRYER, H. C. Lactic Starter Culture Activity in Milk from Cows on Pasture and in Milk from Cows on Dry Feed. J. Milk and Food Technol., 23: 76. 1960.
(6) CLAYDON, T. J., AND KOBURGER, J. A. Stimulation of Lactic Starter Cultures by Filtrates from Milk Cultures of *Pseudomonas fluorescens*. J. Appl. Microbiol., 9: 117. 1961.
(7) CZULAK, J., AND SHIMMIN, P. D. Further Notes on Bitter Flavour in Cheese. Australian J. Dairy Technol., 16: 96. 1961.
(8) DAHIYA, R. S., AND SPECK, M. L. Symbiosis Among Lactic Streptococci. J. Dairy Sci., 45: 607. 1962.
(9) DEUTSCH, A., AND SAMUELSSON, E. G. Amino Acids and Low-Molecular Amino-Acid Derivatives in Cows' Milk. Proc. 15th Intern. Dairy Congr., 3: 1650. 1959.
10) EMMONS, D. B., MCGUGAN, W. A., AND ELLIOTT, J. A. Nitrogen Distribution in Cheddar Cheese as Related to Bitterness and Strain of Starter Culture. J. Dairy Sci., 43: 862. 1960.
11) FOSTER, E. M. The Effect of Heat on Milk as a Culture Medium for Lactic Acid Bacteria. J. Dairy Sci., 35: 988. 1952.
12) GARVIE, E. I., AND MABBITT, L. A. Acid Production in Milk by Starter Cultures—the Effect of Peptone and Other Stimulatory Substances. J. Dairy Research, 23: 305. 1956.
13) GREENE, V. W., AND JEZESKI, J. J. Studies on Starter Metabolism. I. The Relationship Between Starter Activity and the Predrying Heat History of Reconstituted Nonfat Dry Milk Solids. J. Dairy Sci., 40: 1046. 1957.
14) GREENE, V. W., AND JEZESKI, J. J. Studies on Starter Metabolism. II. The Influence of Heating Milk on the Subsequent Response of Starter Cultures. J. Dairy Sci., 40: 1053. 1957.
15) GREENE, V. W., AND JEZESKI, J. J. Studies on Starter Metabolism. III. Studies on Cysteine-Induced Stimulation and Inhibition of Starter Cultures in Milk. J. Dairy Sci., 40: 1062. 1957.
16) HALL, N. J. Influence of *Bacterium coli* on Starter Production. Proc. Intern. Dairy Congr., (Rome), 2: 221. 1956.
17) HAMMER, B. W., AND PATIL, V. H. Proteolysis by *Streptococcus lactis* with Special Reference to Butter Cultures and Butter. Iowa State Coll., Research Bull. 123. 59-91. 1930.
(18) HANKIN, L. Growth Stimulation of *Lactobacillus casei* by Factors from Animal Tissues. Ph.D. thesis, North Carolina State College, Raleigh. 1954.
(19) HARRIMAN, L. A., AND HAMMER, B. W. Variations in the Coagulation and Proteolysis of Milk by *Streptococcus lactis*. J. Dairy Sci., 14: 40. 1931.
(20) HEINEMANN, B. Influence of Cottage Cheese Starter Organisms on Strength of Curd at Time of Cutting. J. Dairy Sci., 40: 437. 1957.
(21) HENNINGSON, R. W., AND KOSIKOWSKI, F. V. A Nutritional Concept of the Germicidal Property of Raw Milk. J. Dairy Sci., 40: 818. 1957.
(22) HETZEL, VON H. F. Papierchromatographischer Nachiveis der Freian Aminosauren des Milchserums Nach Vorreinigung uber Ionenaustauscher. Milchwissenschaft, 13: 269. 1958.
(23) KENNEDY, H. E. Effects of Peptide-Rich Extracts on Acid Production by Oxytetracycline- and Penicillin-Resistant Starters. J. Dairy Sci., 44: 844. 1961.
(24) KENNEDY, H. E., AND SPECK, M. L. Studies on Corn Steep Liquor in the Nutrition of Certain Lactic Acid Bacteria. J. Dairy Sci., 38: 208. 1955.
(25) MCANELLY, J. K., AND SPECK, M. L. The Amino Acid Content of a Peptide Stimulatory for *Lactobacillus casei*. J. Bacteriol., 73: 676. 1957.
(26) NIVEN, C. F., JR. Nutrition of *Streptococcus lactis*. J. Bacteriol., 47: 343. 1944.
(27) RICE, E. B. Studies on Starters. VI. Further Observations on Associative Growth of Lactic Streptococci with Other Bacteria. Dairy Ind., 14: 498. 1949.
(28) SANDINE, W. E., SPECK, M. L., AND AURAND, L. W. Identification of Constituent Amino Acids in a Peptide Stimulatory for Lactic Acid Bacteria. J. Dairy Sci., 39: 1532. 1956.
(29) SPECK, M. L., AND KOBURGER, J. A. Activation of Dry Starter Cultures in Milk. J. Appl. Microbiol. (In press.)
(30) SPECK, M. L., AND LEDFORD, R. A. Acceleration of Cottage Cheese Making by Using Starter Culture Stimulants. J. Dairy Sci., 41: 705. 1958.
(31) SPECK, M. L., MCANELLY, J. K., AND WILBUR, JEANNE D. Variability in Response of Lactic Streptococci to Stimulants in Extracts of Pancreas, Liver, and Yeast. J. Dairy Sci., 41: 502. 1958.
(32) STADHOUDERS, J. Seasonal Variation of Starter Activity. Netherlands Milk Dairy J., 15: 358. 1961.
(33) TEVILEVICH, M. B. Vitamin Requirements and Synthesis of Some Group B Vitamins by *S. lactis* Strains. Fiziol. Biokhim. Mikroorg. pp. 80-86. 1959. (Dairy Sci. Abstr., 24: 145. 1962. Abstr. no. 771.)
(34) VAN DER ZANT, W. C., AND NELSON, F. E. Characteristics of an Endocellular Proteolytic Enzyme System of *Streptococcus lactis*. J. Dairy Sci., 36: 1212. 1953.
(35) VAN DER ZANT, W. C., AND NELSON, F. E. Characteristics of Some Endocellular Pep-

tidases of *Streptococcus lactis*. J. Dairy Sci., 37: 795. 1954.
(36) WILLIAMSON, W. T., AND SPECK, M. L. Proteolysis and Curd Tension in Milk Associated with Accelerated Starter Culture Growth. J. Dairy Sci., 45: 164. 1962.
(37) WILLIAMSON, W. T., TOVE, S. B., AND SPECK, M. L. Purification and Properties of Extracellular Proteinase Produced by *Streptococcus lactis*. Bacteriol. Proc. p. 26. 1962.

V. INDUSTRIAL UTILIZATION OF LACTIC CULTURES

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Lactic cultures are employed in the dairy industry primarily for the manufacture of cultured buttermilk, cultured sour cream, and for all types of cheese. An appreciable amount of culture is also used in the manufacture of butter. Starter distillate may be manufactured by the use of lactic culture, or pure cultures of various citric acid–fermenting species may be used. Some margarine manufacturers also use lactic cultures in their manufacturing procedure.

The dairy industry now has at its disposal more lactic cultures than at any other time in the history of the industry. As a result, dairy plants are selecting cultures for specific purposes. For many years the Cheddar cheese manufacturers were interested mainly in the rate of acid production by a lactic culture. Today, they are still interested in acid production, but they also know that some cultures survive the cooking process better than others, that some cultures produce acid more rapidly than others after salting, and that different cultures produce cheese with different body, texture, and flavor characteristics. The manufacturers of Cottage cheese desire a rapid acid-producing culture, but they also want a culture that does not cause floating curd. The manufacturers of cultured skimmilk select a culture on the basis of biacetyl, volatile acid, and carbon dioxide production, acid development, and by the body and texture characteristics of the finished product.

Propagation of lactic cultures. Facilities. Considerable progress has been made in the dairy industry during recent years in providing facilities that prevent the contamination of cultures with bacteriophage and foreign microorganisms. Isolated rooms for the propagation of mother cultures and for the preparation of intermediate and bulk culture have afforded considerable protection to cultures when they are constructed properly and precautions are taken to prevent their contamination.

The location of the culture area is of primary importance. Many areas have been constructed within the plant, or an addition has been made to the plant. The area must be constructed in such a manner that it will not become contaminated from the manufacturing portion of the plant. A culture area close to the manufacturing operation has the advantage that culture does not need to be transported a long distance, but it has the disadvantage of greater susceptibility to contamination. Some two-story plants having the manufacturing operation on the first floor have found it advantageous to construct a culture area on the second floor. A separate ventilating system for the culture area is an absolute necessity and the system should have sufficient air intake from the outside to create a positive pressure on the area. In the construction of the area and in the selection of equipment, thought must be given to the use of materials that can be cleaned easily and that do not deteriorate when treated with the common bactericides.

Equipment for preparation of bulk culture, even though confined to a culture area, should be selected and operated in such a manner as to prevent all possible contamination. Culture vats with ports, rather than hinged covers are recommended. The air entering the vat after heating should be considered as a possible source of contamination and means should be taken to purify the air by the use of incinerators, passing the air through a bactericidal solution, etc.

The person in charge of the culture area must take all precaution to prevent contamination of the area by personnel entering or leaving the area. In most of the culture areas, the work can be done by one person. Removal of culture from the area, filling and emptying vats, etc., can be done with controls outside of the area.

The detection of culture contamination is not as simple as it might seem. Usually, we think of the common culture contaminants as aerobic spore formers, yeasts, molds, and coliform bacteria. Contamination with various strains of lactic acid–producing streptococci must also be considered. Many plant personnel have found that their facilities for culture propagation, and their techniques, are inadequate for propagating pure cultures of *Leuconostoc citrovorum*. The common contaminant encountered is a lactic acid–producing organism. A good control procedure in the culture laboratory is to determine how long a sample of

sterile milk can be transferred from one flask to another without contamination. If it is difficult for a plant to avoid bacterial contamination, it would be even more difficult to avoid bacteriophage contamination.

Frequency of propagation. At one time it was thought that a lactic culture should be propagated each day, to maintain its activity. Dahlberg and Ferris (6) noted that when lactic cultures were propagated every day or every third day, the appearance, flavor, and acid development were identical. When the cultures were inoculated into milk and incubated at 86 F, acid development was rapid and the same for one-day and three-day-old cultures. In actual cheese manufacture, a freshly coagulated culture produced acid more rapidly in the curd than a three-day-old culture. Cheese manufactured with cultures transferred daily developed more flavor, were of better quality, and ripened in less time than that made with cultures transferred every third day. The old cultures produced cheese with unclean flavors.

Olson et al. (17) state that cultures can be stored at 40 to 50 F for five days or longer without decreasing their rates of acid production in cheese making. Their data indicated that this temperature range was more favorable for storage than lower temperatures such as 32 or 0 to −10 F. The addition of a small amount of calcium carbonate to the containers of milk used for culture propagation prolonged the time that cultures remained active at 40 to 50 F. They recommended that cultures should be propagated at least once after removal from storage, before being used in cheese manufacture.

A number of commercial plants propagate mother cultures two or three days per week. However, these plants generally prepare an intermediate culture from the mother culture and the milk used for bulk culture or cultured buttermilk is inoculated with the intermediate culture. Fewer transfers of a mother culture has certain advantages. There is less likelihood of contamination, less mutation among strains, and a greater time elapse before one strain becomes dominant in a multiple-strain culture.

Culture renewal. The frequency of renewal of cultures in a plant depends upon the ability of plant personnel to maintain the cultures in an uncontaminated condition. It also depends upon the rate of growth of each strain within a multiple-strain culture, and upon their rate of mutation. Some plants having difficulties with bacteriophage can maintain a satisfactory culture for only one or two propagations. Other plants obtain satisfactory results with a culture for several months or even longer. With multiple-strain cultures, renewal should be at least every 2 wk, to prevent strain dominance within the culture. Frequent renewal of cultures is practiced by dairy plants using a culture rotation system. Such plants find that it is more economical to purchase a new culture rather than to propagate a culture that will not be used in the rotation for several days. The number of plants which use cultures but which do not propagate mother cultures is increasing. These plants use a commercial culture to prepare an intermediate culture, and the intermediate culture is used to inoculate the bulk culture.

Control of bacteriophages. Bacteriophages are a common cause of slow acid development by lactic cultures. Moseley and Winslow (15) examined samples of milk, lactic culture, and whey which were obtained from 91 cheese factories in 20 states. The samples were obtained at the time of slow acid development during cheese manufacture. Their study involved 101 cases of culture failure. They also analyzed samples taken from 23 vats in 20 cheese factories that were not experiencing difficulty with slow acid development. Bacteriophages were detected in 93% of the samples taken from plants that had culture failures and in 74% of the samples taken from plants in which acid production was normal. This study, as well as others, have emphasized the importance of bacteriophages as contaminants of dairy plants and their role in slow acid development by lactic cultures.

Use of special media. A survey of the literature indicates that a large number of substances have been tested for their effect on various bacteriophages. Many of the substances tested were added to growth media for the purpose of finding a compound that would permit growth of the bacterial culture but not of the bacteriophage active against it. Although a large number of compounds have been found to inhibit bacteriophages, the inhibition is not sufficient to prevent considerable cell lysis. A compound that might be useful for inhibiting bacteriophages during laboratory investigations might have toxic properties and its use would not be permitted in foods.

Graham and Nelson (8) examined 110 compounds for their effect on lactic streptococcus bacteriophage. Many of the substances were inhibitory to bacteriophage, but most of them were also toxic to the host organism.

The first step in the development of bacteriophage involves the adsorption of the virus particle to the host cell. Adsorption is generally thought of as the attachment of the phage particle to the host cell and the liberation of phage DNA into the interior of the host cell. For a certain bacteriophage-host cell combination, there is an optimum cationic concentration for maximum adsorption. Many of the bacteriophages that have been studied have a definite requirement for calcium ion and this is true of the lactic streptococcus bacteriophage. Cherry and Watson (2) obtained the greatest adsorption when a medium contained 0.02 M

calcium chloride. Collins et al. (3), working with a chemically defined medium, found that eight of the ten lactic streptococcus bacteriophages studied needed soluble calcium salts to multiply on their host cells.

When it became known that calcium ions played an important role in phage adsorption, much research was conducted with compounds which bind calcium ions. In the dairy industry, milk is an important culture medium, especially for lactic cultures. Therefore, it appeared feasible for research workers to study the ability of various compounds to bind the calcium ions in milk and to investigate methods for the removal of calcium.

In 1950, Collins et al. (3) suggested that a medium might be developed for the propagation of many strains of the lactic group of streptococci, with lessened danger of bacteriophage action, from chemically defined nutrients containing no calcium, or by using complex nutrients low in calcium content and supplemented with sufficient phosphate or other calcium-binding ions.

At the Annual Meeting of the American Dairy Science Association in 1959, Hargrove (9) presented a method for limiting bacteriophage development in milk by adding phosphates to bind the calcium. His studies indicated that bacteriophages active against lactic streptococci did not develop in skimmilk containing 2% sodium phosphates (a mixture of three parts $NaH_2PO_4 \cdot H_2O$ and two parts Na_2HPO_4), whereas the bacterial culture grew normally. Kadis and Babel (11) noted that the addition of 2% phosphate decreased the rate of growth of most of the lactic cultures studied by them. Also, the added phosphates failed to prevent the development of bacteriophages active against six of the nine cultures employed in the study.

More recently, Hargrove et al. (10) published data which showed that the kind and concentration of phosphate, pH, and heat treatment had a marked effect on phage inhibition and on the free calcium content of milk. Most of the phage types tested were suppressed by 2% orthophosphate salt (mixture of 30 g KH_2PO_4 and 20 g Na_2HPO_4), but the most resistant types required 3%. Heating the milk after adding phosphate was essential to sufficiently bind most of the free calcium. Usually, the free calcium content of the treated milk ranged from 10 to 30 ppm. The best combination for phage inhibition, minimum precipitation, and economy was obtained when milk was heated with 1.7% orthophosphate salt, pH 6.6, followed by the addition of 0.3% tetrapotassium pyrophosphate.

Galesloot (7) conducted studies with oxalate, orthophosphate, pyrophosphate, and polyphosphate as calcium-binding agents for milk. The use of oxalate gave milk which inhibited bacteriophage development to a greater extent than when the milk was treated with phosphate. Olson (16) also noted that milk treated with 0.5% ammonium oxalate could be used successfully in propagating bacteriophage-infected lactic cultures.

The effectiveness of ammonium oxalate, salts of ethylenediamine tetraacetic acid, and sodium tripolyphosphate in limiting bacteriophage development in milk was studied by Kadis and Babel (12). Of these compounds, ammonium oxalate was the most suitable. Five of the six test cultures used grew well in milk treated with 0.5% oxalate and bacteriophage development did not occur.

Milk, from which the calcium was removed, was suggested for use in propagating lactic cultures by Reiter (18). The product was dried and called PRM (phage-resistant medium). PRM did not permit phage development and lactic cultures were said to grow well in the reconstituted medium. Czulak and Koegh (5) checked the performance of PRM under practical conditions in two Australian factories. A number of single-strain cultures showed reduced acid development in PRM, but the medium was very effective in preventing bacteriophage development. Babel (1) reported that bacteriophages were unable to multiply or cause cellular lysis in PRM when reconstituted with distilled water. Lactic cultures varied in their ability to grow in PRM and growth was not as abundant as in regular skimmilk. Crawford et al. (4) reported that they propagated a single-strain culture successfully in PRM and used the culture for 25 days in a creamery without rotation. The creamery had a heavy infection of bacteriophage.

All of the methods which have been suggested for preventing bacteriophage development in milk have certain limitations. Certain host-virus combinations evidently require only traces of calcium for phage adsorption. There is also some possibility that other cations can replace calcium in permitting adsorption. Further research on the most promising of the calcium-binding agents would be of value to the dairy industry. Also, further improvement in the nutritive properties of a dried calcium-free milk might result in a very useful product.

Culture rotation. Whitehead and Hunter (19) suggested a culture rotation system for controlling bacteriophage in cheese plants. Unrelated strains of lactic streptococci were suggested for use in such a system and each culture in the rotation would be used every four days. Such a procedure would permit considerable dilution of a phage type within the plant because of several clean-ups and applications of bactericide before the culture was used again.

Several dairy plants in the United States follow a culture rotation system. The success of such a system depends upon the accuracy of phage typing the strains used in the rotation. Once a rotation is established, it cannot

be neglected. The development of new phage types within a plant may necessitate a change in the rotation plan and the addition and withdrawal of cultures from the rotation. At the present time, many of the bacteriophages encountered have a rather wide range of host specificity.

Plant sanitation. In any culture control program, sanitation plays a vital role. Many of the culture failures in plants occur at the time of maximum production. During this period sufficient time may not be permitted for a thorough clean-up and effective bactericidal treatment of equipment. The failure of management to provide the necessary time and personnel to maintain proper sanitation procedures is a serious mistake. The concentration of bacteriophage in a plant increases as the duration of production is increased and an expanded sanitation program should coincide with an expanded production schedule.

Standardization of flavor in cultured buttermilk, cultured sour cream, and creamed Cottage cheese. The standardization of flavor in cultured buttermilk and cultured sour cream is not difficult. With these products there is no partition of flavor compounds as there is between the curd and whey in Cottage cheese manufacture. The selection of a suitable culture, one having the ability to ferment citric acid to flavor compounds, is of utmost importance. If the basic principles of culture growth and citric acid fermentation are known by the person responsible for the production of cultured buttermilk and cultured sour cream, little difficulty should be experienced in producing products with adequate flavor from batch to batch.

The amount of biacetyl, volatile acids, and carbon dioxide desired in cultured products varies from one area to another. Frequently, only the selection of a suitable culture is necessary to obtain the desired flavor. It has been definitely established that by increasing the citric acid content of milk or cream more of the flavor compounds can be developed. Standardization of the citric acid content, therefore, plus a controlled culture program can be used to standardize the flavor.

During recent years, pure cultures of *Leuconostoc citrovorum* have been available to the dairy industry. In plants where a general purpose culture is used for cheese manufacture and for cultured products, sometimes the culture is fortified with a pure culture of *L. citrovorum* to increase the amount of flavor compounds in cultured buttermilk and cultured sour cream.

The newly proposed standards for creamed Cottage cheese permit the addition of lactic culture or skimmilk which has been fermented by a culture of *L. citrovorum* or other similar organism. The addition of lactic culture to a Cottage cheese dressing lowers the pH and prevents or delays the growth of a number of psychrophiles. If creamed Cottage cheese containing viable lactic culture organisms is not maintained at low temperatures, a sour product results (13). A method has been published on the standardization of flavor in creamed Cottage cheese by the addition of skimmilk, fermented with *L. citrovorum,* to the creaming mixture (14). A more recent procedure for enhancing the flavor of creamed Cottage cheese makes use of *Streptococcus diacetilactis.* The organism is grown in citrated whey and the fermented whey is added to the creaming mixture.

REFERENCES

(1) Babel, F. J. New Developments in the Propagation of Lactic Cultures: Culture Media and Bacteriophage Inhibition. J. Dairy Sci., 41: 697. 1958.

(2) Cherry, W. B., and Watson, D. W. The *Streptococcus lactis* Host Virus System. II. Characteristics of Virus Growth and the Effect of Electrolytes on Virus Adsorption. J. Bacteriol., 58: 611. 1949.

(3) Collins, E. B., Nelson, F. E., and Parmelee, C. E. The Relation of Calcium and Other Constituents of a Defined Medium to Proliferation of Lactic Streptococcus Bacteriophage. J. Bacteriol., 60: 533. 1950.

(4) Crawford, R. J. M., Gallaway, J. H., and Smillie, D. M. A Note on the Use of a Bacteriophage Resistant Medium in Commercial Cheese Manufacture. Dairy Ind., 24: 183. 1959.

(5) Czulak, J., and Keogh, Barbara. Trials of Phage Resisting Medium. Australian J. Dairy Technol., 12: 54. 1957.

(6) Dahlberg, A. C., and Ferris, F. Influence of the Frequency of Transfer of Lactic Starters upon Rate of Acid Development and Quality of Cheddar Cheese. J. Dairy Sci., 27: 771. 1945.

(7) Galesloot, T. E. The Propagation of Nisin-Producing Starters. Proc. 15th Intern. Dairy Congr. (London), 3(2): 547. 1959.

(8) Graham, D. M., and Nelson, F. E. Inhibition of Lactic Streptococcus Bacteriophage by Crystal Violet and Other Agents. J. Gen. Physiol., 37: 121. 1953.

(9) Hargrove, R. E. A Simple Method for Eliminating and Controlling Bacteriophage in Lactic Starters. J. Dairy Sci., 42: 906. 1959.

(10) Hargrove, R. E., McDonough, F. E., and Tittsler, R. P. Phosphate Heat Treatment of Milk to Prevent Bacteriophage Proliferation in Lactic Cultures. J. Dairy Sci., 44: 1799. 1961.

(11) Kadis, V. W., and Babel, F. J. Effect of Addition of Phosphates to Milk on Development of Bacteriophage and Growth of Lactic Cultures. J. Dairy Sci., 45: 432. 1962.

(12) Kadis, V. W., and Babel, F. J. Effectiveness of Ammonium Oxalate, Salts of Ethylenediamine Tetraacetic Acid, and Sodium Tripolyphosphate in Limiting Bacteriophage Development in Milk. J. Dairy Sci., 45: 486. 1962.

(13) Mather, D. W., and Babel, F. J. Studies

on the Flavor of Creamed Cottage Cheese. J. Dairy Sci., 42: 809. 1959.
(14) MATHER, D. W., AND BABEL, F. J. A Method for Standardizing the Biacetyl Content of Creamed Cottage Cheese. J. Dairy Sci., 42: 1045. 1959.
(15) MOSELEY, W. K., AND WINSLOW, R. L. A Study of Potential Causes of Culture Failures in Cheese Factories. J. Dairy Sci., 42: 906. 1959.
(16) OLSON, H. C. Low-Calcium Media for Control of Bacteriophage in Lactic Cultures. J. Dairy Sci., 43: 439. 1960.
(17) OLSON, H. C., BEACHBOARD, R. F., AND COHENOUR, F. D. The Influence of Propagation Procedures on the Activity of Lactic Cultures. Oklahoma Agr. Expt. Sta., Bull. B-446. 1955.
(18) REITER, B. Inhibition of Lactic Streptococcus Bacteriophage. Dairy Ind., 21: 877. 1956.
(19) WHITEHEAD, H. R., AND HUNTER, G. J. E. Bacteriophage in Cheese Manufacture. Proc. 12th Intern. Dairy Congr. (Stockholm), 2(2): 629. 1949.

VI. CULTURE PRESERVATION

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Interest in the preservation of starter cultures seemingly has intensified during the past decade. We need not speculate on the reasons for this increased interest, but there can be no doubt about the timeliness of this topic.

The ideal preservative method would allow us to take the organisms at the peak of their metabolic activity, to hold them for days or even months in a state of arrested development, and to have them resume their work immediately on restoration to a favorable environment. Unfortunately, this ideal has never been realized, because it is virtually impossible to keep a living organism in a completely inactive state.

Methods of preserving cultures are based on one of two principles: (1) reducing the metabolic rate of the organisms, or (2) separating the cells from their waste products. Bacterial metabolism can be inhibited by reducing the temperature or by removing the available water. Preservation by these methods often can be improved by the addition of glycerol, lactose, milk solids, or other protective materials. The toxicity of acidic waste products can be reduced by adding a buffer, such as calcium carbonate. Alternatively, and preferably, the organisms can be separated from the culture fluid by centrifugation and resuspended in a more favorable medium.

In practice, preservation usually is accomplished by employing a combination of methods. For example, cultures to be lyophilized may first be neutralized to reduce the acidity, then frozen, dried by sublimation, and finally stored at a low temperature.

The choice of preservative method depends in large measure on the purpose for which the culture is to be used. In the dairy plant, it is common practice to refrigerate liquid cultures between transfers. Freezing is useful for extended storage, although shipment of frozen cultures is troublesome. Freeze drying, or lyophilization, has become a common method of preserving individual seed cultures for shipment.

Ultimately, of course, we are interested in whether a culture can be used successfully to make a particular product. However, for evaluating preservative methods it is common practice to apply some kind of activity test, which involves measuring the rate of acid production by the culture under a set of arbitrarily chosen conditions. Unfortunately, it is usually impossible to compare the results of different workers because the methods of testing vary so widely.

Liquid cultures. Most of us will agree with the statement of Hammer and Babel (5), that a normally ripened culture usually can be held at 4 to 8 C for several days without serious change in its activity. At higher temperatures lactic cultures soon lose the ability to produce acid rapidly on transfer (5). Repeated overripening of cultures before refrigerated storage also tends to reduce their activity. For example, Swartling and Lindgren (21) observed that fully ripened cultures often were inactive on subculture after only four days of storage at 5 C, whereas cultures that were incubated barely to curdling before refrigeration were fully active for at least eight days.

For prolonged storage of starter cultures many workers have observed the beneficial effect of adding $CaCO_3$ to the medium. Olson (14) added various insoluble buffers to starter cultures and found that $CaCO_3$ gave the best results. After storage for eight months in the refrigerator two of his cultures had lost activity, one had gained, and two were unchanged. Lindgren and Swartling (9), however, cautioned that holding starter cultures in chalk milk at 3 to 5 C was not a reliable method of preservation for as long as five months.

Heinemann (6) was one of the first to show that glycerol may have a protective effect on starter bacteria. Butter and cheese starters stored at 2 C with 10 or 20% glycerol remained

active up to two months, whereas similar cultures without glycerol lost appreciable activity in this period of time. Olson (14) stated that his cultures maintained fairly satisfactory activity levels for eight months at 7.5 to 10 C when they contained 20% glycerol, 3% salt, and 30% sucrose.

Starter organisms can be preserved in liquid form for several weeks if they are removed from the growth medium and resuspended in a favorable environment. This process naturally is not applicable to cultures growing in milk because the cells can not be separated from the curd. In our laboratory Lamprech (8) has grown *S. lactis* and a *Leuconostoc* culture in broth, harvested the cells by centrifugation, and resuspended them in skimmilk. Samples adjusted to pH 5.0, 6.0, and 7.0, with and without 20% glycerol, were stored at 4 C and tested periodically for viable count and activity.

Both organisms survived better at pH 6.0 or 7.0 than at pH 5.0. Glycerol gave no apparent protection to *S. lactis,* although it was beneficial to the *Leuconostoc* organism. Under the best conditions tried the *S. lactis* suspensions retained about one-half of their original count and over 90% of their original activity for at least 4 wk. After 8 wk the number of viable organisms dropped sharply (Table 1).

TABLE 1

Viability and activity of *Streptococcus lactis* cells stored at 4 C with and without glycerol [a]

Weeks storage	Plate count per milliliter (billions)		Per cent of initial activity	
	With glycerol	Without glycerol	With glycerol	Without glycerol
Initial	27	30		
1	21	31	97	111
2	46	26	94	103
3	14	48	94	100
4	13	17	91	95
5	10	13	86	86
8	10	5	83	78
12	2	1	....	....
21	<1	<1	....	....

[a] *S. lactis* C-2 was grown in broth. The cells were recovered by centrifugation and resuspended in skimmilk at pH 7.0.

Frozen cultures. Freezing can be used to preserve many types of microorganisms (3). Although the freezing process kills part of the cells, it is not unusual to recover at least 75 to 90% of the viable bacteria in a lactic culture after freezing and thawing (7, 12). There is, of course, a gradual diminution in numbers during storage in the frozen state.

Frozen milk cultures of lactic acid bacteria have been held for months and used to propagate new starters. Lindgren and Swartling (9), for example, reported that cultures exhibited essentially all of their initial activity when they were transferred after storage in the freezer for 1 yr. Johns (7) observed a steady decline in the number of viable cells in a frozen culture, but he said that his preparations still were satisfactory for the inoculation of bulk starter as long as the count remained above 500,000 per milliliter, or about three to four months.

Frozen cultures also have been used directly as starter for fermented dairy products (2, 16, 19). Simmons and Graham (19) regularly made good buttermilk and Cottage cheese with frozen culture up to three months old. Activity of the culture compared favorably with that of fresh starters transferred daily. Similarly, Rudnik and Glen (16) used frozen culture up to five months old to inoculate milk directly for Cottage cheese. A total of 39 lots of cheese were made and all were salable. However, the authors did observe that the culture tended to become less active with age.

Freezing does nothing to improve a poor culture. Furthermore, several workers have indicated that the acidity and the age of a culture influence its ability to survive freezing. Johns (7) found it beneficial to neutralize his cultures before freezing. Swartling and Lindgren (21) recorded better performance when cultures were frozen without incubation, or if they were incubated merely to curdling, than when they were fully ripened before freezing.

In our laboratory, also, we have noticed the importance of acidity and physiological age on the activity of frozen cultures. Concentrated suspension of *S. lactis* cells from cultures 15 to 18 hr of age were more active than were cells from older cultures. The rate of freezing and thawing had no effect on survival. However, the organisms survived much better when they were frozen in skimmilk rather than in 0.1% peptone or in distilled water.

Both Heinemann (6) and Richardson (15) have observed a protective effect of glycerol in frozen milk cultures. In our experience glycerol was clearly protective with suspensions of *S. lactis* at pH 5.0, but not at pH 7.0.

At −20 C and pH 7.0 we have held milk suspensions of *S. lactis* and *Leuconostoc* sp. for eight to 12 months without significant loss of viable organisms or acid-producing ability. The suspensions have been used to prepare cultured buttermilk by inoculating pasteurized milk with 20 to 25 million organisms per milliliter in a ratio of 85% *S. lactis* to 15% *Leuconostoc* sp. Acid production was normal even for cells that had been frozen for nine months (Table 2).

Our work with frozen cultures is highly encouraging to one who wishes to preserve the organisms for several months. For maximum activity, cultures in the late logarithmic phase

TABLE 2

Acidity of buttermilk inoculated with frozen starter organisms [a]

Weeks storage at −20 C	Titratable acidity after 17 hr incubation at 21 C
	(%)
1	0.88
3	0.91
5	0.83
14	0.90
42	0.89

[a] *Streptococcus lactis* C-2 and *Leuconostoc* sp. were grown in broth. The cells were recovered by centrifugation, mixed in a ratio of 85% *S. lactis* to 15% *Leuconostoc* sp., resuspended in skimmilk at pH 7.0, and stored at −20 C. After thawing, suspensions were diluted to provide inoculum approximately equal to that of 1% milk culture.

or early maximum stationary phase of growth should be suspended in a favorable medium, such as milk at pH 7.0, before freezing.

Dried cultures. Lactic cultures can be dried successfully either by lyophilization or by spray drying. Freeze drying apparently is the less destructive of the two processes and is readily adapted to the preservation of small amounts of culture.

Freeze drying. This process is widely applied in the preservation of stock cultures (2), and it has been employed for years in the distribution of dairy organisms. Freeze dried cultures can be used for months or years as the seed material for vigorous, active starters (4, 11).

Until recently, little attention was paid to the number of organisms that survived the drying process, most investigators being satisfied if there were enough survivors to initiate growth readily on subculture. In 1959, however, Schubert (18) published figures showing that 17 to 75% of the cells of several lactic streptococci remained alive after lyophilization. McAnelly (12) in our laboratory observed marked differences in the ability of several strains of *S. lactis* and *S. cremoris* to endure the drying process. He obtained average survival values of 42 to 80%, although individual results ranged from 10 to 90%. Yano et al. (23) reported survival values of 60 and 40%, respectively, for *S. lactis* H-61 and *L. bulgaricus* B-1.

Several factors, including age of culture and the nature of the suspending medium, influence the ability of lactic organisms to survive freeze drying (12, 22). Watts (22), for example, lyophilized a milk culture at various stages in the growth cycle up to 19 hr. Samples dried at 9 and 12 hr, which represented the late logarithmic and early maximum stationary phases of growth, respectively, showed the highest survival values—76 and 84%. On rehydration their acid-producing ability approached that of the undried culture. Neutralizing the culture before drying was not beneficial.

Once they are dried, lactic organisms must be protected from moisture and kept at a low temperature if they are to remain alive and active. According to Yano et al. (23), death during storage is logarithmic. In the range of 10 to 37 C they obtained Q_{10} values of 2.2 to 3.5 for the death rates of several lactic organisms. Cells stored at 0 C for eight to 15 months showed a gradual increase in the lag phase following rehydration, but acid development returned to normal on subculturing (13).

Watts (22) observed highly significant decreases in activity of a lyophilized culture within 2 wk at 25 C and within 4 wk at 5 C. Cultures stored at −28 C showed no significant change in activity within 12 wk. Buttermilk and Cottage cheese prepared by direct inoculation of the milk with Watts' lyophilized culture that had been stored for 22 wk at −28 C was equal in quality to similar products made with a fresh, active culture.

Spray drying. Several investigators have considered the possibility of drying large quantities of culture, with the idea of using it to replace the usual liquid bulk starter in the production of fermented dairy products. For this purpose spray drying would be preferable to freeze drying because of the lower cost.

Prior to 1955, Mamaeva (10) spray-dried a mixture of lactobacilli and yeasts that was used for the manufacture of koumiss, but recovered only a fraction of 1% of the cells in a viable condition. Nevertheless, on rehydration the organisms grew rapidly and showed acceptable rates of gas and acid production. Under the best storage conditions tried, the dried culture was said to be sufficiently active for use up to about six months. Tablets prepared from spray-dried culture are said to be available in Russia for the preparation of yogurt in the home (1).

Attempts to spray-dry ordinary milk cultures of lactic acid bacteria have not been very encouraging. After two trials, Richardson (15) abandoned the process because the product was difficult to rehydrate and it was less active than lyophilized culture. In a more extensive study, Sapp and Hedrick (17) spray-dried a large number of cultures at 16 hr of age, when the acidities were 0.80 to 0.85%. To measure the effect of experimental variables the authors ran an activity test, which involved inoculating 1 g of the dry culture into 99 ml of pasteurized skimmilk and titrating the acidity after 16 hr at 72 F.

To obtain cultures with sufficient activity, it was necessary to operate the dryer at an outlet air temperature of 135 to 165 F. Neutralization of the cultures before drying reduced rather than increased the activity of the dry product. Cultures dried at 12, 16, and 24 hr of age showed practically the same activity, but cultures dried at 8 hr were less active.

In our laboratory we have spray-dried several strains of lactic streptococci with fair success. *S. lactis* C-10, the culture used for most experiments, consistently gave survival values of 53 to 63%. For these trials we grew the organism in a broth medium, recovered the cells by centrifugation, and resuspended them in 5% nonfat dry milk adjusted to pH 7.0. Rehydration of the dry culture was not difficult.

Under our conditions of drying we have found consistent differences between the survival values of *S. lactis* and *S. cremoris*. As Table 3 shows, *S. lactis* cultures were more resistant both to spray drying and to storage in the dry state.

TABLE 3

Survival of *Streptococcus lactis* and *Streptococcus cremoris* during spray drying and storage [a]

Organism	Per cent survival [b]	
	After drying	After 14 days storage
S. lactis C-10	53	49
S. lactis 16	48	49
S. cremoris 1	18	10
S. cremoris HP	9	2

[a] The cultures were grown in broth. The cells were recovered by centrifugation and resuspended in 5% nonfat dry milk at pH 7.0. The inlet air temperature of the dryer was 78 to 79 C. The dry cultures were stored under nitrogen at −18 C.

[b] Per cent of viable cells in the original suspension.

Spray-dried cultures die rapidly at temperatures above freezing. Within 1 wk at 72 F or above, the activity of samples prepared by Sapp and Hedrick (17) dropped below 0.70, which was considered the minimum satisfactory value. Most of their preparations still were active after 1 wk at 40 F, but none was satisfactory after 3 wk. Dry cultures stored at −15 F retained their initial activity much longer.

Our results with *S. lactis* C-10 confirm, in principle, those of Sapp and Hedrick. Under our conditions this organism better survived both spray drying and storage if it were dried in 5% nonfat dry milk than if it were dried in the dextrin–ascorbic acid–thiourea diluent described by Splittstoesser and Foster (20). Addition of phosphate to the milk suspension was harmful to the organisms. Packaging under nitrogen allowed slightly better survival than packaging in air. Within the range 2.4 to 4.4% the moisture content of the product had no effect on survival during storage for four months. Table 4 shows typical results for *S. lactis* C-10. Under the best conditions we have been able to store the dry culture for at least four months without losing more than 15% of the original viable count.

TABLE 4

Effect of storage conditions on survival of spray-dried *Streptococcus lactis* [a]

Storage conditions		Per cent survival [b]	
Temperature	Atmosphere	After drying	After 60 days
4 C	Nitrogen	53	37
4 C	Air	53	23
−18 C	Nitrogen	53	48
−18 C	Air	53	38

[a] Organisms were dried as described in Table 3.

[b] Per cent of viable cells in the original suspension.

Like others, we have been interested in the performance of our cultures after rehydration. To measure activity we have inoculated milk with a known number of viable dry cells and followed their development by both plate counts and acid titrations. Plotting the resulting values provides curves that can be compared with similar ones obtained with a fresh, active culture.

In our experience the shapes of the curves prepared for the dried cultures are practically identical to those for fresh cultures once growth begins. The only appreciable difference is in the length of the lag phase. The difference in activity between dried cells and fresh cells seems to be an inherent characteristic of the particular dried culture. For example, with one lot of powder the lag of the dry culture was 5 hr longer than that of the fresh culture. Once growth began, however, both produced acid at essentially the same rate and to the same extent. With two other lots of powder the lag periods of the dry cultures were only one-half to one hour longer than those of the fresh cultures. These differences, apparent immediately after drying, seem to persist throughout storage.

Though our work is not extensive, we are encouraged to believe that it is possible to spray dry lactic organisms without seriously impairing their ability to grow and produce acid rapidly on rehydration. Furthermore, the dry cultures can be stored under nitrogen in the freezer for at least several weeks without significant loss of viability or acid-producing capacity.

SUMMARY

Small amounts of culture can be preserved for short periods simply by refrigeration, or for longer periods by freezing or lyophilization. Best results are obtained if the cultures are young and vigorous when they are frozen or dried.

Large quantities of culture can be frozen successfully and held for several months without undue loss of activity. Attempts to spray-dry

milk cultures for direct use as the inoculum in making fermented products have not been encouraging. Starter cultures can, however, be grown in broth, the cells can be recovered by centrifugation, and resuspended in skimmilk at pH 7.0. The resulting suspension can be frozen or spray-dried without excessive loss of viable organisms. According to present knowledge, dry cultures must be stored at freezing temperatures to prevent excessive loss of activity. This requirement reduces any advantage that drying might have over freezing when applied to highly concentrated cell suspensions.

Additional work is needed on the effect of glycerol and of neutralizing the acidity of cultures before freezing or drying. Reports in the literature give conflicting indications.

REFERENCES

(1) BANNIKOVA, L. A. New Method for the Preparation of Starter Cultures. (In Russian) Doklady Vsesoyuz. Konf. moloch. Delu, p. 361. 1958. (Dairy Sci. Abstr., 21: 109. 1959.)

(2) CARDWELL, J. T., AND MARTIN, J. H. Uniformly Good Cultured Buttermilk. Milk Dealer, 48(12): 58. 1959.

(3) CLARK, W. A. The Use of Low Temperatures in Preserving Some Microorganisms and Other Biological Materials. Proc. Low Temperature Microbiology Symposium. 1961. p. 285-293. Campbell Soup Company, Camden, N. J.

(4) CZULAK, J., AND HAMMOND, L. A. Freeze-Dried Starter Cultures. Australian J. Dairy Technol., 8: 89. 1953.

(5) HAMMER, B. W., AND BABEL, F. J. Bacteriology of Butter Cultures: A Review. J. Dairy Sci., 26: 83. 1943.

(6) HEINEMANN, B. Preserving the Activity of Lactic Cultures. J. Dairy Sci., 41: 705. 1958.

(7) JOHNS, C. K. Preserving the Activity of Frozen Cheese Starter Culture. Canadian Dairy and Ice Cream J., 35(1): 32. 1956.

(8) LAMPRECH, E. D. Production and Storage of Dairy Starter Organisms. Ph.D. thesis, University of Wisconsin. 1962.

(9) LINDGREN, B., AND SWARTLING, P. Maintenance of Active Lactic Cultures. (In Swedish). Medd. Svenska Mejeriern. Riksfören, No. 61, 1960. (Dairy Sci. Abstr., 23: 379. 1961.)

(10) MAMAEVA, P. Dry Koumiss Culture. (In Russian) Konevodstvo, 25: 46. 1955. (Dairy Sci. Abstr., 18: 639. 1956.)

(11) MAXA, V., AND TEPLY, M. The Activity of Dried Dairy Cultures and Their Use Under Manufacturing Conditions. (In Czech). Prumysl Potravin, 11: 589. 1960. (Dairy Sci. Abstr., 23: 230. 1961.)

(12) MCANELLY, J. K. The Effects of Drying on Survival of *Streptococcus lactis*. Ph.D. thesis, University of Wisconsin, 1959. (L. C. Card No. Mic. 58-5479) 96 pp. Univ. Microfilms. Ann Arbor, Michigan. (Dissertation Abstr., 20: 4245. 1960.)

(13) MORICHI, T., YANO, N., IRIE, R., AND KEMBO, H. Studies on the Lyophilization of Lactic Organisms. II. Physiological Characteristics and Acid Producing Activities of Lyophilized Cultures. (In Japanese). J. Agr. Chem. Soc. Japan, 35: 150. 1961. (Dairy Sci. Abstr., 23: 380. 1961.)

(14) OLSON, H. C. Preservation of Lactic Cultures. J. Dairy Sci., 42: 388. 1959.

(15) RICHARDSON, G. H. Studies Involving the Preservation of a Lactic Culture. Ph.D. thesis, University of Wisconsin. 1960. (L. C. Card No. Mic. 60-3255) 145 pp. Univ. Microfilms. Ann Arbor, Michigan. (Dissertation Abstr., 21: 429. 1960.)

(16) RUDNIK, A. W., JR., AND GLENN, W. E. The Use of Frozen Culture as a Direct Inoculum for Making Cottage Cheese. J. Dairy Sci., 43: 845. 1960.

(17) SAPP, C. W., AND HEDRICK, T. I. Factors Affecting the Activity of Spray-Dried Cheese Culture. Quart. Bull. Mich. Agr. Expt. Sta., 43: 96. 1960.

(18) SCHUBERT, W. F. Freeze-Drying of Starter Cultures. Queensland J. Agr. Sci., 16: 409. 1959.

(19) SIMMONS, J. C., AND GRAHAM, D. M. Maintenance of Active Lactic Cultures by Freezing as an Alternative to Daily Transfer. J. Dairy Sci., 42: 363. 1959.

(20) SPLITTSTOESSER, D. F., AND FOSTER, E. M. The Influence of Drying Conditions on Survival of *Serratia marcescens*. Appl. Microbiol., 5: 333. 1957.

(21) SWARTLING, P., AND LINDGREN, B. Must Starters Be Subcultured Every Day? (In Swedish). Svenska Mejeritidn., 52: 631. 1960. (Dairy Sci. Abstr., 23: 230. 1961.)

(22) WATTS, J. C. A Study of the Factors Influencing the Activity of a Lyophilized Lactic Starter. Ph.D. thesis, University of Wisconsin. 1955.

(23) YANO, N., MORICHI, T., IRIE, R., AND KEMBO, H. Studies on the Lyophilization of Lactic Starter Organisms. I. Survival in the Lyophilization Process and Death of the Dried Bacteria During Storage. (In Japanese). J. Agr. Chem. Soc. Japan, 34: 1046. 1960. (Dairy Sci. Abstr., 23: 380. 1961.)

ASSOCIATION AFFAIRS

RESEARCH AND EDUCATION IN A FREE SOCIETY [1]

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I am privileged to join with you in commemorating the centennial of our Nation's Land-Grant College system and the Department of Agriculture.

It is doubtful if any of the founding fathers could have foreseen the tremendous impact of their action a hundred years ago in setting up these institutions. The expectations of 1862 have been realized beyond the most extravagant dreams of those who created them.

We know, of course, that these institutions have given us the means to expand agricultural knowledge and to apply it for the welfare of mankind. But they have also altered the structure of our national economy and shown to the world what research and education can accomplish in a free society.

I should like to look briefly into the origins of these two institutions that have so greatly affected our nation and much of the world. Then, I should like to point to some of the achievements in research and education over the years . . . and some of the problems we are trying to solve in these two areas.

The 17th and 18th Centuries in our country reflected a growing consciousness of the need for experimentation in farming. Enterprising farmers were using seeds and plants from other countries to grow better crops and a greater variety. Agricultural societies sprang up to disseminate general scientific information and to encourage experimentation. Agricultural journals began to be published. And by 1813, several states appropriated funds for agricultural experiments.

This growing need to experiment—combined with a favorable political, cultural, and social climate—produced a remarkable concept of research and education. This concept found expression in the establishment of the institutions we are honoring today.

Education—according to this unique idea that gained support through the years—should be made available to all, not merely the elite. Science rather than tradition should be the basis for farming operation. Research and education—so went the thought—are too deeply interwoven to go their separate ways and must, therefore, function closely together.

These were striking new thoughts—radical departures from accepted ideals of education and farming.

The concept grew and took firm roots in a free and inventive society. At the same time, agriculture was becoming commercialized. Food processing advanced, transportation improved, and farmers' problems became more complex. Aid to agricultural education and a Federal agency to serve farmers were sought eagerly on all sides.

The proposals gained support. And, in 1862, both were realized in the establishment of the land-grant colleges and the Department of Agriculture.

Although the scope of the law setting up the Department was broad, the organizational structure and activities evolved gradually during the course of a century. Research and education continued as major concerns through the years. But the Department was given new responsibilities from time to time in response to changing needs and demands of the people.

Meanwhile, growth of the land-grant colleges proceeded rapidly, in the face of many difficulties. Perhaps the most important was the lack of exact knowledge in agricultural subjects. To meet this need, the Hatch Act—passed in 1887—made Federal funds available to the states to operate an agricultural experiment station as part of each land-grant institution. Thus, the groundwork was laid for state and federal cooperation in agricultural research.

After the Hatch Act came other federal provisions to supplement state appropriations and to extend and strengthen agricultural research. The Adams Act of 1906 provided for original research and experiments . . . the Purnell Act of 1925 strengthened economic and social research . . . the Bankhead-Jones Act of 1935 provided additional financial support to the state experiment stations and made available special funds for basic research conducted largely at nine regional laboratories throughout the country . . . and the Research and Marketing Act of 1946 further strengthened research on production and utilization of farm products and expanded research and services relating to marketing and distribution.

These laws provided the framework within which the Department and the land-grant colleges grew and expanded through the years. These schools continually modified and adjusted their programs to fit man's ever-changing environment. In doing so, they made major contributions to our national welfare.

The land-grant system had much to do with our rapid advance in scientific research and development, with our great material progress,

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and with making our agriculture the most productive in the world. In fact, probably no single institution in the country has contributed more richly or permanently to the development of our resources and to the enlightenment of our people generally. For example, the land-grant schools enroll more than 20% of the nation's college students and grant 40% of the doctoral degrees.

Another factor added to our rapid advances in research and our material progress. And that was the cooperative extension work, which brought research results to the people on the farms and put new ideas and techniques to practical use.

In addition, the cooperative relationships that we have maintained through the years with the state experiment stations have been a strong factor in achieving our research success. These close working relationships are of the greatest importance. They provide a basis for effective research on a local, regional, and nationwide basis.

The Department and the states have definite responsibilities in regard to research. The states are free to investigate any problem of interest to the people of their states. The Department of Agriculture directs most of its efforts toward problems of regional or national significance, generally in cooperation with one or more states.

Occasionally, however, some work can best be done at one location where expensive equipment and other facilities are available, even though the problem involves many other areas. Research is likely to involve principles that are the same in Maine as they are in California.

Furthermore, as agriculture grows more complex, problems become more complex and require greater expenditures of money and manpower. Some states would find it difficult or impossible to assume the heavy financial burdens of solving costly agricultural problems.

Thus, an increasing amount of the research in agriculture is being carried out cooperatively—by the states, by a growing segment of industry and private organizations, and by the federal government.

A classic example of such cooperation in research is the story of hybrid corn. The first work on hybrid corn in this country was done in the state agricultural experiment stations. The real potential of hybrid vigor was opened up at the Connecticut station. Work here resulted in the double crosses—the basis for all corn hybrids.

Within a few years, corn breeders in 12 states, in industry, and in the Department decided to combine their efforts. This cooperation made possible the fullest possible use of all available information on breeding hybrid corn—and has been largely responsible for its phenomenal spread throughout the country.

This is one example of hundreds of similar cooperative projects under way throughout the country in state, federal, and industrial laboratories. Regardless of where the research is done or who does it—I believe it is increasingly important to recognize that our individual efforts are part of a much larger effort in the service of agriculture. Our impact as a team working together can be much greater than the sum of our individual efforts.

If we think along these lines, I believe we will tend to think less in terms of whether a given function is a state or federal activity—and think more in terms of pooling our resources and talents in a common effort toward the goal of human advancement.

Now, let us see what this state-federal partnership has meant in terms of greater farming efficiency.

When the land-grant colleges and the Department of Agriculture were founded in 1862, one farm worker supplied enough to support five people. Today, a farm worker supplies enough for 27 people. Compare this with the four or five that one Russian farmer can feed.

Because our agriculture is efficient, Americans spend only one-fifth their take-home pay for food. Again, compare this with the more than 50% that the Russians spend for food—and food that is not as nutritious and well-balanced as ours. Only recently, in fact, drastic increases in the price of butter and meat were announced in Russia—an admission that these two products are in short supply.

Now, let us look at what the state-federal research partnership has meant in terms of total progress. We'll start first with the dairy industry.

We have fewer but larger dairy herds . . . fewer but more productive cows . . . more specialized and more efficient units. Research has helped at every step of the way to bring about these developments.

For example, studies on artificial breeding have been a strong factor in improving our dairy herds. About 35% of our 25 million cows are artificially bred and the figure grows yearly. As artificial breeding has grown, research has been intensified to improve the quality of bulls—and to develop better techniques to evaluate them.

Research has developed improved feeding methods and high-energy rations that enable us to feed cattle better than ever before.

Advances have come through a combination of many other things as well—record-keeping, programs to control disease, more effective marketing methods, and better chemicals, medicines, and equipment.

One of our major concerns now is to increase the efficiency of dairy operations. A great deal of effort is being made to increase mechanization, streamline farm and chore operations, and reduce the hard work and hours of labor needed for dairying.

In other areas of livestock research, we have developed meat-type hogs and small family-size turkeys. These are what consumers want—and we can look for much more tailoring of livestock in the future to meet specific consumer demands. Methods of performance testing of beef cattle are making important contributions to increased efficiency of production. We have learned more about animal diseases and parasites and have successfully kept some of the most serious ones out of the country through our combined research and regulatory activities.

In crops research, scientists have changed the plants that farmers grow in their fields. They have supplied new germ plasm and adapted foreign crops to our climate and methods of farming. They have bred crops for resistance to various diseases and insect pests and tailored many crops for mechanized operations on the farm.

Our soil and water studies have helped us to slow down soil deterioration and shown us ways we can use water more efficiently.

In entomology research, we have developed chemicals for almost every major agricultural pest we have. At the same time, we are also investigating nonchemical means of control to overcome growing insect resistance to chemicals and to eliminate the problem of residues.

State-federal research has given us new techniques of mechanization . . . added to knowledge of human nutrition and consumer use of farm products . . . helped to protect our forest resources . . . found wider markets for farm products . . . and improved methods of handling and marketing them.

These accomplishments are a small fraction of the many produced during the past 100 years. Increases in all areas of agriculture have been dramatic indeed. But the job is by no means done.

Our gains simply mean that we have to work harder to hold on to what we have already won. We will need an increasing amount of research to keep up with the ever-present and ever-changing diseases, insects, parasites, and countless hazards that drag down efficiency and cut into the incomes of farmers.

But beyond this, we must look ahead and determine how agriculture is to supply future needs for food and fiber. The possibilities for doing so through continuing research are enough to excite the imagination.

In the field of dairy research, much remains to be done. We need to take full advantage of dairy cattle as meat producers. When you consider that 50% of the dairy calves born are bull calves and only a small number are retained for breeding purposes, you can realize the potential for increasing our meat supply by utilizing the rest of the animals. They can be made to produce carcasses of acceptable market quality.

We will also need to place more research emphasis on breeding dairy cattle to produce milk of high protein content.

And, finally, we need to know more clearly how changes in the production of plants used for feed affect the characteristics of the milk produced. There is some evidence that increased fertilization of these plants may affect the vitamin A requirements of cattle. We will have to take another look at vitamin A needs of dairy cattle, and look into the possibility of supplementing their feed.

We have not capitalized fully on the genetic capabilities inherent in the crops that we grow.

We have a long way to go to reach full mechanization and top efficiency in nearly every phase of agricultural operation.

We have not solved the many problems of processing, marketing, and utilization that deny us the maximum benefits from agricultural production. Nor have we found out how to harness the full productive capacity of our agriculture to the changing demands of our market.

We need to know exactly how insects develop resistance to insecticides and how, in fact, insecticides actually kill insects. We must know how viruses reproduce in plants and animals. We need to take full advantage of the opportunities offered in animal genetics to alter livestock for desirable traits.

We have to marshal all our research resources to conserve and maintain the quality of our water supplies. Agriculture is the nation's number-one user of water—and uses it inefficiently. This cannot go on indefinitely.

These are some of the things that we will be working toward in the years ahead. Whether we succeed in our efforts will be determined in large part by the kind of people developed by the land-grant colleges for our activities.

The land-grant schools in the past have been extraordinarily successful in producing technical leadership for agriculture as well as for other fields. But there is today a growing and urgent need for more and better trained scientists. And they are increasingly difficult to come by in agriculture for many reasons.

For one thing, enrollment of agricultural students is dropping off steadily.

Another difficulty is that the various space programs offer tremendous competition for some of our best young minds.

Moreover, there is a common misconception in the public mind that agriculture is not important these days—or, at any rate, less so than it was at one time. It is true that fewer people than ever before work on farms today to produce our abundance. But this does not mean that agriculture is less important—only that it is more efficient. If our food supply were to be curtailed only moderately, agriculture would rapidly assume critical importance in the eyes of every American. Our economy is built upon the productivity of our agriculture and the assurance of continued productivity.

So both of us—state and federal agencies—must exert all our efforts to attract the bright young minds that we will need to fulfill our mutual responsibilities in agriculture.

The pressures bearing down on state and federal agencies to fulfill these responsibilities will demand the best that is in us. We must be increasingly flexible and imaginative in our planning—and take into consideration international as well as national aspirations and problems.

We will, in fact, have to project ourselves more deeply into the world picture. We will have to learn how we can be most helpful in establishing the broad foundations for a productive agriculture in underdeveloped areas of the world.

But—above all—there must be a continuation of the remarkable cooperation that has served us so well in the past. The strength that lies in free men working together in a concerted effort is incredible.

Science and education—operating in a free society and through the land-grant schools and the Department of Agriculture—have removed from us the heaviest burdens of the past. It is surely our obligation to use science and education to help all mankind achieve a better life.

THE LAND-GRANT SYSTEM AND THE AMERICAN SOCIETY [1]

RUSSELL I. THACKREY, *Executive Secretary*
Association of State Universities and Land-Grant Colleges, Washington, D. C.

R. I. Thackrey

My job this morning, as I understand it, is to discuss with you the contributions of the Land-Grant system to the American society. This is, of course, a highly appropriate topic for us to concern ourselves with at this time. As you know, Abraham Lincoln signed the Morrill Land-Grant Act into law just 100 years ago this next month. If silver and golden anniversaries have special significance, centennials surely provide an even greater temptation for stock-taking, review, reassessment, and the like.

Such occasions carry with them an almost irresistible urge to luxuriate in nostalgia and self-congratulations. Yet all of you, from your experiences with your research, know the tremendous value of periodically standing back from your work to take a new look at it from the prospective of first principles. In our day-to-day operations, we tend to confuse the ends with the means, to substitute expediency for principle, to care for the urgent at the expense of the important. Leadership degenerates into management, and we find ourselves, if we are not careful, ministering to a corpus from which the spirit has departed.

What are these first principles for the Land-Grant system? To determine this—and to establish the prospective for our review—we have to look at least briefly at conditions as they existed in America during the first half of the Nineteenth Century.

America at that time was a land of almost boundless enthusiasm. It was a muscular, broad-shouldered young democracy—crude perhaps, but powerful, idealistic, yet restless to get on with the work that needed to be done. And there was work to be done—lots of it. There were millions of acres of fertile land to be tilled, mines to be opened, cities to be built, railroads to be laid. There was, in fact, a continent to be conquered and, perhaps even more important, a democracy to be maintained and extended.

At the same time, however, colleges and universities willing and able to provide educational programs fitted to work that needed to be done were sadly lacking. The importance of this deficiency cannot be overestimated.

Five years before the Land-Grant Act was signed, the distinguished British statesman and historian, Thomas Babington Macaulay, flatly predicted the collapse of the United States in the Twentieth Century. "Either some Caesar or Napoleon will seize the reins of government

[1] Presented at the 57th Annual Meeting of the American Dairy Science Association, University of Maryland, College Park, June, 1962.

with a strong hand or your republic will . . . be laid waste by barbarians," he wrote. This eventuality was inevitable, he thought, because we had entrusted the "supreme authority in the state . . . to the majority of the citizens told by the head; in other words, to the poorest and most ignorant part of society." By way of contrast, he wrote, in England the "supreme authority is in the hands of a class, numerous indeed, but select; of an educated class which is, and knows itself to be, deeply interested in the security of property and the maintenance of order."

This was not an irresponsible statement by an ill-informed partisan. All history, all experience seemed to prove Lord Macaulay's prediction sound. And, as a matter of fact, it looked somewhat as though many people in America believed as Macaulay did. The existing colleges in America at that time were patterned largely after the centuries-old British institutions and took, as their function, the same objective the British institutions served: the training of an educated elite for civic and social leadership. Well over half the program at these institutions was given over to the history, poetry, and philosophy of Rome and Greece; and academic preparation was limited to the four traditional learned professions: law, medicine, the ministry, and teaching. Instruction was carried out by recitation and rote memory, all students studied exactly the same subjects, and almost no emphasis was given to the discovery of new knowledge. Collegiate opportunity was shrinking in proportion to the population, and effective governmental control was held largely by the graduates of these institutions.

A few far-sighted men felt there was another solution to the problem that Macaulay had outlined—a solution that did not involve the gradual imitation of Britain's aristocratic class system. These men could see no reason why higher education could not serve the purposes of democracy just as well as the purposes of aristocracy. Couldn't the "majority of the citizens told by the head" itself become an "educated class?" In fact, couldn't higher education, made available to all willing and able to benefit from it, eliminate the concept of "class" and guarantee free mobility within the American society? Did higher education have to be limited to the existing learned professions? Couldn't there also be designed an education for doing—for carrying out the work that needed to be done to convert the wilderness into a nation of free and prosperous men? Did Americans have to remain poor and ignorant?

John Adams seemed to forecast something of this when he wrote, in 1780, that "the science of government is my duty to study, more than all other sciences . . . I must study politics and war that my sons have liberty to study mathematics and philosophy, geography, natural history and naval architecture, navigation, commerce, and agriculture, in order to give their children the right to study painting, poetry, music, architecture. . . ."

By the middle of the Nineteenth Century, America was ready for the second of these three stages. A new education for a new society was needed—an education for doing as well as for being—an education for the sons of the farmers, the mechanics, the merchants, and all the other leaders of this new world in which they would not have to abandon their fathers' vocations but would find these vocations lifted to professional dignity. Higher education needed to be brought into harmony with the realities, the objectives, and the ambitions of a new society.

Among the people who saw this need and worked to do something about satisfying it, two names stand out—Jonathan Baldwin Turner of Illinois and Justin Smith Morrill of Vermont. Working separately, insofar as we know, each of them formulated a plan to use the vast nationally owned public lands and the prestige of the Federal government to bring about an educational revolution—a revolution that was to do for education what Jacksonian egalitarianism had done in law and politics.

The result of their efforts, and of the efforts of those who worked along with them, was the Land-Grant Act. This Act offered each state 30,000 acres of land (or its equivalent in scrip for those states in which no public lands were available) for each member of Congress. This land was to be sold, and the proceeds were to be used as a permanent endowment to pay professors' salaries in at least one college in every state accepting the conditions of the Act. The fact that those conditions specified that "agriculture and the mechanic arts," along with "military tactics," were to be emphasized has been widely misinterpreted. One hundred years ago, most of the people in our country—something like three-fourths of them— were farmers, and the greatest unfilled professional need was for engineers. These were also precisely the academic areas the existing colleges and universities were most reluctant to add to their programs. To make certain that they were not neglected, Justin Morrill wrote them into his act. But the charter of the Land-Grant institution was as broad as the needs of America. The Act stated, in fact, that "other scientific and classical studies were not to be excluded," which meant, Justin Morrill said, that they were to be included.

In this way, these institutions were to provide for the "liberal and practical education of the industrial classes in the several pursuits and professions of life." In 1859, Abraham Lincoln said, "Let us hope . . . that by the best cultivation of the physical world beneath and around us, and the best intellectual and moral world within us, we shall secure an individual, social, and political prosperity and happiness,

whose course shall be onward and upward, and which, while the earth endures, shall not pass away." One hundred years ago, the land-grant colleges became America's best means of realizing this hope.

The most important idea, then, behind the establishment of the land-grant colleges—the principle out of which they were born and which gave them form and shape—was that of democracy: democracy not just in terms of the students admitted but also in terms of the instructional programs offered. These were to be institutions at which any young person would have a free opportunity to receive as complete an education as his abilities and his desires permitted. Further, he was to have the privilege of studying for almost any major profession of interest or value to man.

However, the pattern was not yet complete. Those who came to teach in these new "people's colleges" soon ran out of things to teach in the new fields. Since, for the most part, colleges had refused to consider these new fields as proper subjects for collegiate concern up to this time, no body of organized, definitive knowledge existed in them. And so, in 1887, a second federal act was passed, this one providing federal funds to start research in agriculture in each of the land-grant institutions. The agricultural experiment stations founded as a result of this act, historian Allan Nevins tells us, "became . . . the most effective agencies of inquiry on the globe." This act is credited by many with being the major stimulus for making research an accepted function of higher education, not simply at the land-grant institutions but also at the older colleges and universities as well.

Along with the advance of research came the necessity for disseminating the results in order that it might be put directly to use in the lives of the people. This activity was carried out in many ways by many agencies. Finally, in 1914, Congress passed a third act, this one to provide for federal-state cooperation in extension work, carried out through the land-grant colleges. This resulted in the world's largest and most effective adult education program. Agricultural research and extension carried out in the land-grant institutions, in fact, have been so effective that they are sometimes criticized for having created our embarrassing farm-surplus problem. There is, however, another way of putting this matter: if the 180 million people in America have plenty to eat, and if they spend far less to get it in terms of hours worked than they ever have, that, too, is the responsibility of the land-grant institutions and the U. S. Department of Agriculture, whose centennial we also celebrate this year. This is a problem that almost every other country in the world, including Russia, would be glad to share with us. I must say that I like having enough to eat, and I like to know that we are able to help the hungry people of the world.

These, then, are the first principles on which the land-grant institutions were established. For more than a quarter of a century, they had to endure pitifully inadequate finances, the apathy of students and their parents, the indifference and even contempt of their more traditional—and hence more respectable—sister institutions, and uncertain leadership and instruction. But they survived and grew robust as they demonstrated that Abraham Lincoln's act of faith in people, taken during some of the worst days of the Civil War, was not misplaced.

Today there are 68 land-grant institutions, with one in Puerto Rico and at least one in each of the 50 states. Together, they account for about 3½% of all American institutions of higher education, but they enroll almost a fifth of all the students in the country. They award two-fifths of all the doctoral degrees granted, including practically all those awarded in agriculture and over half of those granted in the biological sciences, health professions, engineering, and home economics. Three-fifths of all the living U. S.-educated Nobel Prize winners have earned degrees from these institutions, and the research contributions include a vast array of discoveries—the Babcock test for measuring the fat content of milk, streptomycin, the ion microscope that permits man to photograph atoms, and hundreds of others.

The record is clear. As one historian has said, together with the separate state universities, the land-grant institutions now "constitute the most impressive set of agencies for higher education in the world." But probably even more impressive than their individual achievements has been the revolution that, through their example and leadership, they have brought about in American higher education generally. Look at the changes they have made—changes such as—

The establishment of research as a basic function of higher education.

The elevation of the useful arts, sciences, and professions to academic respectability and dignity and the introduction of the concept of education for doing as well as for being.

The development of the concept of adult continuing education, of sharing knowledge with those who can make use of it regardless of age, formal enrollment, or any other restriction except that of need and desire to know.

The construction of a successfully working model for federal-state cooperation in instruction, research, and extension that secures all the advantages of such cooperation without incurring any of the dangers that many seem to fear.

The establishment of the principle of service, taken in the best possible sense—the principle that an institution of higher education should serve as a useful academic and scientific complement to the society in which it exists.

Most important of all, the development of

the concept that higher education should be made freely available to all capable of benefiting and willing to benefit from it—that educational opportunity should be kept open to all who have talent.

Now, what about the future? What problems do the land-grant institutions face, and what connection is there between these problems and the first principles that I have tried to outline?

There remains, of course, the central problem of any institution that attempts to serve a society responsible. The university's function is to give society not what it wants, but what it needs, and the two do not always coincide. Disinterested thought and scientific research challenge vested interests and traditional ideas and tend, therefore, to alienate the support the universities need to carry out their work. Yet it is essential that the universities maintain their critical function, even if they must threaten the stability of society as it exists at any given time.

Much the same sort of thing is true of the balance between education for living and education for making a living. One of the persistent charges hurled at the land-grant institutions is that they are vocational. Of course, almost all higher education has historically been vocational, if vocational is taken to mean training for a specific profession or calling. The distinction here seems to be, actually, the vocation one is talking about. Traditionally, some vocations or professions are more equal than others. As I have said, part of the land-grant mandate was to change this by dignifying all useful major vocations by providing university-level preparation for them. In this way, the land-grant system has been responsible for the expansion of professional preparation that has contributed so importantly to the development of the American economy and culture. If the universities repudiate professional education, they will cease to survive, simply because they will lose contact with reality and will lose the confidence of the society that supports them. If, on the other hand, they repudiate the cultivation of nonpractical values, they will cease to merit the title of university. They must somehow continue to provide education for being and for doing. As knowledge is advanced and specialization deepens, the job becomes increasingly difficult.

There are other questions. But the central question remains the same as it was 100 years ago: How can the land-grant institutions continue to contribute to the maintenance of an open and mobile society by providing equal opportunity for all with the talent and the will to develop it?

There are those, and they have powerful voices, who would undo most of what has been accomplished in an attempt to avoid, rather than to solve, this problem. Some would restrict the numbers going to the university by examinations given at the equivalent of about the sixth-grade age in our own schools. Every country that has this system is trying to get away from it because it does not work. Actually, the fact is now being rediscovered that testing is far from infallible, that it might easily discriminate for the good memorizer and against the truly creative boy or girl, and that it is a poor indicator in comparison with test by performance. Instead of increasing opportunity, it restricts it. Today, college admissions officers are apt to say that all who qualify are admitted. But qualification is determined in too many instances by the college board cut-off score used, and this, in turn is determined by the ratio between the number of applicants for admission and the number of students that the institution can accept. In this way, the potential student who was qualified five years ago might easily not be qualified this next fall, even though the program might be essentially unchanged. Qualification is determined, therefore, by arbitrary resource limitations, and not by student ability. This is like the old story of the Procrustean bed, in which the man was cut to fit the bed, instead of the bed fitted to the man.

Others say that access to higher education is just fine, but that since university graduates earn more than nongraduates, each student should pay the full cost of his own education. Those whose parents are wealthy presumably have no problem, whereas others—so the theory runs—should buy their education on the charge-account system. Education would be open to all, but some would start out life with a heavy burden of debt and others would not. Such a system would make it important that young men without money would marry girls with money, and vice versa. Perhaps in time this would lead to a change in the marriage vow to read, "With all my college debts I thee endow."

To be fair about it, many who advocate making the student pay the full cost of his education say that those who can't afford it should be given scholarships. Make those who can pay, do so, and give scholarships to the rest, runs the argument. Consider the effects of such a policy. It would in time result in one of two things: either hundreds of thousands of students would be denied access to higher education, or we would have to have hundreds of thousands of scholarships, each paying many times the average amount of the scholarships available today. There are no two ways about it.

The best scholarship system ever devised for keeping opportunity open is the public university, open to all and supported by all. Certainly, scholarships are needed, because living costs alone are beyond the reach of many able students. Certainly, the beneficiaries of education should pay for it, but the beneficiaries include all of us—all of us who appreciate the privilege of enjoying human dignity and the freedom of democracy, all of us who share in

the American standard of living, all of us who benefit in any way by living in a modern industrial economy. But they should pay for it when they are most able to do so, through taxation and voluntary gifts; and not at the time they are least able to do so, as students and during the early (and difficult) post-college years. And we should never permit a condition to exist in which America's supply of educated people will depend on how much money the student is likely to make in the years after his graduation. That is the one way to make certain that we will not have enough scientists, enough teachers, enough qualified civil servants, to name just a few examples. An individual might find higher education desirable; a society finds it necessary.

All the suggested solutions to this problem are based on the implied assumption that America somehow cannot afford to continue to provide free and open access to higher education to all the young people who have the will and the ability to profit from it. This, of course, is nonsense. We can afford it if we want to afford it. Many people have noticed that a society can almost always achieve that which it wants to achieve—and it very often deserves what it gets. Education is, actually, not an expenditure but an investment. This is true even in the grossest material terms. Increased expenditures do not follow an increase in the level of an economy; the level of the economy follows increased investment in education.

The central issue, then, is how adequately do the American people want to invest in democracy? In the potential of the American people for intellectual and moral growth? In the development of more effective human beings? The land-grant institutions—and others which share their ideals—stand now as they stood 100 years ago—as America's best hope of achieving and maintaining an open and mobile society. How well they accomplish their task will depend, to a critical extent, on how highly the American people value the maintenance of such a society. Macaulay's prediction cannot be completely discounted until the year 2001. History may still prove him to be right.

CALL FOR PAPERS FOR THE 1963 ANNUAL MEETING OF THE AMERICAN DAIRY SCIENCE ASSOCIATION

S. D. MUSGRAVE, Chairman, Program Committee, A.D.S.A.
Department of Dairying, Oklahoma State University, Stillwater

The 58th Annual Meeting of this Association will be held June 16-19, 1963, in the Memorial Center of Purdue University, Lafayette, Indiana.

All members of the Association, including graduate student affiliates, are entitled and encouraged to submit papers for consideration for presentation. Participation by members of industry and by senior members of the Association is especially encouraged. The Program Committee favors the general policy that an individual present only one paper and that his name should appear as author on no more than two. This committee, with the Association officers and membership, wishes to stimulate excellence in research and in presentation and realizes that these restrictions may penalize certain members engaged in full-time research. Therefore, a member may exceed these limits if he or his department ranks the affected abstracts in order of preference for oral presentation.

Papers submitted for the Annual Meeting must be confined to research that has not been reported elsewhere. Abstracts of papers accepted for publication by a scientific journal before the annual meeting are not acceptable for presentation at this meeting. If the total number of acceptable papers submitted by the membership for presentation in a section program is too great to include in the program, the committee will assign some papers to be read by title. In this event, consideration will be given to evident quality of research to be reported and number of abstracts per author and per department. Abstracts arriving late may be read by title or rejected.

Attention is called to the Dairy Manufacturing Extension Section program, and interested members are urged to participate. This is a subsection of the Manufacturing Section.

Mimeographed copies of pertinent details and data are desirable for distribution at the time of presentation. At least 300 copies should be made available. They should be numbered with the assigned program number in the upper right corner of the first page. This can be supplemented with slides for projection, provided the author can adhere to the assigned time of 12 to 14 min for presentation of each paper. For papers presented in joint sessions or in a symposium, it is suggested that 500 copies of the mimeographed material be made available.

ABSTRACT PREPARATION

The Program Committee continues to encourage improvement in quality of material to be presented and in the method of presentation. Good experimental design and interpre-

tation are essential. Exact compliance with these instructions for preparation of abstracts will simplify the task of the Program Committee, improve the program, and ease your own work load. Careful editing of abstracts before submission is essential! Each year a number of abstracts must be returned for correction or clarification.

1. Submitted titles and abstracts must be in the hands of appropriate section officers by March 1. This deadline must be met to permit publication of titles with the complete program in the April JOURNAL and abstracts in the May or June issue.
2. Four copies of each abstract must be typed double-spaced on 8½- by 11-inch paper. The original (on bond paper) and the first carbon should be mailed to the chairman, the second carbon to the vice-chairman, and the third carbon to the secretary of the Section where the paper is to be presented. The original copy will be used for publication in the JOURNAL.

Student affiliates entering the Graduate Student Presentation Contest should check below for additional instructions on submitting abstracts.

3. Abstracts must not exceed 200 words by actual count. Those exceeding 200 words must be returned to the author for revision.
4. The style and abbreviations of the JOURNAL OF DAIRY SCIENCE must be used. Please refer to abstracts in the May, 1962 JOURNAL for guidance.
5. Only initials of authors should be used, except in unusual cases, where it may be necessary to use the complete name.
6. When more than one author is listed, indicate who will present the paper by an asterisk after his name.
7. The title should indicate clearly the nature of the research. It should not be repeated in the text. The abstract should indicate, insofar as possible, the design and major results of the investigation. Only completed research should be reported. Brief, essential statistics will make the abstract more meaningful.
8. The following form with no caps for the title is correct: Utilization of carbohydrates posterior to the rumen-reticulum of the bovine. J. T. Huber and N. L. Jacobson, Iowa State University.
9. If the author lists an address for an experiment station other than the university, such as a USDA research branch or a commercial company, the complete address should be provided, as in the following example: A study of dye reduction methods as platform tests for the detection of antibiotics. Burdet Heinemann, Producers Creamery Co., Springfield, Missouri.
10. All symposium papers should be typed double-spaced and organized according to the acceptable style of the JOURNAL OF DAIRY SCIENCE. The author should send the first copy directly to the JOURNAL editor, E. O. Herreid, before the annual meeting.

GRADUATE STUDENT PRESENTATION CONTEST

This contest will be conducted in both the Production and Manufacturing Sections. A cash award will be given to the winner in each section. Each institution is entitled to enter one participant in each contest. Participants must be student affiliate members. Complete rules for the contest will be sent to department heads. Those wishing to enter the contest must submit copies of their abstracts to the section officers as outlined above. In addition they must include a letter indicating their desire to enter the contest. This letter must be signed by the major professor and the department head. A carbon copy of this letter and four additional copies of the abstract should be mailed to the contest representative so as to be received by March 1.

For Production Section Graduate Student Competition: Dr. J. L. Albright, Department of Dairy Science, University of Illinois, Urbana.

For Manufacturing Section Graduate Student Competition: Dr. A. V. Moore, Department of Dairy Science, The Agricultural and Mechanical College of Texas, College Station.

Names and addresses of officers of sections to whom titles and abstracts should be sent are:

EXTENSION SECTION

Chairman: C. D. McGrew, Department of Dairy Science, Ohio State University, Columbus 10.

Vice-Chairman: D. E. Voelker, Department of Animal Husbandry, Iowa State University, Ames.

Secretary: C. H. Parsons, Department of Dairy and Animal Science, University of Massachusetts, Amherst.

PRODUCTION SECTION

Chairman: L. H. Schultz, Department of Dairy Husbandry, University of Wisconsin, Madison.

Vice-Chairman: V. R. Smith, Department of Dairy Science, University of Arizona, Tucson.

Secretary: P. M. Reaves, Department of Dairy Science, Virginia Polytechnic Institute, Blacksburg.

MANUFACTURING SECTION

Chairman: D. M. Graham, Pet Milk Co., Research and Development Center, Greenville, Illinois.

Vice-Chairman: E. L. Thomas, Department of Dairy Industries, University of Minnesota, St. Paul.

Secretary: M. E. Ellertson, Carnation Research Laboratory, 8015 Van Nuys Boulevard, Van Nuys, California.

(The Dairy Manufacturing Extension Section is a subsection of the Manufacturing Section. A. L. Rippen, Department of Food Science, Michigan State University, East Lansing, is chairman, and J. C. White, Professor of Dairy and Food Science, Cornell University, Ithaca, New York, is secretary of this subsection. All abstracts for papers in this subsection should be submitted through the regular channels of the Manufacturing Section, and should be identified for presentation at the Manufacturing Extension Section meeting and a courtesy copy sent to A. L. Rippen, Chairman.)

INVITATION FOR NOMINATIONS FOR OFFICERS OF THE AMERICAN DAIRY SCIENCE ASSOCIATION

R. E. Hodgson
Animal Husbandry Research Branch, USDA, Beltsville, Maryland

It is the duty and responsibility of every member of A.D.S.A. to participate in the selection of officers and directors for 1963-64.

This year we must elect:

(1) A Vice-President from the field of Dairy Production and who will automatically become President in 1964-65.

(2) One director from the field of Dairy Production to serve for three years.

(3) One director from the field of Dairy Manufacturing to serve for three years.

As a member of A.D.S.A. you are specifically requested NOW to submit your nominations for each of these three very important offices. Suggest nominations of persons who have demonstrated their interest in ASSOCIATION AFFAIRS by having served as sectional officers or having worked on important committees. Our Association budget is now close to $100,000, annually. Therefore, it is important that the nominees for Vice-President be familiar with the business affairs of the ASSOCIATION.

On January 1, 1963, Chairman Hodgson will assemble the nominations and request from the nominators a biographical sketch of each nominee for the consideration of the committee.

Two candidates will be selected for each of the three offices and after receiving their acceptances the list of six candidates will be published in the April Journal for 1963. Ballots will be sent to the membership by Secretary Judkins.

Members of the Nominating Committee are:

E. L. Jack, University of California, Davis

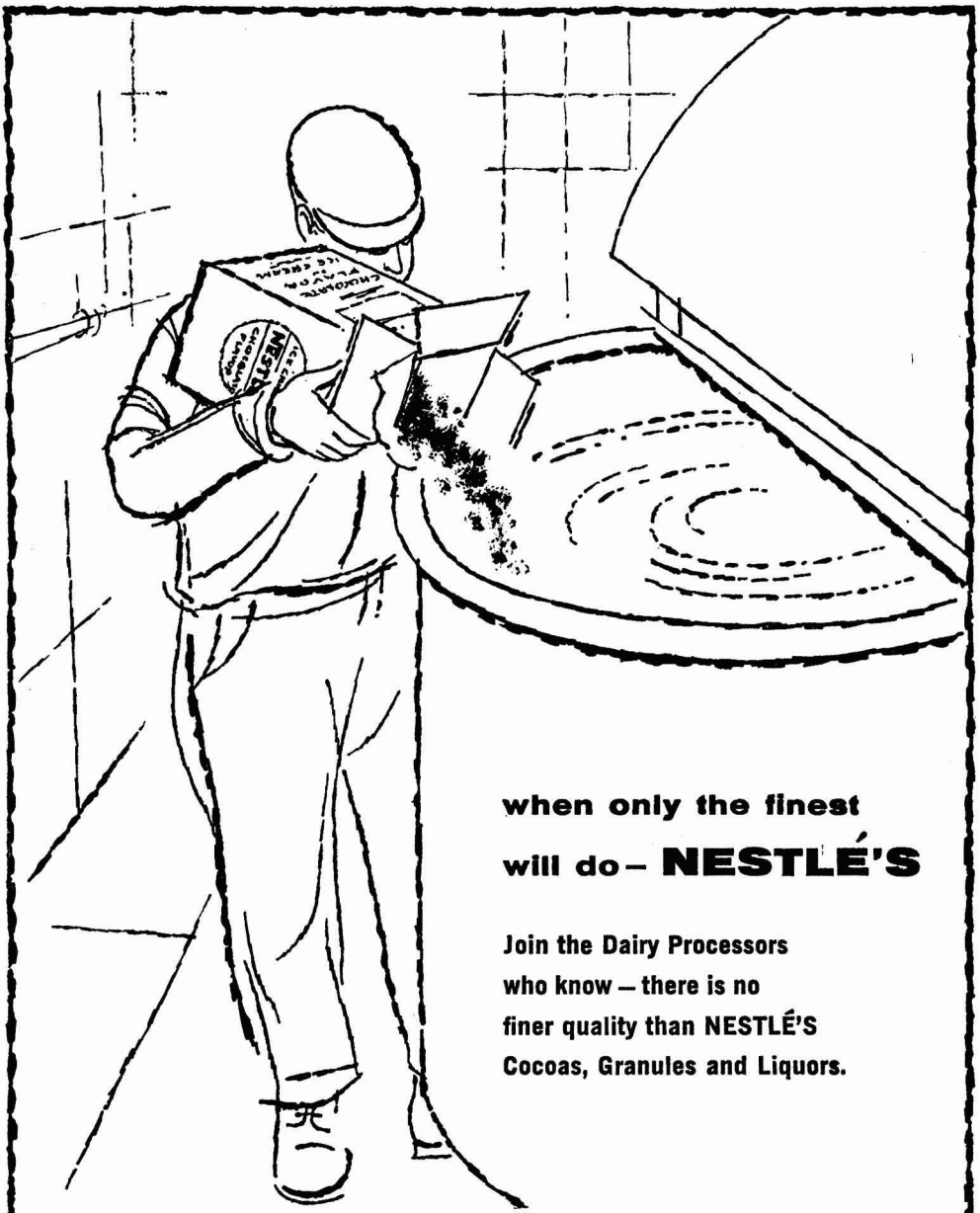
D. M. Graham, Pet Milk Co., Greenville, Illinois

L. H. Schultz, University of Wisconsin, Madison

C. D. McGrew, Ohio State University, Columbus

R. E. Hodgson, Chairman, Animal Husbandry Research Branch, USDA, Beltsville, Maryland

MEMBERS, this is an urgent request for your help and cooperation. Please send your nominations to Chairman R. E. Hodgson by December 31, 1962, OR IF MORE CONVENIENT SEND THEM TO ANY MEMBER OF THE COMMITTEE.



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