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LYDIA JANE ROBERTS

(1879 — 1965)

When you looked at the clock that earth-bound day
Did you see then it was your time to go
To lunch, or to a meadow sweet with May
In Michigan, where bloodroot petals glow?
— Black hawthorns clot the Midway drop white blooms
Late snowflakes sting swirl-sprinkle Dillon's hills
Around cold winds blow dark Kentucky looms
Spin mountain cabins — stop
A great heart stills.

Tread softly here, Miss Roberts now must sleep,
Nor dare disturb the hopes her lessons bring
To parents everywhere who too would reap
The joys they earn when bright-eyed children sing.
Then shall you smile, dear lady, while clocks give
Us time to learn your patterns, and so live.

From a drawing by Albert Schmid, Chicago, after a photograph

LYDIA JANE ROBERTS

Lydia Jane Roberts

— A Biographical Sketch

(June 30, 1879 — May 28, 1965)

Early in the year 1899 the United States Senate gave its consent to the Treaty of Paris. By that action this country obtained possession of certain territories formerly governed by Spain, among them the Island of Puerto Rico. In June of that year another event occurred. In the little town of Mt. Pleasant, Michigan, a serious young teacher, tall for a woman, with wavy brown hair and clear blue eyes that seemed to glow when she smiled, stepped to the platform of the normal school there, now known as Central Michigan University. Proudly, she received what was called a Limited Certificate. This document, awarded after the successful completion of one full academic year of work following graduation from high school, entitled the holder to teach in any rural school in the State. In this manner, a few days before her twentieth birthday and a few months before the start of the Twentieth Century, Lydia Roberts was officially launched on the teaching career she was to follow — in Michigan and Montana until 1915, in Chicago until 1944, and then in Puerto Rico — until the day she died.

Actually, Miss Roberts had begun to teach as soon as she was graduated from high school. She never married. She had no other occupation. She was a teacher, the kind who constantly seeks additional information, who obtains information not available in books by means of experiments designed to acquire it, and who provides a permanent record of the results of observations and experience to help guide others who will teach in future years. In the beginning of her long career, she taught second and third graders how to read and write and how to work and play together. In time she taught university students how they in turn could teach mothers to feed their children and their families, in order to live healthier and more rewarding lives. Nutrition became her field of specialization, particularly the problems of securing good growth and well-being of children, but her thinking encompassed all needs of all children. She indicated the scope of her thinking once, early in her career as a nutritionist, in these words:

> When all mothers have adequate prenatal care; when all children have proper supervision up to the age of 6 years by child-welfare agencies or by private physicians; when all schools, through proper medical attention, health instruction, school lunches, and healthful schoolroom conditions, insure suitable nutritional and health care of every school child; when all parents have some fundamental training in the care, feeding, and management of children; then the ideal—continuous conditions favorable to normal nutrition and growth for all children from conception throughout the growing period—will come near being realized. Not till then can we hope to resolve the problem of the malnourished child and thus to grow a healthy, well-nourished generation.
>
> (From "What is Malnutrition?" Publication no. 59, U.S. Children's Bureau, Washington, D. C., 1919.)

Near the end of her productive life she was able to provide a scientific demonstration of what a welfare program could accomplish, if it were coordinated about a central and controlling program of nutrition education. This demonstration, which will be described later in this sketch, was performed in Puerto Rico.

Early years

What factors in the early life of Miss Roberts contributed to the development of her character? Lydia J., as her close friends often referred to her, was born on June 30, 1879, in Hope Township, a rural area in Barry County, Michigan. She had two older sisters and a younger brother, the children of Warren and Mary Roberts.

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Her father was a carpenter and, not long after the birth of Lydia J., he moved his family to an adjoining county, where they settled in the town of Martin. Still small, Martin was then reached by about a day's journey with horse and buggy along the dirt road connecting Kalamazoo with Grand Rapids, starting from either of these cities. It was here in a general farming area that Lydia, with her brother and sisters, spent her childhood and attended grammar and high schools.

A close reading of some of her writings might show that Miss Roberts had undergone the rewarding experience of having lived as a child among people whose livelihoods depended on the soil and the weather, plus their own hard work. She wrote, for example, of a malnourished child as one who had, among other signs, hair which was rough—"like that of a poorly cared for farm animal" is the way she put it. She wrote that little boys, if in good health, were active and sometimes got into mischief, and she added that this was to be expected if they were the healthy young animals Nature intended them to be.

When Miss Roberts was graduated from normal school, the states west of the Mississippi were growing rapidly, school teachers were in demand, and she, a self-reliant young woman, wanted to travel. She always did like to travel, not to look at scenery but to observe how people lived from day to day. After her initial experience as a teacher in Michigan, she went to Montana, and taught in Miles City and in Great Falls; she also taught in the State of Virginia once, for a very short time. In 1909 she was awarded a Life Certificate by the Mt. Pleasant, Michigan, Normal College. This Certificate, usually given after two years of training beyond high school, entiltled its recipient to teach in any school, rural or urban, in the elementary school systems of Michigan. Along with the Limited Certificate, it is no longer awarded, the last one having been granted in 1939. But in 1909, with this additional recognition of achievement, Miss Roberts went to Dillon, Montana. During the next six years she taught third grade pupils in a Dillon school, and at the same time she served as a critic teacher in the normal school there, now known as Western Montana College, and drew part of her salary from each institution. She resigned her dual positions in 1915 in order to enroll as an undergraduate, with advanced standing, at the University of Chicago. She was then 36 years old.

First years in Chicago

Lydia J. went to Chicago, according to her sister Lillian, with whom she lived for many years, for the express purpose of learning how to feed children. She had done some summer work in Montana at a children's institution, and what she saw there made her want to learn more about the relationship of diet to health. At the University, she majored in Home Economics, which at the time was an interdepartmental discipline. The chairman of the department was an assistant professor, Dr. Katharine Blunt, a biochemist and a remarkably vigorous woman. When Miss Roberts received her B.S. degree in 1917, with Phi Beta Kappa honors, the country was at war, and food was very much in the news. Encouraged by Miss Blunt, she stayed on at the University and completed the work for her M.S. degree, which she received a year later. At the same time Miss Blunt was promoted to an associate professorship, the Department of Home Economics and Household Administration became a full-fledged department, and Miss Roberts was named an assistant professor.

It was in 1918 that Miss Roberts' first professional paper was published, with Elizabeth Miller (Koch). It told how to prepare soybeans on a small scale in the home, and then use the processed meal in the preparation of foods for diabetics. This paper is noteworthy because it is one of the few which Miss Roberts contributed on foods. Nearly all her papers were on human nutrition, mostly on the feeding of children. The title of her Master's thesis, which was published in the *Journal of Home Economics* for 1919, was "A Malnutrition Clinic as a University Problem in Applied Nutrition." It is a paper which deserves comment, because it illustrates a characteristic of Miss Roberts' career: She was on hand, prepared and willing to participate, when important programs or activities in nutrition

were being initiated. Further, when she did participate in a program, she handled each of her assignments with distinction, carried the work to a successful conclusion, and wrote a finished report of what had been accomplished.

The malnutrition clinic which Miss Roberts wrote about for her Master's thesis had its start in 1917, as a result of a luncheon which Miss Blunt had with the director of the Central Free Dispensary of the Rush Medical College. What Miss Blunt wanted were facilities which would permit her graduate students in home economics to work with children. Suitable arrangements were indeed made. Miss Blunt assigned Ann Boller (Beach) as a nutritionist in the Dispensary, and assigned Miss Roberts, largely because of her excellent scholastic record and her experience as a teacher of young children, the task of developing a course of instruction, using the children of the clinics for this purpose. For a year, Miss Roberts worked with the children on an individual basis, during which time she developed a course in child feeding. She had from the beginning and for many years afterwards the sympathetic cooperation of a beloved pediatrician, Dr. Walter H. O. Hoffman, who did the medical examinations of the children and helped in all possible ways the work of Miss Boller and Miss Roberts. This Dispensary activity was in a real sense a pioneering venture, although its sponsors had been encouraged by glowing reports of what Dr. William R. P. Emerson of Boston had accomplished, when nutrition education was made part of the health program in elementary schools.

The year 1918, when Miss Roberts received her M.S. degree, was a favorable one for nutrition activities. The rejection of many young men for military service on grounds of physical unfitness had aroused the nation, as it was to arouse the nation during World War II many years later. A principal cause of poor physical fitness was considered to be poor dietary habits. Under government auspices, Professor E. V. McCollum of Johns Hopkins University talked on foods and nutrition around the country. At one of these lectures he made use of the term "protective foods" for the very first time. It is of interest that this occurred at the Kent Theatre of the University of Chicago, in reply to a question by a student in the audience. Interest in nutrition continued after the war. A so-called Children's Year was declared, and there were increased activities within the U. S. Children's Bureau. In some of these activities, Miss Roberts, as a member of the staff of the department at the University, became importantly involved.

She planned two survey studies of nutritional status for the Bureau, supervised their performance, interpreted the data obtained and wrote the accounts of what was done. One report was called, "The Nutrition and Care of Children in a Mountain County of Kentucky," and the other, "Children of Preschool Age in Gary, Indiana. Part II. Diet of the Children," these being Bulletin no. 110 and part of Bulletin no. 122 of the Children's Bureau, respectively. They are interesting to read today, especially in connection with the studies which Miss Roberts was to write about many years later, of work in Puerto Rico.

Chicago—the years of rapid growth

From 1919 to 1928, when Miss Roberts served as an assistant professor of home economics at the University of Chicago, she read and studied continuously. Opportunities for practical work in nutrition kept increasing, as various interested organizations witnessed what was being accomplished at the Central Free Dispensary. The Department of Home Economics and Household Administration was growing; in a few years it could boast of a staff of 20 full-time persons who offered more than 60 courses for undergraduates and graduates. For a number of years, the University of Chicago was the only institution in the world where an ambitious girl could go to earn a Ph.D. degree in Home Economics—under professors who were not only recognized scholars but superb teachers as well. Like a frugal housekeeper, Miss Roberts made use of all the information she and her many students garnered, if not in some report of original research, then perhaps in an article for the general public. During these years

she was winnowing the material in the literature on the nutrition of children and saving the wisdom of her experiences for the book which represented the climax, without a doubt, of her endeavors during these years.

This book, entitled *"Nutrition Work with Children,"* was completed late in 1926 and published by the University of Chicago Press in 1927. It is one of the masterpieces of the literature on nutrition. It was extensively revised and enlarged in 1935. The third edition was completely rewritten according to the original plan by Ethel Austin Martin, and published in 1954 under the title *"Roberts' Nutrition Work with Children."* The first edition was accepted as the dissertation for the Ph.D. degree in home economics, which was awarded to Miss Roberts in 1928; at the same time she was promoted to an associate professorship in the department. She was then 49 years old.

Her book contains many practical hints about teaching good food habits. She was regarded by her pupils as the most inspiring teacher they had, and it must be remembered that the University of Chicago had some pedagogic giants in those days. One of her minor yet valuable contributions to teaching was the development of outline drawings of servings of foods, as an aid to the learning of food values and meal planning. The National Dairy Council later produced these models in colors on cardboard, and they were and are widely used.

One of the striking characteristics of Miss Roberts was her ability to make and to retain the friendships of her associates. Yet she brooked no nonsense and demanded the best possible effort from her students. Her demeanor barred familiarities. She had a way of bringing out the best in her students and, almost without exception, they adored her. Her sense of humor, always evident, helped put others at ease, and speeded a chastised pupil happily on to better work. It was a feminine type of humor, reminding one of a little girl, watching the antics of little boys out of the corners of her eyes, quietly chuckling, and then sharing her amusement with her friends afterwards.

She once during these years wrote a letter to University officials, a letter which has been preserved with no indication that its humor was suspected or appreciated by the deans and other administrators who gravely considered its recommendations. In this letter Miss Roberts complained about students arriving tardily for her ten o'clock lecture. She had talked with the laggards, she wrote, and found that most of them had been detained by the instructors of their nine o'clock classes. One male student was dismissed on time but, though he ran all the way from the medical school, almost invariably he would be several minutes late. Miss Roberts then offered a number of possible solutions. One was that students be prohibited from scheduling classes "back to back." Another was that the University allow more time between classes. She had other suggestions, but the one which probably caused the most consternation was that the University provide a fleet of buses on which to transport students from one building to another around the campus. There are some attachments to this letter, which is in the file of so-called Presidential Papers in the Library of the University of Chicago, and there are indications that a notice was sent to all instructors, urging them to dismiss their classes more promptly in the future.

Chicago–the transition years

In retrospect, the years 1929 and 1930 represent a transitional period in Miss Roberts' career, though they began routinely enough. She was firmly established in the niche she had made for herself in Miss Blunt's department when, in the late Spring of 1929, some events occurred which were to have far-reaching effects. Miss Blunt, the woman who had started the publication of a series of monographs, in order to build up a literature on home economics, the series to which Miss Roberts' book was a notable addition, accepted a call to become president of Connecticut College for Women, at New London. At about the same time, it was announced that a young man, Robert M. Hutchins, would leave a minor post at another institution in Connecticut, and come to Chicago to fill the vacancy which

for some time had existed in its presidency. Even before he left New Haven, Mr. Hutchins was made acutely aware of the necessity of his filling Miss Blunt's vacated position. The administrative officers at the Midway felt at first that a person should be looked for outside Chicago, to head up a department that had grown so rapidly—almost alarmingly so, some of them may have thought.

The new President was subjected to letters, suggestions, and even interviews with volunteer candidates for the position before he got to Chicago. He made a sensible decision; a committee of three persons in the department was appointed to handle its administrative affairs, while further consideration could be given to the problem of making a permanent appointment. The committee, of which Miss Roberts was a member, promptly elected her their chairman, and the administration's search continued while the members of the staff went about their day-by-day routines. During the next several months some excellent persons were considered, some came to visit the institution, but nothing happened to fill the vacancy.

Early in 1930, the Federation met in Chicago at a time when the city was trying to dig itself out of a crippling snow storm. Some of the home economists who attended this meeting had received their training at the University of Chicago. There is good reason to believe that they held some informal conferences which were not included on the official program of the Federation, and which Miss Roberts knew nothing about. They drafted a letter, which each signed, and sent it to President Hutchins. In effect, they wrote that no matter how long the University might search, no better qualified candidate could be found than Miss Roberts. They called attention to her qualifications in an exceedingly well-written letter. They mentioned some of the studies she had done with children, and the esteem in which she was held by persons who knew her professionally. There were 30 signatures to this letter. Shortly afterwards the President received additional letters, from Miss Blunt and other individuals, each strongly recommending Miss Roberts. All these letters were acknowledged, somewhat perfunctorily. Then in May, 1930, in answer to a strong plea by a dentist with whom Miss Roberts had done some research, a man of influence at the University, President Hutchins wrote that Miss Roberts' name would be presented to the Board of Trustees in June, with the recommendation that she be appointed head of the department. In due course this was done and at the same time Miss Roberts was promoted to a full professorship.

White House Conference on Child Health and Protection. One of the points in Miss Roberts' favor which her friends had emphasized was that she had been appointed to three committees of the White House Conference on Child Health and Protection, called by President Herbert Hoover because of his intense interest in all children, and financed wholly from private funds. The reports of this conference were published in over 30 volumes by the Century Company in 1932; the conference was planned as soon as Mr. Hoover became President, and it got under way during 1929. Many distinguished scientists contributed to this endeavor; others served also, according to their abilities. The following reminiscences, hazy though they are because of the passage of time, provide some information that may be of interest.

> I was a member, along with Miss Roberts, of the Committee on Nutrition, under the chairmanship of Kenneth D. Blackfan. Late in 1929, or more likely, early in 1930, I attended a meeting of the Committee at the Children's Hospital in Boston. Considering that from this time on my contacts with Miss Roberts were many, I am sorry to say that I have no recollection of meeting her in Boston. I am not sure that she attended the meeting, although some woman from Chicago did. She sat at the other end of a long table, and on my side, so that I did not get a good look at her when she was called on to present her comments. Her subject was the caloric requirements of children. She passed around some mimeographed sheets which contained a great mass of information having to do with everything that had ever been written on the subject. I was impressed, and I think others there were also impressed, especially some of the clinicians whose presentations of their own assignments had been casually sketchy. It was obvious that this person knew what she was talking about, for she could evaluate each of the reports summarized on her sheets, and talk knowingly about research

that ought to be done. Later on, at this same meeting, Dr. Alfred T. Shohl suggested that the effects on children of drinking tea, coffee and cocoa be discussed in our final report. The woman at the other end of the table, the same one who had discussed calories, and who was either Miss Roberts or one of her associates, told us that somebody in Chicago had done this already for another purpose. In due course this material appeared as a separate chapter in our book, volume 3 of the series, edited by Dr. Hallowell Davis ["*Reports of the White House Conference on Child Health and Protection*"]. I remember on the train ride back to Cleveland, Doctor Shohl's telling me of the excellent work being done by the Chicago women. He did not need to tell me that the Roberts' report on calories had stimulated at least one member of the Committee to put extra effort into his own assignments.

Chicago — the harvest years

From 1930 until her retirement in 1944, Miss Roberts led a life of continuous professional activity and accomplishment. She kept up her teaching duties in child feeding. She inherited or secured competent persons to handle other phases — Dr. Evelyn Halliday, for instance, in foods and their preparation, Dr. Hazel Kyrk in economics, and Dr. Margaret Hessler Brookes, whose field involved experimental work with animals. A study of Miss Roberts' bibliography, which has been compiled and published by Ethel Austin Martin (in the *Journal of the American Dietetic Association,* 1966), will show her continued interest in experimental studies of the nutritional needs of children. These, her major studies, began with the research on caloric requirements with Bernice Wait (Woods) and proceeded to studies of protein, mineral and vitamin needs, with other students over many years. The research associated with her name was eminently practical; the results could be applied directly to problems of feeding children and adults, and they often were made available just when pressing need for the information had become known.

Many of the research publications during these golden years in the history of home economics at the University of Chicago, papers which dealt principally with human requirements, are cited today whenever the subject of the body's needs for nutrients arises. In addition to studies of human requirements during health, there was a series of reports on the effects of liberal additions of milk to diets already adequate; for this particular work, Miss Roberts received a Borden Award in 1938. All the papers from her department were characterized by thoroughness and accuracy. The actual laboratory work was done by the students. The names of numerous young women who subsequently went on to careers of distinction of their own appeared as collaborators. It is a temptation to mention each of these students and their contributions — to discuss, for example, the extraordinary studies of iron balances made over periods of months in order to obtain data on the iron losses in consecutive menstrual cycles — but space limitations make this impossible.

During these years Miss Roberts not only directed research and ran the affairs of her department, but she also had a full roster of other obligations. She not only taught students at the University and those who attended summer sessions, but she also wrote in whole or in part numerous articles and government bulletins for the general public. She served on several committees and boards having to do with problems of nutrition. Her services on the Council on Foods and Nutrition of the American Medical Association and on the Food and Nutrition Board of the National Research Council deserve special mention, because she contributed importantly to some of the good things which these bodies have accomplished.

Council on Foods and Nutrition. Miss Roberts was made a member of the Council, then called the Committee on Foods, in 1934. She served continuously until 1948, when she resigned because of the pressure of her duties in Puerto Rico. For many years she was depended on, along with Mrs. Mary Swartz Rose, to present the views of nutritionists in Council deliberations. Except on minor details, both women usually agreed. Dr. Ruth Cowan Clouse, when a member of the Council staff, often took questions directly to Miss Roberts, over week-ends for example, when she would be a guest at the cottage in the Indiana dunes which Miss Roberts shared with Miss Halliday. When on Mondays Dr. Clouse would come into the office, she

would have with her a well thought-out plan for the consideration of the Council as a whole, on matters such as strained meats in the diets of infants, what the Council policy should be towards commercial desserts for young children, or perhaps, how the nutritive values of certain foods might be properly described in advertising.

The Council considered many problems which confronted industry during those years, especially questions about the addition of vitamins and minerals to different food products. When the nutritional improvement of flour and bread was first discussed, it was Russell M. Wilder and George R. Cowgill, and others, who worked most actively to establish what came to be known as the enrichment program. So when the fortification of foods with vitamins and minerals was announced as the subject of a symposium which Dr. L. A. Maynard planned for the meeting of the American Institute of Nutrition in 1940, and Dr. Roberts was invited to participate, it was with some trepidation that some of the Council members went to listen to what she would say. It may be recalled that at this meeting Dr. W. H. Sebrell, Jr. spoke vigorously in opposition to the addition of vitamins and minerals to foods; he changed his views later. Of the four speakers at this symposium, the first time the subject was considered by a scientific group except for the A.M.A. Councils, Miss Roberts voiced the Council's views; she alone was unequivocally in favor of the addition of selected vitamins and minerals to selected foods. Her forthright acceptance of the idea meant a great deal at the time, which was a crucial period in the entire enrichment program, before it had actually been accepted by the milling and baking industries.

The Food and Nutrition Board. Miss Roberts was made a member of the original Board, then called a Committee, in 1940. This was in the days of military preparedness before this country became an acknowledged participant in World War II. It was a curious circumstance that permitted Miss Roberts to serve as a member when she was appointed. For various reasons, a policy prevailed then of not asking anyone to serve who was over the age of 60; this eliminated E. V. McCollum, Henry C. Sherman, John R. Murlin and Mary S. Rose, probably the four leading authorities on nutrition in the world. It should have eliminated Miss Roberts, but because she never recorded her birth year for *American Men of Science,* nobody knew her age exactly. The policy of the committee, which was never publicized so that Miss Roberts never knew it existed, was soon changed, and each of the "oldsters" mentioned served, and served importantly, as members of the group, except Mrs. Rose, who was suffering what proved to be her fatal illness.

So it came about that at the first meeting of the Board, to use its later name, Miss Roberts, who did not belong there, sat in on a discussion of the needs for dietary standards. Something like a yardstick was essential to help interpret food consumption figures for large groups and populations, as well as to help plan agricultural production goals. The League of Nations, under the leadership of Mrs. Rose, had previously formulated certain standards, but much new information had become available. The Food and Drug Administration, under the leadership of Dr. Elmer M. Nelson, had considered requirements in connection with the labeling of foods for special dietary purposes, but the regulations had not yet been published. It was felt that the matter should be re-examined from a broad viewpoint, because of the important and diverse uses to which the standards would be put.

Accordingly, Dr. Wilder, the chairman, appointed a committee of three members, with Miss Roberts as chairman, and asked that they retire, pool their information, and report their recommendations about dietary standards before the entire committee the following morning. Miss Roberts has described her consternation when she heard this request; amusingly, she asserted that she and Hazel Stiebeling and Helen S. Mitchell tackled their assignment in a hotel room while the men on the committee were "seeing the town." Miss Roberts was joking when she said that. She knew that Dr. Wilder had assignments for everyone, and that he often continued meetings after dinner, and called them for Saturday afternoons and all day Sundays,

if need be, in those days. Nor does she tell the whole story. She did report promptly as requested, and she did furnish figures for about ten important nutrients, including calories, to serve as a rough guide for immediate use. At the same time she emphasized the importance of the assignment, and expressed a desire that it be handled in a manner that would produce figures which the experts who might use them would be willing to accept. She was well aware of the difficulties likely to be encountered by anyone attempting this chore. At this first meeting, one member wanted the allowance for thiamine set at 3 milligrams a day for the adult. It was under these conditions that a committee on dietary allowances was established, with a very sprightly 61-year-old as its chairman. The use of the word "allowances" in place of "requirements" was incidentally her terminology.

Miss Roberts served as chairman of this committee until she went to Puerto Rico. The committee has continued to function, with different personnel, in order to consider newer evidence as it became available. The tone which Miss Roberts sounded for the operations of the original committee was responsible for the general acceptance of these standards from the beginning. She permitted full discussion in her committee, then did most of the collation and the attempts at reconciliation herself. She had a test which she often applied. She would match suggested figures for allowances against the estimated contributions of a diet which she knew, from actual experience, to be adequate, in order to decide whether the figures for the allowances were "reasonable." When discrepancies occurred, extra weight might be given to the evidence of experience; this was a way to avoid mistakes from using inaccurate or incomplete data, which were all too common before improved methods of assay had been developed. She sought the adivce of qualified persons throughout the country, so that many persons can truthfully claim to have had a part in the development of the first edition of the Recommended Dietary Allowances. It was a democratic procedure but also a tedious job of work. After her committee was satisfied, she had the additional task of presenting her report to the Board, when further discussion would ensue. She never faltered, she never lost patience, she considered every suggestion and subjected all questions to serious evaluation. Revisions were made when she and her committee considered them to be justified and doubtless, as Elmer Nelson once remarked, the recommended allowances were as good a set of figures as any competent body could develop.

Miss Roberts also served on other committees of the Broard, notably as a member of the Committee on Nutrition in Industry. She helped this committee keep its sights focused on the importance of foods, at a time when some persons would have simplified matters, as they thought, by dispensing multi-vitamin preparations to workers in industry. One can almost underline the phrases in the two reports of this committee which were published, for which she alone could have been responsible. Who but Miss Roberts would have written, in a discussion of the importance of having dietitians manage in-plant feeding practices, the following comments:

> The dietitian should be one who is able to deal easily and agreeably with people and to be interested in their welfare. Her primary job is to humanize the science of nutrition. To do this she must be able to make the diet so simple that anyone can understand it. Above all, it must be practical.

Puerto Rico

One day in Washington, at a Board meeting late in 1942, Dr. M. L. Wilson of the Department of Agriculture (sometimes referred to as the Father of the Board) approached Miss Roberts and asked her what she was going to do during during her "free quarter" at the University. He went on to say that there was a problem in Puerto Rico, which ordinarily imported much of its food, because of the diversion of coastal shipping during the war. Would Miss Roberts go to Puerto Rico, study the food and nutrition situation there, and report back to Washington? Miss Roberts would. She went there early in 1943 and, when she returned, she turned over a detailed report with practical suggestions about what could be done to assure better nutritional well-being, including information about the personnel and facilities of

the Island for carrying out her recommendations. One of the first steps taken by the Washington officials was to arrange for Miss Roberts to return to Puerto Rico in June, to conduct one of her famous workshops in nutrition education.

A so-called workshop is a pedagogic technique for the instruction of advanced students and workers in practical nutrition, and it is one of Miss Roberts' important contributions. Its methods follow essentially the Socratic method of asking questions, defining problems, and developing answers. Participants bring their problems and the group works out their solution under the guidance of the leader. This teaching method, borrowed from others in the field of education, was perfected by Miss Roberts and first used by her in a course held in Michigan in 1940, under the auspices of the Kellogg Foundation and the University of Chicago.

The workshop in Puerto Rico was attended by all the qualified nutritionists and workers in related fields on the Island. Some of her observations on the problems peculiar to Puerto Rico, and similar areas at the time, were described by Miss Roberts in an address before the American Dietetic Association on October 20, 1943. The date is mentioned in order to indicate the rapidity with which matters were taken care of under war-time conditions; by then, actions based on Miss Roberts' original report were well under way.

Back in Chicago, Miss Roberts was visited one day early in 1944 by the Chancellor of the University of Puerto Rico. He asked her to come to Puerto Rico as a member of the staff of the university. He could not have selected a more propitious time to have made this request. That was the year when Miss Roberts would be obliged to retire from the faculty of the University of Chicago on account of age.

She accepted the invitation as a challenge, and went to Puerto Rico as soon as the academic year was over. When she left the university with which she had been associated as student and professor for 29 years, she simply walked away. Her sister Lillian, who worked as a secretary, took care of removing the books, papers, and personal belongings from the office, so that the quarters would be available for Thelma Porter, Miss Roberts' former pupil and her successor at the University of Chicago. For two years, Miss Roberts continued to regard Chicago as her home, and she returned at intervals to the residence where Lillian continued to live — with the phone number still listed in the name of Lydia J. On one such occasion she suddenly announced that henceforth Puerto Rico would be her home. She broke off all remaining connections with affairs in the Chicago area, and although she returned for an occasional visit, Puerto Rico was indeed her home from that time on.

With characteristic energy and enthusiasm, Miss Roberts rejuvenated the teaching of home economics at the University in Rio Piedras, strengthened the nutrition education programs of the Island, and stimulated young men and women as she had previously stimulated others in Chicago. Friends have often remarked that she had two careers, one in Chicago and the other in Puerto Rico. They overlook her earlier career as a teacher of elementary grades. If Miss Roberts is regarded as a teacher, she had but one career, to which she devoted her energies during her entire adult life.

One of the first projects which engaged her attention in Puerto Rico was a thorough study of the food habits of the people. A report of this study, made over a five-year period, was published as a book with Rosa L. Stefani, who later succeeded Miss Roberts in her position at the University of Puerto Rico. The title of this book, published in 1949, was "*Patterns of Living in Puerto Rican Families.*" A summary account of this work appeared in *Nutrition Reviews* for November, 1950, and a paper on "A Basic Food Pattern for Puerto Rico," one of the results of this study, appeared in the *Journal of the American Dietetic Association* in 1954. Ten years later, in the same journal, there was another article entitled "A Cooperative Nutrition Research Program for Puerto Rico. 1. Background and General Plan." Through such reports the many friends of Miss Roberts knew something of what she was doing. Those who visited her on the island and there were many, learned about additional journal articles and pamphlets for which she was responsible; some of these had been

translated into Spanish. Numerous honors came to Miss Roberts during these years. They have been listed in Mrs. Martin's splendid tribute to her teacher and friend, published in the dietetic journal in 1965. Miss Roberts enjoyed receiving these awards — they showed an interest in nutrition on the part of those who gave them — but she probably derived more pleasure from the successful completion of a nutrition education project for an entire Puerto Rican community; this achievement, which has been alluded to several times in this sketch, may now be described.

Doña Elena

Miss Roberts wrote the story of the crowning achievement of her career in a book entitled, *"The Doña Elena Project."* It bears a sub-title, "A Better Living Program in an Isolated Rural Community." The book was published by the University of Puerto Rico in 1963. Persons concerned with the problems of developing nations, to whom this book has been shown, have been unable to put it down until they have read it through. It is a book which is bound to become recognized as a classic in the literature on nutrition.

Doña Elena was an isolated community of about 100 families, situated about five miles away from an adequate road to the towns and cities. The people lived in poverty more severe than that encountered by Miss Roberts in her early work among the hill people of Kentucky. The emphasis of the welfare program which was carried out in Doña Elena was on nutrition education. Other measures were taken but — this was important — they were tied in with the nutrition program. The community was provided with a good road. Electricity was brought in. These improvements the people knew they wanted. Because they got them they paid more attention to what those in charge of the nutrition education program had to say about foods — for these persons were looked on as responsible for getting a good road and electric power lines. All activities of a welfare nature were under the direction of a nutritionist and her husband, who happened to be an agronomist. This young couple moved into the community, where they taught by example as well as by precept. The local school, which had been poorly attended, was made a place the children were loath to stay away from. Three meals a day were served to all children on school days, affording a real opportunity to make nutrition a part of their daily lives. The children learned to eat certain foods and their families soon became aware of the importance of what was being taught. The Williams-Waterman Fund in New York sent Dr. Elmer Severinghaus to conduct a survey of nutritional status, both early in the program and after about a year, and the health officers of the Island cooperated throughout. The people, under guidance, did many things for themselves. Latrines, once non-existent, were constructed. Kitchens, once primitive, were improved. Home gardens were started, but not without difficulty. One reason was that most of the men were seasonally employed on tobacco plantations and, during the growing season, they had little time or inclination to care for a garden in the little plots available to them. Somehow, enthusiasm was generated and maintained, the people were instructed about the kinds of food plants to grow and how to use the harvest, and success was slowly obtained. To provide another source of meat, a scarce food in the dietary, boys were given rabbits to breed, after first promising to distribute the young from the first litters to other boys to start additional colonies. The agricultural services introduced improved strains of milk cows, and chickens that would lay more eggs. Every agency that could contribute did so, but always the focus of attention was on what the people must do for themselves to improve their standard of living.

The results were so striking, in better health, in improved growth of children, in the appearance of the community, but especially in the mental attitude of the people, that the legislature of the Island enacted a law in 1960 for the purpose of extending these benefits to others. This Law established an organization to provide for all communities on the Island needing improvement, a program similar to that which had been demonstrated in Doña Elena. In 1965 it was reported that about 30 communities had suitable pro-

grams under way. More were planned as soon as personnel and facilities would permit. Similar programs have been either considered for other Latin American countries by their governments, or have been put into effect. The Doña Elena project showed government officials how the people can learn to do things for themselves, and it showed nutritionists what an ideal nutrition education program can accomplish.

Who should receive credit for what was accomplished? Of course, Miss Roberts planned it all and saw that it was done. But there is no doubt that others could have accomplished what she did. The young people who were locally in charge of the project, Alejandro and Carmen Santiago, deserve much credit. So do others who participated, and there were many. If any of these persons had failed, there is no doubt that the results would not have been as favorable as they were. Who was the indispensable person?

The answer is probably in Miss Roberts' book, where she described how the project was begun. The Governor of the Island and Miss Roberts had a talk, in which the Governor expressed his concern for rural families that had not benefited from his industralization program. Miss Roberts then stated:

> In the course of the discussion which followed I chanced to tell him of a "pipe-dream" I had long had. This was to select a remote rural community of some 100 or so families, make a detailed study of all aspects of living conditions for every family, and then on the basis of the findings have all agencies concerned plan and carry out a concerted program to help raise the standard of living in every family.... The Governor was enthusiastic about the idea and decided that it should be put into action.

This discussion took place in 1956. Not even the hurricane which struck the Island that year could delay Miss Roberts, once the project was approved. The Governor, Luis-Muñoz Marin, continued to be helpful; naturally, all other officials, including the superintendent of schools, cooperated. The really indispensable person, therefore, can be none other than the Governor. Yet who, except Miss Roberts, could have secured his backing for a "pipe-dream"?

During her post-Chicago career, which lasted 21 years, Miss Roberts continued to write guidelines for practical nutrition work, not only for Puerto Rico, but for people in other areas. During "vacations" she made surveys for the World Health Organization — in Uganda, East Africa, for example — and for other organizations — in Costa Rica, Jamaica, and elsewhere.

"Now cometh rest . . ."

On the morning of May 28, 1965, Miss Roberts was seated at her desk, at work as usual, even though she was again "retired" from university obligations. It had been a fruitful morning. She had just completed the arrangements for a new book, a text on nutrition for Latin American students, to be translated into Spanish. When it appears in English and in Spanish, Ethel Austin Martin, ever faithful, will have rendered one more service for her dear friend and mentor, for she will have guided the manuscript through its printing. While seated at her derk at the University, shortly before lunchtime this day, Lydia J. suddenly collapsed. She was taken, unconscious, to the hospital, where she died two hours later; her fatal illness was said to have been the result of an aneurysm of the aorta. Her body was flown to Martin, Michigan, and there, in the little cemetery of her old hometown, Lydia Jane Roberts was buried. All these things happened so quickly that it was some time before her many friends could realize that this gracious lady was now obliged to rest from her labors of a lifetime, and that it would be up to others to carry on her efforts in behalf of little children everywhere.

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The Biological Value of Selenium in Bovine Milk for the Rat and Chick [1,2]

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ABSTRACT Chemical and biological assays of experimentally produced dried skim milks demonstrated that the lactating cow could incorporate selenite placed in the rumen into milk. Rat and chick assays were designed to test the biological potency of milk selenium as compared with selenite for growth and the prevention of death due to liver necrosis or exudative diathesis. The basal diets contained Torula yeast, and 4 to 10% dried skim milk was added at the expense of the carbohydrate. Selenium source comparisons were made only within the same level of dried skim milk supplementation; selenium was supplied either by induced high selenium milk or by "normal" low selenium milk plus an equated amount of Na_2SeO_3. In the rat assays the high protein quality of the added skim milks produced a greater protective effect than could be attributed to their selenium content. However, controlled comparisons showed no significant differences between the selenium sources. In the chick assay, the milk source was of greater biological value than the selenite source for the prevention of exudative diathesis.

Selenium administration has been effective in preventing liver necrosis in the rat (1), exudative diathesis in the chick (2, 3) and muscular dystrophy in the lamb (4–6). Feeding selenium to the ewe and allowing placental or mammary transfer of the selenium to the lamb has prevented enzootic muscular dystrophy, or white muscle disease. McConnell et al. (7, 8) and Jones and Godwin (9) have demonstrated that lactating females incorporate injected inorganic ^{75}Se into protein fractions of milk. Numerous investigators have also isolated seleno-organic compounds from animal tissues after administering inorganic selenium.

Historically, the most interesting organic source of selenium has been a purified material (Factor 3) isolated by Schwarz (1, 10) from swine kidney powder. The activity of the selenium in Factor 3 preparations as determined by activation analysis has exceeded by threefold the potency of other selenium compounds tested in a rat liver necrosis assay (11). To date no evidence has appeared to explain the apparent biological potency of Factor 3-selenium.

The possibility that dietary selenite may be converted by rumen microflora or animal tissues to a more biologically active form which would be secreted in milk prompted this investigation. A bioassay comparing selenium in dried skim milk with an inorganic selenium for the prevention of vitamin E and selenium deficiency syndromes in the rat and the chick are reported herein. A preliminary report of this work has appeared previously.[3]

EXPERIMENTS AND RESULTS

Preparation and analyses of dried skim milk. To obtain controlled quantities of the dried skim milks needed, milk was collected from 2 Holstein cows which were fed a locally obtained grass-legume hay. This "normal" milk was considered low selenium milk and served as the control. Immediately following this collection period 16 mg of selenium, as Na_2SeO_3 solution, were introduced into the rumen via a fistula twice daily for a 21-day preliminary period and a subsequent collection period. This quantity of selenium was equivalent to the addition of approximately 2 ppm selenium to the total ration. The

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[1] This investigation was supported in part by Public Health Service Research Grant no. NB-03517 from the National Institute of Neurological Diseases and Blindness.
[2] The authors are indebted to E. E. Cary and W. H. Allaway for the selenium analysis of the milk samples.
[3] Mathias, M. M., D. E. Hogue, J. K. Loosli and M. L. Scott 1963 The biological activity of selenium in bovine milk for the chick and rat. Federation Proc., 22: 377 (abstract).

TABLE 1

Composition of the experimental dried skim milks

Dried skim milk	Moisture [1]	Crude protein [2]	Fat [3]	Selenium [4]
	%	%	%	*ppb*
Low selenium	4.3	34.1	0.61	60
High selenium	3.2	36.2	0.72	280

[1] Determined by toluene distillation.
[2] Kjeldahl nitrogen × 6.38.
[3] Determined by the Mojonnier method.
[4] Determined by fluorometric analysis (13).

milk collected during this period was designated high selenium milk. About 300 kg of each milk were collected.

The milk was frozen at the time of collection; the fat was subsequently removed following thawing and elevation to a suitable temperature. The skim milk was then dried by lyophilization to avoid possible selenium loss (12), mixed and stored at − 10° until used for diet preparation.

The chemical analyses of the dried skim milks are shown in table 1. The probable low content of vitamin E in the milk, due to the removal of most of the milk fat, was not considered important in the chick and rat bioassays of selenium. The selenium content measured fluorometrically (13) was used to calculate the selenium contributed by the dried skim milk to the various diets. Values on the high Se milk obtained by activation analyses were variable and consistently lower than those obtained by the fluorometric procedure. Allaway and Cary (13) found general agreement between the 2 methods for many feeds but were unable to obtain similar values for this particular sample.

Experimental diets. The basal diets (table 2) were patterned after Schwarz for rats (1) and after Nesheim [4] for chicks. The experimental dried skim milks were incorporated in the diets at the expense of the carbohydrate source. The inorganic selenium was added as Na_2SeO_3 in an aqueous solution.

Four basic diet series were usually included in each experiment as follows: 1) basal with or without additions of Na_2SeO_3; 2) basal with low selenium milk; 3) basal with high selenium milk; and 4) basal with low selenium milk plus Na_2SeO_3 so that the calculated selenium content equaled that of series 3. Each experiment included a standard "assay" curve (diet series 1) without added milk as a reference, thus it was possible to determine the effect of the milk per se. The last 2 diet series made possible direct comparisons of selenium source (that is, Na_2SeO_3 or milk) with all other components of the diet equal. The number of dietary treatments included in each experimental diet series varied because of the factors discussed with the appropriate experiment.

Experiments with rats. Weanling male rats of the Holtzman strain allotted at

TABLE 2

Composition of basal diets

	Rat	Chick
	%	%
Torula yeast [1]	30.0	58.50
Carbohydrate [2]	55.8	29.57
Cellulose	3.0	—
Stripped lard [3]	5.0	5.00
Vitamin mixture	2.2 [4]	1.22 [5]
Mineral mixture	4.0 [6]	4.70 [5]
Amino acid mixture	—	1.00 [5]
Ethoxyquin	—	0.0125 [7]

[1] Kindly supplied by Lake States Yeast and Chemical Division, St. Regis Paper Company, Rhinelander, Wisconsin.
[2] Carbohydrate source for the rat diet was sucrose and for the chick diet, glucose monohydrate; dried skim milk was added at the expense of the carbohydrate.
[3] Distillation Products Industries, Rochester, New York.
[4] Vitamins in dextrose, amount/kg of diet: vitamin A, 19,800 IU; vitamin D, 2,200 IU; (in milligrams) ascorbic acid, 990; inositol, 110; menadione, 49.5; p-aminobenzoic acid, 110; niacin, 99; riboflavin, 22; pyridoxine·HCl, 22; thiamine·HCl, 22; Ca pantothenate, 66; biotin, 0.44; folic acid, 1.98; and choline chloride, 1.65 g and vitamin B_{12}, 29.7 μg (obtained from Nutritional Biochemicals Corporation, Cleveland).
[5] Mathias et al. (14).
[6] Phillips and Hart IV (Phillips, P. H., and E. B. Hart, J. Biol. Chem., *109*: 657, 1935) (plus cobalt) contained: (in per cent) K_2HPO_4, 32.2; $CaCO_3$, 30.0; NaCl, 16.7; $MgSO_4 \cdot H_2O$, 10.2; $CaHPO_3 \cdot 2H_2O$, 7.5; $FeC_6H_5O_7$, 2.75; $MnSO_4$, 0.51; KI, 0.08; $CuSO_4$, 0.03; $ZnCl_2$, 0.025; and $CoCl_2$, 0.005 (obtained from General Biochemicals, Chagrin Falls, Ohio).
[7] 1,2-Dihydro-6-ethoxy-2,2,4-trimethyl quinoline, Monsanto Company, St. Louis.

[4] Nesheim, M. C. 1959 Studies on the effect of selenium and other factors on vitamin E deficiency in the chick. Ph.D. Thesis, Cornell University.

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random to individually suspended wire cages were maintained in an air conditioned animal room (22°) and fed the appropriate diet with water supplied ad libitum. Groups of 5 rats each were designated according to vertical cage areas and two of these groups of 5 rats assigned at random to each treatment. The rats were observed at least 3 times daily. All the dead animals were autopsied. Most of the dead rats showed gross evidence of liver necrosis.

Experiment 1 was conducted with all animals fed the experimental diets for 48 days. In experiment 2, the basal diet was fed for 14 days to all animals to reduce the body stores of vitamin E and selenium (11) and then the experimental diets were fed for an additional 35 days.

Results from preliminary experiments established the criteria for the biological evaluation of selenium and a response curve was obtained covering intakes of selenium ranging from zero to 25 ppb added selenium. Holtzman rats fed the basal diet survived an average of 26 days. Deaths did not begin to occur in Dunning Fischer 355 descendants [5] until the basal diet had been fed for 57 days.

The results of the first rat bioassay are summarized in table 3. The addition of the dried skim milks decreased mortality and significantly ($P < 0.05$) stimulated weight gains above the response expected solely from their selenium content. For an adequate control of this protective factor, the 4, 6 and 8% levels of dried skim milk in the high selenium series and the low selenium plus Na_2SeO_3 series were compared to provide a contrast solely of selenium sources. Corresponding growth and survival data of the rats fed the 2 selenium sources were very similar within

[5] Charles River Breeding Laboratories, Brookline, Massachusetts.

TABLE 3

Effect of source and level of selenium on growth and survival of rats (exp. 1)

Treatment	Dietary selenium			Avg wt gain [1]	Avg survival time [2]	Deaths
	Na_2SeO_3	Milk	Total			
	ppb	*ppb*		*g*	*day*	*%*
			Standard "assay" curve			
Basal	—	0	0	80.0	28.9	80
+ 5 ppb Se	5	0	5	90.0	31.0	80
+10 ppb Se	10	0	10	115.6	34.6	50
+15 ppb Se	15	0	15	145.0	—	0
+20 ppb Se	20	0	20	147.4	—	0
+25 ppb Se	25	0	25	174.6	—	0
+30 ppb Se	30	0	30	176.4	—	0
			Basal with low selenium dried skim milk (DSM)			
4% DSM	—	2	2	121.5	29.6	78 [3]
6% DSM	—	4	4	108.3	30.1	70
8% DSM	—	5	5	149.0	32.5	20
16% DSM	—	10	10	169.7	42.0	10
			Basal with high selenium dried skim milk			
4% DSM	—	11	11	153.8	30.8	40
6% DSM	—	17	17	163.8	—	0
8% DSM	—	22	22	187.3	—	0
			Basal with low selenium dried skim milk + Na_2SeO_3			
4% DSM + 8 ppb Se	8	2	10	146.3	33.0	30
6% DSM + 12 ppb Se	12	4	16	167.8	—	0
8% DSM + 16 ppb Se	16	5	21	181.1	—	0
Estimated SD [4]				22	5	14

[1] Mean weight gained by the rats surviving the 48 days of experiment.
[2] Survival time is the number of days following the start of the experiment that the rat lived. Rats surviving to the end of the experiment were not considered in the average.
[3] Only 9 rats were considered.
[4] Estimated standard deviation of an observation calculated from the error mean square in the analysis of variance.

essentially equal selenium additions suggesting an equal biological value. The selenite form of selenium represented 80% of the total selenium in diets containing the low selenium milk plus Na_2SeO_3.

In the second rat bioassay, an attempt was made to increase the severity of the selenium deficiency and more combinations of low selenium dried skim milk and Na_2SeO_3 were included. The results are shown in table 4. Dried skim milk additions again provided greater protection than could be attributed to their selenium content alone. No significant differences were observed between the 4% low selenium dried skim milk plus Na_2SeO_3 treatments which were calculated to contain 10 ppb selenium and the 4% high selenium dried skim milk treatment which was calculated to contain 11 ppb. Thus, little or no difference in the degree of protection was detected between the 2 sources of selenium.

Experiments with chicks. Male one-day-old White Rock chicks [6] were allotted at random to thermostatically controlled, electrically heated pens with wire-mesh floors. Two pens of 10 chicks each were assigned at random to each treatment. The diets and tap water were supplied ad libitum. The chicks were observed weekly in experiment 3 and semi-weekly in experiment 4 for incidence of exudative diathesis. Experiment 3 was conducted for 42 days. In experiment 4, all chicks were fed the basal diet for 2 weeks as a depletion period. The chicks were then fed the experimental diets for an additional 20 days.

The results of the first chick bioassay (exp. 3) are summarized in table 5. The results for the standard "assay" curve show that the protectiveness of selenium markedly increased between 10 to 20 ppb selenium. A preliminary experiment and the work of Nesheim and Scott (16) indicated that the inflection point of the curve usually occurs at a higher level of selenium addition (between 20 and 40 ppb).

The incidence of death and exudative diathesis in chicks fed the diet containing high selenium milk was significantly lower ($P < 0.05$) than for those fed the diet containing a similar amount of selenium of which 77% was in the Na_2SeO_3 form

[6] Cobbs Chick Hatchery, Concord, Massachusetts.

TABLE 4

Effect of source and level of selenium on growth and survival of depleted rats (exp. 2)

Treatment	Dietary selenium			Avg wt gain [1]	Avg survival time [2]	Deaths
	Na_2SeO_3	Milk	Total			
	ppb	*ppb*		*g*	*day*	*%*
			Standard "assay" curve			
Basal	—	0	0	64.0	26.9	80
+ 5 ppb Se	5	0	5	—	34.2	100
+10 ppb Se	10	0	10	116.9	28.7	30
+15 ppb Se	15	0	15	134.5	—	0
+20 ppb Se	20	0	20	139.6	42.0	10
+25 ppb Se	25	0	25	155.6	—	0
			Basal with high selenium dried skim milk (DSM)			
4% DSM	—	11	11	107.6	34.7	30
8% DSM	—	22	22	167.2 [3]	—	0
			Basal with low selenium dried skim milk (DSM) + Na_2SeO_3			
4% DSM+ 4ppb Se	4	2	8	114.3	32.7	30
4% DSM+ 8 ppb Se	8	2	10	124.0	30.0	20
8% DSM+ 8 ppb Se	8	5	13	155.8	—	0
8% DSM+16 ppb Se	16	5	21	176.8	—	0
Estimated SD [4]				20	7	8

[1] Mean weight gained by the rats surviving the 49 days of experiment.
[2] Survival time is the number of days following the start of the complete experiment that the rat lived; rats surviving to the end of the experiment were not considered in the average.
[3] Only 9 rats were considered.
[4] Estimated standard deviation of an observation calculated from the error mean square in the analysis of variance.

TABLE 5

Effect of source and level of selenium on growth, survival and incidence of exudative diathesis in chicks (exp. 3)

Treatment	Total dietary selenium added [1]	Avg wt gain [2]	Avg survival time [3]	Deaths	Exudative diathesis
	ppb	*g*	*day*	*%*	*%*
		Standard "assay" curve			
Basal	—	—	26.0	100	100
+ 5 ppb Se	5	—	26.2	100	100
+10 ppb Se	10	418	30.5	80	95
+20 ppb Se	20	720	28.2	25	30
+30 ppb Se	30	782	—	0	0
		Basal with 10% dried skim milk (DSM)			
Low Se DSM	6	220	27.1	95	100
High Se DSM	28	765	—	0	0
Low Se DSM + 20 ppb Se as Na_2SeO_3	26	761	32.3	32 [4]	26 [4]
Estimated SD [5]		130	4	9	6

[1] The amount of selenium was calculated from the amount of Na_2SeO_3 added plus selenium content of the dried skim milks as determined by the fluorometric procedure.
[2] Mean weight gained by chicks surviving the 42 days of experiment.
[3] Survival time is the number of days following the start of the experiment that the chick lived; chicks surviving to the end of the experiment were not considered in the average.
[4] Only 19 chicks were considered.
[5] Estimated standard deviation of an observation calculated from the error mean square in the analysis of variance.

and the remainder was from an equivalent amount of low selenium milk. The close similarity between the data for the standard "assay" curve treatments and the low selenium dried skim milk treatments, when compared by their selenium content, suggests that no protective factor was detected in the milk other than selenium.

In experiment 4, the depletion period produced a more severe selenium and vitamin E deficiency as evidenced by the increased range of the standard "assay" curve (table 6). Comparison of the results associated with the milk selenium and the appropiate selenium levels in the standard "assay" curve also suggests that the selenium in the low selenium milk was available to the chick. The biological value of selenium, as established by average weight gain, average survival time, and incidence of death or exudative diathesis, tended to be greater for the milk source than for the low selenium milk plus Na_2SeO_3 source.

DISCUSSION

The chemical analysis and the biological assays of the experimental dried skim milks give further evidence that the lactating female incorporates dietary selenium into milk proteins. Calculations based upon the milk production of the experimental cows showed 0.7% of the added selenium was incorporated into their milk. Fluorometric selenium determinations of the protein fractions of the high selenium dried skim milk indicated the casein fraction (dilute HCl precipitate at pH 4.6) contained 580 ppb selenium or 59% of the total milk selenium and the whey portion (remaining trichloroacetic acid-insoluble material) contained 350 ppb selenium or 13% of the total milk selenium. The remaining 28% was considered to be present as selenite, selenate or free seleno-amino acids.

These values agree closely with those of McConnell et al. (7, 8) and Jones and Godwin (9) who investigated the mammary route for the secretion of injected inorganic ^{75}Se. The latter workers further demonstrated that lactating mice fed alfalfa, containing ^{75}Se-labeled protein, could transfer appreciable quantities of radioactivity to their suckling young via the milk.

Mathias et al. (14), in a later experiment, reported a similar biological activity for selenium supplied either in the selenite form or in alfalfa which contained

TABLE 6

Effect of source and level of selenium on growth, survival and incidence of exudative diathesis in depleted chicks (exp. 4)

Treatment	Dietary selenium			Avg wt gain [1]	Avg survival time [2]	Deaths	Exudative diathesis
	Na_2SeO_3	Milk	Total				
	ppb	*ppb*		*g*	*day*	*%*	*%*
			Standard "assay" curve				
Basal	—	0	0	—	21.3	100	100
+10 ppb Se	10	0	10	138	24.2	90	95
+15 ppb Se	15	0	15	281	25.6	80	95
+20 ppb Se	20	0	20	350	24.1	55 [3]	65 [4]
+25 ppb Se	25	0	25	366	25.4	60	70
+30 ppb Se	30	0	30	354	22.0	50	55
+40 ppb Se	40	0	40	488	24.5	10	20
			Basal with 10% dried skim milk (DSM)				
High Se DSM	—	28	28	500	25.9	40	40
Low Se DSM+ 10 ppb Se	10	6	16	228	24.8	65	90
Low Se DSM+ 20 ppb Se	20	6	26	430	23.4	60	60
Estimated SD [5]				—	4	12	13

[1] Mean weight gained by the chicks surviving the 34 days of the experiment.
[2] Survival time is the number of days following the start of the experiment that the chick lived. Chicks surviving to the end of experiment were not considered in the average.
[3] Range: 40–70%.
[4] Range: 50–80%.
[5] Estimated standard deviation of an observation calculated from the error mean square in the analysis of variance.

most of the selenium in the protein fraction. The bioassay was essentially the same as used in the experiments reported here except that smaller quantities of alfalfa were necessary because a very high selenium content was induced by soil fertilization. To assay the dried skim milk, additions of 4 to 10% were needed; subsequently, it was necessary to consider other endogenous factors. In the rat experiments but not in the chick experiments, an unidentified protective factor(s) was apparent. The rat diet was deficient in sulfur-amino acids and the addition of 8% dried skim milk would have supplied approximately 0.1% sulfur-amino acids which may have been responsible for most of the protective effect (16).[7] Controlled comparisons between selenium sources in the rat experiments indicated only small differences in protection, suggesting that the milk selenium did not possess an exceptionally high biological potency.

This was not the case in the chick experiments. Selenium in milk was more effective in increasing survival and reducing the incidence of exudative diathesis than selenite-selenium. The possible nutritional or physiological etiology of highly potent forms of selenium has not been fully elucidated. Schwarz (11) tested many organic sources of selenium in a rat assay and found that seleno-amino acids afforded the same protectiveness as selenite. A highly purified Factor 3 preparation was, however, much more effective. At a concentration of approximately 36 ppm, Factor 3 protected 50% of the rats from liver necrosis; the corresponding LD_{50}, calculated from selenium activation analysis, was 7 ppb selenium. For the seleno-amino acids and selenite, the LD_{50} was approximately 20 ppb selenium (11).

From results of the chick and rat assays of selenium in alfalfa (14) and in milk reported here, only the chick assay of high selenium milk suggested the presence of an exceptionally highly potent form of selenium. However, any conclusions from the bioassays depend strongly on the accepted method of selenium analysis and on species specificities. Also, possible synergisms, such as those between selenium and sulfur-amino acids or anti-

[7] Unpublished data, M. M. Mathias and D. E. Hogue, 1964.

oxidants,[8] can confound the bioassay procedures.

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Use of Free Plasma Amino Acid Levels for Estimating Amino Acid Requirements of the Growing Rat [1]

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ABSTRACT The validity of using plasma amino acid levels for estimating amino acid requirements of the growing rat was investigated. A series of diets was prepared (equivalent to 10% protein, provided by whole egg and an amino acid mixture simulating egg proteins) having graded levels of the amino acid under test. These test diets were fed to groups of 10 male Wistar rats, 28 to 30 days old, for 3 days. The amino acid under test was determined in the plasma of individual rats by microbiological assay. The dose-response curve relating plasma level and dietary level of the test amino acid was drawn. The requirement was considered to be the dietary level at which the corresponding plasma level was equal to the normal fasting level. Requirements expressed as percentage of diet were estimated to be: lysine, 0.65; tryptophan, 0.13; threonine, 0.42; leucine, 0.82; isoleucine, 0.50; and histidine, 0.20. Estimates of amino acid requirements of the rat were in close agreement with average values obtained by other methods reported recently in the literature.

Amino acid requirements vary with caloric and protein level of the diet and with age and physiological state of the animal. Nevertheless under specific conditions amino acid requirements are relatively fixed values and except for lysine are well established for the growing rat. Rao et al. (1) used both growth and protein efficiency ratio (PER) values in determining relative requirements for amino acids, whereas Bender (2) calculated net protein utilization (NPU) for this purpose. Although these methods appeared to be satisfactory in work with laboratory animals, similar studies with humans have yielded variable results (3) and there is a need for other criteria for judging adequacy of dietary amino acid levels.

Several years ago, Almquist (4) suggested that the relationship between plasma amino acid (PAA) levels and dietary protein might provide a direct method for establishing amino acid requirements. Morrison et al. (5) fed lysine-deficient diets supplemented with graded levels of lysine, to growing rats for 21 days and measured levels of free lysine in the plasma. The curve relating plasma lysine to dietary lysine was sigmoid, remaining almost flat until the diet contained a nearly optimal amount of lysine for growth and then deflecting sharply upwards. A similar type of response curve was reported by Zimmerman and Scott (6) for plasma and dietary levels of lysine, arginine and valine in the chick. Since the dietary level at which the test amino acid content of plasma began to increase markedly coincided with the requirement to sustain optimal growth, these authors concluded that the plasma technique can be used to determine the chick's requirement for amino acids.

In short-term studies (7) with rats, however, it appeared that there was a more direct relationship between plasma and dietary levels of the limiting amino acid and it was suggested that amino acid requirements could be "titrated" by addition of graded levels of the limiting amino acid to the diet until plasma level of this acid was equivalent to that characteristic of a fasting animal that had been maintained with an optimal diet. Such a level would have a plasma amino acid (PAA) score of 100 (7).

The utility of the plasma method for evaluating amino acid requirements was tested with young rats because the amino acid requirements of these animals have

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TABLE 1

Composition of diets

	Whole egg diet	Test diets
	g/100 g diet	
Whole egg powder [1]	23.0	13.8
Cornstarch	72.0	77.0
Mineral mixture, USP 14	4.0	4.0
Vitamin mixture [2]	1.0	1.0
Amino acid mixture [3]		4.2

[1] Whole egg powder contained approximately 30% fat.

[2] The vitamin mixture (1.0 g) supplied: vitamin A, 200 IU; vitamin D, 100 IU; and (in milligrams) *dl*-α-tocopheryl acetate, 40; menadione, 0.5; thiamine, 0.5; riboflavin, 1.0; pyridoxine, 0.4; pantothenic acid, 4.0; niacin, 4.0; choline 200; inositol, 25; *p*-aminobenzoic acid, 10; biotin, 0.02; folic acid, 0.2; and vitamin B_{12}, 2 μg.

[3] The amino acid mixture which was calculated from the average amino acid content of egg proteins as reported in the Amino Acid Handbook (11) and the Amino Acid Content of Foods (12) had the following composition: (in milligrams) L-arginine·HCl, 270; L-histidine·HCl hydrate, 96; L-lysine·HCl, 344; L-tyrosine, 164; L-phenylalanine, 232; L-cystine, 92; DL-methionine, 132; DL-serine, 312; DL-threonine, 400; L-leucine, 376; L-isoleucine, 276; L-valine, 296; DL-aspartic acid, 328; glycine, 144; L-proline, 180; L-tryptophan, 64; and L-glutamic acid, 504. The amino acid under test was omitted from mixture. Test diets containing graded amounts of the test amino acid were kept isonitrogenous by varying the level of glutamic acid.

been reasonably well established by independent methods.

EXPERIMENTAL

Weanling male Wistar rats [2] were kept in community cages and fed a commercial stock diet for 2 to 3 days. Six groups of 10 rats were then placed in individual screen-bottom cages in an air conditioned room maintained at 23 to 24°. Each animal was given 6.5 to 7.5 g of whole egg diet (graduated according to body weight) the composition of which is given in table 1, at 8:30 AM daily; rats that did not eat all their daily ration within 24 hours were given slightly less food on the next day. Rats in group 1 were kept on this regimen for 5 days, food being removed from cages at 4:30 PM on the fifth day and animals were fasted until 8:30 AM the next day at which time they were anesthetized, decapitated and blood of individual rats was collected separately in heparinized tubes. The other 5 groups of rats were given the whole egg diet for only 3 days and then were fed appropriate test diets (table 1) in a similar manner for 3 days. The range of concentrations of the test amino acid in diets included the normal requirement level of that amino acid as calculated from literature reports (1, 2). These animals were killed between 2:30 and 3:30 PM, six hours after the third daily presentation of the test diet.

In general, the food intake habits of animals receiving the whole egg diet and those receiving the various test diets were similar. Rats fed the diets containing the lowest dosage levels of the test amino acid, however, may have consumed food less rapidly than rats of the other group; on the final day (third day of test diets) these rats consumed an average of approximately 4.0 g of diet, whereas those fed more optimal diets ate approximately 5.0 g.

Blood plasma was deproteinized and levels of amino acids in plasma of individual rats were determined by microbiological assay as described previously (8).

RESULTS AND DISCUSSION

As shown in figure 1 (A) there was an almost linear relationship between free lysine in plasma and the dietary level of lysine. The fasting lysine level was 60 μg/ml and interpolation from that point on the response curve showed that a dietary level of 0.65% was required to produce a corresponding lysine level in plasma of rats fed the test diets. In table 2 amino acid requirements of young male rats (10% level of protein) as observed in the present study are compared with values reported by Rao (1) and Bender (2). These estimates of lysine requirement varied widely. The intermediate value of 0.65% found in this study is close to the amount provided by a whole egg protein diet and Mitchell (9) has suggested the amount in egg is very close to the rats' requirement. Data reported by Bressani and Mertz (10) also indicate a lysine requirement of approximately 0.60% at the 10% level of dietary protein.

The PAA response curve of tryptophan is shown in figure 1 (B); the indicated requirement was 0.13%. Agreement regarding the tryptophan requirement (given in table 2) was good. Bender's basal diet contained 5% protein supplied by egg and 5% amino acids in a mixture simulating the composition of egg protein. In calculating the amount of tryptophan supplied by egg proteins, Bender used the

[2] Obtained from Woodlyn Farms, Guelph, Ontario.

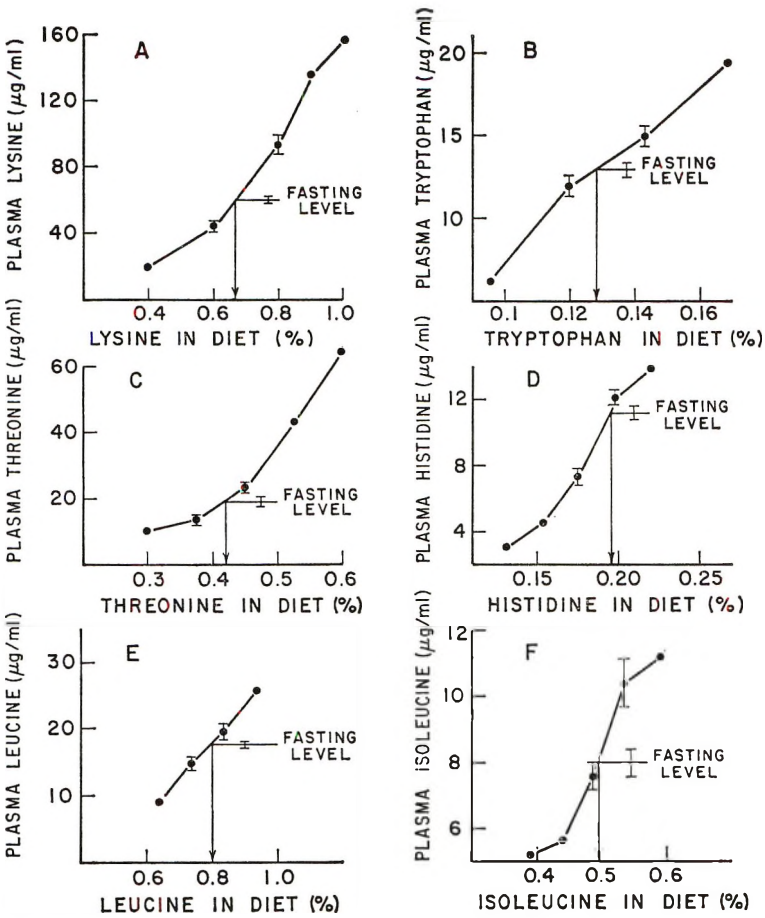

Fig. 1 Relationship between plasma and dietary levels of individual amino acids. Vertical bars indicate SE.

tryptophan content of egg reported in the Rutgers collaborative study (11) which appears to underestimate the tryptophan content of egg. If the earlier Rutgers' value had been used in the present study, the estimate of the tryptophan requirement would have been 0.10, that is, the same as the requirement reported by Bender.

PAA dose-response curves for histidine, threonine, leucine and isoleucine are shown in figures 1 (C) to 1 (F). The 3 estimates of histidine requirement were in good agreement. Two experiments were run with leucine because variation at the highest dosage level was excessive in the first experiment; nevertheless the 2 estimates of leucine requirement agreed within 5% (the curve for the second experiment is shown in figure 1 (E)). The leucine requirement reported by Rao et al. and the isoleucine value found by Bender were approximately 15% lower than estimated requirements obtained in the other two laboratories.

In the earlier reports of Morrison et al. (5) and Zimmerman and Scott (6) the dose-response curves remained almost flat initially and then deflected sharply upwards, whereas in the present study the relationship was more direct. This difference, with the rat at least, reflects the duration of the period the deficient diets were fed. With the longer feeding period the limiting amino acid fell to an inordinately low level in the plasma; advantage was taken of this phenomenon in the PAA score

TABLE 2

Estimated amino acid requirements of the growing rat [1]

Amino acid	Present study	Rao et al. (1)	Bender (2)
Lysine	0.65	0.9	0.52
Tryptophan	0.13	0.11	0.10
Histidine	0.20	0.25	0.18
Threonine	0.42	0.50	0.41
Leucine	0.82 [2]	0.70	0.78
Isoleucine	0.50	0.55	0.43

[1] Expressed as percentage of diet at a protein level of 10%.

[2] Mean value of 2 experiments (estimates were 0.84 and 0.80). The first test was not considered reliable because variation among animals was great; variation was not large in the second experiment and the leucine requirement was estimated to be 0.80.

method (7) for predicting the limiting amino acid in dietary proteins.

Previous studies with rats (5), (7) and chicks (6) have indicated that PAA levels may be useful for titrating the amino acid requirements of animals. The present study shows the PAA method reliable in deriving dietary amino acid requirements for the growing rat and suggests that it might be successfully applied to the study of other animals including man.

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Protein as a Carbohydrate Precursor in the Chick [1,2]

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ABSTRACT Experiments were conducted to study the effectiveness of extra protein in promoting growth of chicks fed "carbohydrate- and glycerol-free" diets. Diets containing 15.4, 13.2 and 11.0 kcal metabolizable energy/g protein were fed. Results indicated that the requirement of the chick for carbohydrate can be met by protein but that the protein mixture used (soybean protein, methionine and glycine) was much less efficient than glucose in meeting this requirement. Pair-feeding studies were conducted to determine whether chicks fed "carbohydrate- and glycerol-free" diets divert amino acids from protein to carbohydrate synthesis. Results showed that little protein was diverted from protein to carbohydrate synthesis when protein was supplied by a mixture of soybean protein, methionine and glycine. Protein retention with and without supplemental glucose was 52 and 50%, respectively. However, deletion of excess glucogenic amino acids from the diet by replacing protein with a mixture of amino acids resulted in a marked reduction in nitrogen retention and growth which was overcome by the addition of glucose. These results indicate that excess glucogenic amino acids in soybean protein contribute to the carbohydrate requirement of the chick. The ability of the chick to synthesize carbohydrate from fatty acids, if present at all, is extremely limited.

Previous studies with the chick showed that deletion of glycerol from a "carbohydrate-free" diet by substituting soybean fatty acids for soybean oil resulted in a marked depression in rate of growth which could be largely overcome by the addition of glycerol or glucose in the amount required for theoretical conversion of fatty acids to triglyceride (1). The following experiments were conducted to 1) determine the effectiveness of extra protein in promoting growth of chicks fed "carbohydrate- and glycerol-free" diets; 2) determine whether chicks fed "carbohydrate- and glycerol-free" diets divert amino acids from protein synthesis to synthesis of carbohydrate; and 3) determine the role which glucogenic amino acids play in promoting growth of chicks fed "carbohydrate- and glycerol-free" diets.

MATERIAL AND METHODS

To determine the effectiveness of extra protein in promoting growth of chicks fed "carbohydrate- and glycerol-free" diets (exp. 1), duplicate groups of 10 male crossbred (Dominant White × Plymouth Rock) chicks were fed diets containing 15.4, 13.2 and 11.0 kcal metabolizable energy/g protein in which non-protein energy was supplied by either soybean oil or soybean fatty acids. Since soybean protein contains approximately 3.83 kcal metabolizable energy/g, the protein in these diets supplied 24.9, 29.0 and 34.8% of the total energy, respectively. For comparative purposes, chicks also were fed soybean fatty acid diets containing 15.4 kcal metabolizable energy/g protein supplemented with 0.035, 0.105 and 0.210 g glucose/g fatty acids. Glucose added at these levels supplies approximately 1.0, 2.9 and 5.7% of the total calories, respectively.

The composition of the "carbohydrate-free" diet containing 15.4 kcal metabolizable energy/g protein is shown in table 1. This diet contained sufficient protein to promote rapid growth but at this level protein was not in excess (2). Diets containing 13.2 and 11.0 kcal metabolizable energy/g protein (table 1) were formulated from this diet by the isocaloric replacement of part of the soybean oil by the mixture of soybean protein, methionine and glycine. Diets in which non-pro-

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[2] Reported in part at the 1964 meetings of the American Institute of Nutrition: Renner, R., and A. M. Elcombe 1964 Metabolic effects of feeding "carbohydrate-free" diets to chicks. Federation Proc., 23: 50 (abstract).

TABLE 1

Composition of "carbohydrate-free" diets

	Metabolizable energy/g protein, kcal		
	15.4	13.2	11.0
	g	*g*	*g*
Constants			
Limestone	1.49	1.49	1.49
Dicalcium phosphate	1.70	1.70	1.70
Sodium chloride	0.60	0.60	0.60
Mineral mixture [1]	1.50	1.50	1.50
Vitamin mixture [2]	0.52	0.52	0.52
Antioxidant [3]	0.025	0.025	0.025
Variables			
Soybean protein [4]	23.59	27.64	33.16
Methionine	0.81	0.95	1.14
Glycine	0.63	0.74	0.89
Soybean oil	28.54	26.75	24.31
Cellulose [5]	7.19	6.03	5.13

[1] Mineral mixture supplied: (in mg/100 g diet) KH_2PO_4, 930; $MgSO_4 \cdot 7H_2O$, 495; KI, 0.29; $FeSO_4 \cdot 7H_2O$, 28; $CuSO_4 \cdot 5H_2O$, 0.78; $ZnCl_2$, 12.5; $CoCl_2 \cdot 6H_2O$, 0.17; $Na_2MoO_4 \cdot 2H_2O$, 0.83; Na_2SeO_3, 0.022; and $MnSO_4 \cdot H_2O$, 37.

[2] Vitamin mixture supplied: (in mg/100 g diet) thiamine, 1.0; riboflavin, 1.0; Ca pantothenate, 4.0; biotin, 0.04; pyridoxine, 2.0; niacin, 8.0; folacin, 0.3; menadione, 0.3; vitamin B_{12}, 0.005; choline chloride, 210; vitamin A palmitate, 1000 USP units; vitamin D_3, 150 ICU; vitamin E acetate, 3.31 IU; and chlortetracycline, 1.

[3] Ethoxyquin.

[4] Promine, Central Soya, Chemurgy Division, Chicago 60639.

[5] Solka Floc, S.W.-40-A, Brown Forest Products Limited, Montreal, Quebec.

tein energy was supplied by soybean fatty acids or soybean fatty acids plus glucose were formulated from the respective soybean oil-containing diet by substituting soybean fatty acids or soybean fatty acids plus glucose isocalorically for all but 2 parts of the soybean oil, using the values 9.21, 8.65 and 3.64 kcal/g for the metabolizable energy content of soybean oil, soybean fatty acids and glucose, respectively. The soybean fatty acids were prepared by alkaline hydrolysis of degummed soybean oil as described previously (1). Analyses showed that the soybean protein contained 95.9% crude protein, 0.17% crude fat, 2.36% ash and 0.27% crude fiber on a dry matter basis. All diets contained approximately 2.35 kcal metabolizable energy/cm ^{3}.

The chicks were fed to 4 days of age the "carbohydrate-free" diet containing 15.4 kcal metabolizable energy/g protein in which non-protein energy was supplied by soybean oil. They were then allotted on the basis of body weight to the experimental groups and fed the experimental diets to 28 days of age. The chicks were maintained in electrically heated, thermostatically controlled, battery brooders with raised wire-screen floors in a temperature-controlled laboratory. Feed and water were supplied ad libitum. Data on growth and feed consumption were obtained weekly and feed wastage was determined daily.

Two experiments (exps. 2 and 3) were conducted to compare nitrogen retention when chicks were pair-fed diets in which non-protein energy was supplied by soybean oil, soybean fatty acids or soybean fatty acids plus glucose. In each experiment, duplicate groups of 10 male crossbred (Dominant White × White Rock) chicks were fed each of the experimental diets. The diets were similar in composition to those in experiment 1. Analysis showed that the diets containing soybean oil, soybean fatty acids, soybean fatty acids plus 0.035 or 0.210 g glucose/g fatty acids contained 14.0, 14.2, 14.9 and 14.3 kcal metabolizable energy/g protein, respectively, in experiment 2 and 13.9, 12.8, 13.0 and 13.0 kcal metabolizable energy/g protein, respectively, in experiment 3.

The housing, weighing and methods of allotment of chicks were similar to those of experiment 1, except that chicks were assigned to their respective groups at seven rather than at four days of age. After allotment the nutrient intakes of chicks receiving the diets containing soybean fatty acids plus glucose or soybean oil were restricted to the intake of chicks fed a comparable diet containing soybean fatty acids with no added glucose. Chicks being pair-fed received their daily allotment of feed once every 24 hours.

During the fourth week of the experiment, excreta were collected from each experimental group at 24-hour intervals for the determination of metabolizable energy. Chromic oxide was incorporated in each of the diets at a level of approximately 0.3% as an index substance to eliminate the need for quantitative collection of excreta and quantitative measurement of feed intake. The methods of processing excreta, conducting chemical analyses for moisture, nitrogen, combustible energy and chromic oxide and com-

TABLE 2

Composition of amino acid diet

	%
Amino acid mix [1]	20.59
Ammonium acetate	2.05
Soybean oil	15.00
Cornstarch	47.23
Vitamin mix [2]	0.56
Mineral mix [3]	9.25
Antioxidant [4]	0.025
Chromium oxide	0.30
Cellulose [5]	5.00

[1] Amino acid mixture supplied: (in grams) L-lysine·HCl, 1.40; L-leucine, 1.20; DL-isoleucine, 1.60; DL-valine, 1.64; DL-methionine, 0.55; L-arginine·HCl, 1.33; L-histidine·HCl·H_2O, 0.41; DL-threonine, 1.30; L-tryptophan, 0.225; L-tyrosine, 0.63; L-cystine, 0.35; glycine, 1.60; L-proline, 1.00; DL-phenylalanine, 1.36; and L-glutamic acid, 6.00.

[2] Vitamin mixture supplied: (in mg/100 g diet) thiamine, 1.0; riboflavin, 1.0; Ca pantothenate, 4.0; biotin, 0.04; pyridoxine, 2.0; niacin, 8.0; folacin, 0.3; menadione, 0.3; vitamin B_{12}, 0.005; choline chloride, 220; inositol, 10; p-aminobenzoic acid, 0.2; ascorbic acid, 25; vitamin A palmitate, 1000 USP units; vitamin D_3, 150 ICU; vitamin E acetate, 3.31 IU and chlortetracycline, 1.

[3] Mineral mixture supplied: (in mg/100 g diet) $CaHPO_4·2H_2O$, 4670; $CaCO_3$, 750; $KHCO_3$, 1900; $NaHCO_3$, 1600; $MnSO_4·H_2O$, 33; $FeSO_4·7H_2O$, 33; $MgSO_4$, 250; KI, 0.26; $CuSO_4·5H_2O$, 1.67; $ZnCO_3$, 11.5; $CoCl_2·6H_2O$, 0.17; $NaMoO_4·2H_2O$, 0.76; and Na_2SeO_3, 0.022.

[4] Ethoxyquin.

[5] Solka Floc S.W.-40-A, Brown Forest Products Limited, Montreal, Quebec.

puting metabolizable energy from these data have been described previously (3, 4).

At the termination of the experiment the chicks were killed without loss of blood. After cooling, the contents of the gastrointestinal tract were removed and the entire carcasses from each experimental group were frozen, ground, mixed and an aliquot dried by lyophilization. In order that tissue gains could be determined, a representative group of chicks was killed at the beginning of the experiment and prepared for analysis in a similar fashion. Carcass samples were analyzed for protein, fat and moisture as described by Hill and Anderson (3).

To determine the role which glucogenic amino acids play in promoting growth of chicks fed "carbohydrate- and glycerol-free" diets (exp. 4), a semipurified diet in which nitrogen was supplied by an amino acid mixture was used. The composition of the basal diet is shown in table 2. The experimental diets were formulated from this diet by replacing starch and 13 parts of the soybean oil isocalorically with soybean fatty acids or soybean fatty acids plus glucose (0.105 g/g fatty acids), using the values 8.65 and 3.64 kcal/g for the metabolizable energy content of soybean fatty acids and glucose, respectively.

The amino acid mixture was patterned after that of Dean and Scott (5) except that glutamic acid was partially replaced by ammonium acetate. Where DL-mixtures were used the amino acid was included at twice the level recommended for the L-forms except for methionine. The mineral mixture used, was low in chloride; the chloride requirement was met by the hydrochlorides of the essential amino acids.

The housing, feeding, weighing and methods of allotment of chicks were similar to those in experiments 2 and 3. Until allotment at 7 days of age chicks were maintained with a "carbohydrate-free" diet containing 24.7 kcal metabolizable energy/g protein in which non-protein energy was supplied by soybean oil and protein by a mixture of soybean protein, methionine and glycine. The experimental diets were fed from 7 to 21 days of age. At the termination of the experiment the chicks were killed without loss of blood. The carcasses were prepared for analyses as in experiments 2 and 3. Samples of the carcass and feed were analyzed for nitrogen and moisture so that nitrogen retention could be calculated.

RESULTS

Data showing 4-week weights, caloric consumption and caloric efficiencies of chicks fed diets containing graded levels of protein in which non-protein energy was supplied entirely by soybean oil or soybean fatty acids are summarized in table 3. Also included are comparable data for chicks fed soybean fatty acid diets supplemented with glucose. Analysis of variances and application of Duncan's multiple range test (6) to the data on growth showed that increasing the protein content of diets containing soybean oil did not increase the rate of growth; however, when non-protein energy was supplied entirely by soybean fatty acids, chicks grew significantly faster ($P < 0.05$) when fed a diet containing 11.0 kcal metabolizable energy/g protein than when diets containing 13.2 or 15.4 kcal metabolizable energy/g protein were fed. However, even at the highest protein level, growth of

TABLE 3

Effect of level of protein on growth, caloric consumption and caloric efficiency in chicks fed "carbohydrate-free" and "carbohydrate- and glycerol-free" diets (exp. 1)

Treatment			Avg wt, 4 weeks	Calories consumed [1]	
	Energy source				
kcal/g protein		*g glucose/g SFA* [2]	*g*	*kcal*	*kcal/g gain*
11.0	Soybean oil	—	507 [a,f]	2273	5.24 [a,b]
11.0	SFA	—	414 [c]	1908	5.33 [a,b]
13.2	Soybean oil	—	492 [e,f]	2245	5.11 [a]
13.2	SFA	—	367 [a,b]	1800	5.43 [a,b]
15.4	Soybean oil	—	490 [e,f]	2207	5.09 [a]
15.4	SFA	—	335 [a]	1567	5.59 [b]
15.4	SFA	0.035	425 [c,d]	1915	5.22 [a,b]
15.4	SFA	0.105	400 [b,c]	1837	5.59 [b]
15.4	SFA	0.210	460 [d,e]	2167	5.36 [a,b]

[1] Calculated using calculated energy values for the diets.
[2] Soybean fatty acids.
[3] Values are averages of duplicate groups. Values without a common letter in their superscript are significantly different.

chicks fed the soybean fatty acid diet was significantly less than that of chicks fed the comparable diet containing soybean oil.

To formulate diets containing 13.2 and 11.0 kcal metabolizable energy/g protein, 0.210 and 0.551 g of extra protein/g fatty acids, respectively, were added to the diet containing 15.4 kcal metabolizable energy/g protein. Results of this experiment show that the addition of 0.035 or 0.105 g glucose/g fatty acids to the diet containing 15.4 kcal metabolizable energy/g protein was as effective in promoting growth as was the addition of 0.551 g extra protein/g fatty acids. Calculations indicate that protein was only 0.035/0.551 to 0.105/0.551 (6 to 19%) as effective as glucose in meeting this requirement. These results indicate that the requirement of the chick for carbohydrate can be met by protein but that protein is much less efficient than glucose in meeting this requirement, at least when growth is used as the criterion.

Results of two replicate experiments conducted to compare growth and nitrogen retention when chicks were pair-fed diets in which non-protein energy was supplied by soybean oil, soybean fatty acids or soybean fatty acids plus glucose are summarized in table 4. Analysis of variance and application of Duncan's multiple range test (6) to the combined data from the 2 experiments showed that when chicks were pair-fed, the addition of either 0.035 or 0.210 g glucose/g soybean fatty acids caused a small but statistically significant increase in growth ($P < 0.01$) and nitrogen retention ($P < 0.05$); however, in individual experiments the increase in growth and nitrogen retention brought about by the addition of either 0.035 or 0.210 g glucose/g fatty acids was not great enough to be significant ($P > 0.05$). Analysis of the combined data also showed that chicks pair-fed diets in which non-protein calories were supplied by soybean oil grew faster and retained more nitrogen than chicks receiving soybean fatty acids plus glucose. In individual experiments this effect was noted only in experiment 3.

Summarized in table 5 are data showing weight gain, nitrogen intake and nitrogen retained by chicks fed amino acid diets in which non-protein energy was supplied by soybean fatty acids and soybean fatty acids plus glucose. The diets contained glucogenic amino acids in sufficient quantities to promote growth but were not in excess. The data show that when chicks are fed a "carbohydrate- and glycerol-free" diet containing limited amounts of glucogenic amino acids, growth practically ceased. The addition of 0.105 g glucose/g fatty acids, that is, the amount required for theoretical conversion of fatty acids to triglycerides, caused a marked increase in rate of growth. Even when the feed intake

TABLE 4

Growth and protein retention when chicks are pair-fed diets containing graded levels of glucose (exp. 2 and 3)

Treatment		Exp. no.	Avg wt, 4 weeks	Protein gain	Protein intake	Protein retention [1]
Source of energy	Level of glucose					
	g/g SFA [2]		*g*	*g*	*g*	
SFA	—	2	355	54.6	109.4	50
		3	355	57.4	113.2	51
			355 [3,a]	56.0	111.3	50 [a]
SFA	0.035	2	366	54.8	104.8	52
		3	369	60.4	112.8	54
			368 [b]	57.6	108.8	53 [b,c]
SFA	0.210	2	365	56.9	109.5	52
		3	370	59.0	112.0	53
			368 [b]	58.0	110.8	52 [b]
Soybean oil	—	2	366	55.8	104.8	53
		3	396	62.2	112.0	56
			381 [c]	59.0	108.4	54 [c]

[1] (Gain in carcass protein, g/protein consumed, g) × 100.
[2] Soybean fatty acids.
[3] Underlined values are averages of duplicate experiments. Values without a common letter in their superscript are significantly different.

TABLE 5

Response of chicks to glucose when fed a "carbohydrate- and glycerol-free" diet containing limited amounts of glucogenic amino acids (exp. 4)

Treatment		Feeding regimen	Wt gain, 7–21 days	Nitrogen intake	Nitrogen retention [1]
Energy source	Level of glucose				
	g/g SFA [2]		*g*	*g*	%
SFA	—	ad libitum	12 [3,a]	3.00	22 [a]
SFA	0.105	pair-fed	30 [b]	3.07	47 [b]
SFA	0.105	ad libitum	111 [c]	6.36	60 [c]

[1] (Gain in carcass nitrogen, g/nitrogen consumed, g) × 100.
[2] Soybean fatty acids.
[3] Values are averages of duplicate groups. Values without a common letter in their superscript are significantly different.

of chicks fed the glucose-supplemented diet was restricted to that of chicks fed the unsupplemented diet, the addition of glucose increased both rate of growth and nitrogen retention.

DISCUSSION

Studies in a variety of species of animals have been interpreted as indicating that roughly 50% of an average protein can be converted to carbohydrate (7, 8). Results of the present studies indicate, however, that in the chick extra dietary protein is less than 50% as effective as glucose in meeting the chick's requirement for carbohydrate. A possible explanation for this difference may lie in the fact that the chick excretes uric acid as the major end product of nitrogen metabolism and may therefore divert part of the carbohydrate derived from protein to synthesis of the glucogenic amino acid glycine, an intermediate required for uric acid synthesis. Thus, the net yield of carbohydrate from extra dietary protein in the chick would be lower than in species excreting urea as the major end product of nitrogen metabolism.

The finding that chicks fed "carbohydrate- and glycerol-free" diets containing soybean fatty acids utilized the nitrogen in soybean protein almost as efficiently as

chicks fed comparable diets containing 0.035 or 0.210 g glucose/g fatty acids indicated that very little dietary protein was diverted from protein to carbohydrate synthesis. These results show that the reduction in chick growth observed under ad libitum feeding when glycerol is deleted from a "carbohydrate-free" diet by substituting soybean fatty acids for soybean oil (1) was not due to impaired nitrogen retention. Similar conclusions were reached by Brambila and Hill (9) from comparisons of nitrogen intakes and excretion by chicks at 2 weeks of age.

The foregoing results are in contrast with the marked increase in nitrogen retention and growth which was observed in pair-feeding studies when glucose was added to a "carbohydrate- and glycerol-free" diet in which protein was supplied by a mixture of amino acids containing limited quantities of glucogenic amino acids. Results showed that the addition of glucose increased nitrogen retention from 22 to 47%. These results show that in the absence of excess glucogenic amino acids and carbohydrate, amino acids are diverted from protein to carbohydrate synthesis. Since chicks fed "carbohydrate- and glycerol-free" diets containing soybean protein do grow, although at a reduced rate, without appreciably altering protein retention, they must derive carbohydrate from excess glucogenic amino acids in soybean protein. Thus, the ability of the chick to synthesize carbohydrate from fatty acids, if present at all, is extremely limited.

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Metabolic Effects of Feeding "Carbohydrate-free" Diets to Chicks [1,2]

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ABSTRACT Experiments were conducted to study the metabolic effects of feeding chicks "carbohydrate-free" and "carbohydrate- and glycerol-free" diets. Diets containing 15.4 kcal metabolizable energy/g protein were fed since at this level protein is present in sufficient quantities to promote rapid growth, but is not in excess. Results showed that chicks fed "carbohydrate-free" diets in which non-protein energy was supplied by either soybean oil or soybean fatty acids maintained normal levels of blood glucose and muscle glycogen but showed a marked depression in level of liver glycogen. Studies also showed that blood levels of ketone bodies increased and level of liver fat decreased when chicks were fed "carbohydrate- and glycerol-free" diets containing soybean fatty acids, but remained normal when soybean oil served as the sole source of non-protein energy. Further studies showed that the ketogenic effects of feeding a "carbohydrate-free" diet containing soybean fatty acids could be overcome by the addition of protein in amounts to decrease the calorie-to-protein ratio from 15.4 to either 13.2 or 11.0 kcal metabolizable energy/g protein.

Results of a previous study (1) have shown that calories from fat can completely replace calories from carbohydrate in the diet of the chick without altering rate of growth. Further studies (2, 3) have shown that removal of glycerol from a "carbohydrate-free" diet by substituting soybean fatty acids for soybean oil, results in a marked depression in rate of growth. The following experiments were conducted to study the metabolic effects of feeding chicks "carbohydrate-free" diets and "carbohydrate- and glycerol-free" diets.

EXPERIMENTAL

Four experiments were conducted in which duplicate groups of 10 male White Plymouth Rock or Dominant White × White Plymouth Rock chicks were fed carbohydrate-containing, "carbohydrate-free" and "carbohydrate- and glycerol-free" diets in which protein supplied 24.9% of the total calories (15.4 kcal metabolizable energy/g protein) and in which non-protein energy was supplied by glucose, soybean oil, soybean fatty acids and soybean fatty acids plus graded levels of glycerol or glucose. The composition of the diets, the feeding and housing of the chicks have been described previously (2). In experiment 3, chicks were also fed "carbohydrate-free" and "carbohydrate- and glycerol-free" diets in which protein supplied 34.8 and 29.0% of the total calories (11.0 and 13.2 kcal metabolizable energy/g protein, respectively). The latter diets were formulated by the isocaloric replacement of part of the soybean oil or soybean fatty acids by the protein mixture containing soybean protein, methionine and glycine using the values 9.21, 8.65 and 3.83 kcal/g for the metabolizable energy content of soybean oil, soybean fatty acids and the protein mixture, respectively. Analysis showed the soybean protein to contain 95.9% protein, 0.17% ether extract, 2.36% ash and 0.27% fiber on a dry-matter basis.

At 4 weeks of age, samples of blood, liver and muscle were taken from representative chicks in each experimental group for the determination of blood glucose, blood ketone bodies, liver glycogen, liver fat and muscle glycogen.

Blood samples were obtained by heart puncture using potassium oxalate to prevent coagulation and sodium fluoride to inhibit glycolysis.

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TABLE 1

Level of glucose in blood of chicks fed diets with and without carbohydrate

Treatment			Blood glucose		
Source of energy	Glucose	Glycerol	Exp. 1	Exp. 2	Exp. 3
	g/g SFA [1]	*g/g SFA*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*
SFA	—	—	197 [2]	181 [3]	212 [4]
SFA	0.035	—	—	195	—
SFA	0.105	—	—	181	—
SFA	—	0.108	231	—	—
SFA	0.210	—	—	246	—
Soybean oil	—	—	210	—	203
Glucose	—	—	230	229	242

[1] Soybean fatty acids.
[2] Values are averages of duplicate determinations on 8 chicks (4/replicate group). Chicks were fed experimental diets from zero to 28 days of age.
[3] Values are averages of duplicate determinations on 12 chicks (6/replicate group). Chicks were fed experimental diets from 4 to 28 days of age ((2) exp. 1).
[4] Values are averages of duplicate determinations on 8 chicks (4/replicate group). Chicks were fed experimental diets from 4 to 28 days of age ((2) exp. 4).

Samples of liver and breast muscles were taken immediately after killing chicks with sodium pentobarbital, frozen with solid carbon dioxide, wrapped in aluminum foil and stored in plastic bags at − 17° until analysis was performed.

Blood glucose was determined colorimetrically by the ferricyanide method of Folin and Malmros (4) using sodium lauryl sulfate instead of gum ghatti as the emulsifier (5).

Total blood ketone bodies were determined using a modification of the method of Bakker and White (6). This modification, as suggested by Werk et al. (7), consisted of heating the tubes in an oil bath at 110° to 120° for 10 minutes and for a further 30 minutes after the addition of potassium dichromate. This procedure is based on the conversion of acetoacetate and β-hydroxybutyrate to acetone, which is then determined colorimetrically after the addition of salicylaldehyde.

Glycogen was isolated from liver and muscle using the method of Good and coworkers (8). The precipitate was washed with 65% ethanol as suggested by Fong et al. (9). The glycogen was dissolved in water and determined colorimetrically by the anthrone method (10).

Liver fat was determine on pooled samples containing 5 or 6 livers. The samples were lyophilized and then extracted for 16 hours with a 2:1 mixture of chloroform and methanol using a Goldfisch apparatus. The dried extracts were then re-extracted with light petroleum (30°–60°). This method is similar to the method of Feigenbaum and Fisher (11).

RESULTS AND DISCUSSION

Levels of blood glucose in chicks fed carbohydrate-containing and "carbohydrate-free" diets containing 15.4 kcal metabolizable energy/g protein are summarized in table 1. Analysis of variance (12) of the data in each of the experiments showed that neither level of dietary carbohydrate nor source of non-protein calories affected the level of blood glucose significantly ($P > 0.05$). These results indicate that chicks can maintain level of blood glucose when "carbohydrate- and glycerol-free" diets containing soybean fatty acids are fed. Since previous studies have shown that chicks fed soybean fatty acids retain almost as much protein as chicks receiving comparable diets containing glucose (13) they must have derived glucose either from excess glucogenic amino acids in soybean protein or from fatty acids.

In the human, Kekwick and Pawan (14) and Azar and Bloom (15) showed that level of blood glucose was maintained when high fat, low carbohydrate diets were fed; however, in their experiments, an increased excretion of urinary nitrogen indicated that amino acids were being diverted from protein to synthesis of other compounds. In contrast with both the chick and the human, Mayes (16) and Samuels et al. (17) reported that level of

TABLE 2

Level of blood glucose in chicks fed "carbohydrate-free" diets from zero and 7 days of age

Treatment	Blood glucose at 28 days	
	Fed diet from day zero	Fed diet from day 7
	mg/100 ml	*mg/100 ml*
Soybean oil	188 [1]	—
SFA [2]	184	181
SFA + 0.105 g glucose/g SFA	203	199

[1] Values are averages of duplicate determinations on 10 chicks.
[2] Soybean fatty acids.

blood glucose was lowered significantly when rats were fed high fat, "carbohydrate-free" diets.

The finding that chicks fed "carbohydrate- and glycerol-free" diets in which non-protein calories are supplied by soybean fatty acids maintain normal levels of blood glucose is in contrast with results reported by Brambila and Hill (3). They found the level of blood glucose to be reduced when chicks were fed "carbohydrate- and glycerol-free" diets. That this difference is not due to our practice of maintaining chicks with a "carbohydrate-free" diet before placing them on experiment is shown by the data in table 2. Whether this difference is due to the incorporation of 2% soybean oil in our diets has yet to be determined.

Summarized in table 3 are data showing levels of ketone bodies in blood of chicks fed carbohydrate-containing, "carbohydrate-free" and "carbohydrate- and glycerol-free" diets. Analysis of variance and application of Duncan's multiple range test (12) to the data in experiment 1 showed that deletion of carbohydrate from the diet by substituting soybean oil for glucose did not increase level of ketone bodies significantly ($P > 0.05$). Results showed, however, that levels of blood ketone bodies rose significantly ($P < 0.05$) when glycerol was deleted from the "carbohydrate-free" diet by substituting soybean fatty acids for soybean oil. The addition of glycerol in the amount required for theoretical conversion of fatty acids to triglyceride reduced ketone bodies, significantly ($P < 0.05$). These results indicate that gluconeogenesis was of sufficient magnitude to prevent the accumulation of ketone bodies when a "carbohydrate-free" diet containing soybean oil was fed, but not when a "carbohydrate- and glycerol-free" diet containing soybean fatty acids was fed.

In a subsequent experiment, the effect on blood level of ketone bodies of the addition of graded levels of glucose or protein to the "carbohydrate- and glycerol-free" diet was studied (table 3, exp. 2). In agreement with previous results, levels of blood ketone bodies increased significantly when soybean fatty acids formed the sole source of non-protein energy in a diet containing 15.4 kcal metabolizable energy/g protein. The addition of 0.035 g glucose/g

TABLE 3

Level of ketone bodies in blood of chicks fed diets with and without carbohydrate

Source of energy	Treatment			Blood ketone bodies [1]	
		Glucose	Glycerol	Exp. 1	Exp. 2
	kcal/g protein	*g/g SFA* [2]	*g/g SFA*	*mg/100 ml*	*mg/100 ml*
Glucose	15.4	—	—	5 [3,a]	6 [4,a]
Soybean oil	15.4	—	—	12 [a]	
SFA	15.4	—	—	32 [b]	24 [b]
SFA	15.4	0.035	—	—	7 [a]
SFA	15.4	0.105	—	—	8 [a]
SFA	15.4	—	0.108	12 [a]	—
SFA	15.4	0.210	—	—	5 [a]
SFA	13.2	—	—	—	14 [a]
SFA	11.0	—	—	—	10 [a]

[1] Total ketone bodies as acetone.
[2] Soybean fatty acids.
[3] See table 1, footnote 2. Values without a common letter in their superscript are significantly different.
[4] See table 1, footnote 3. Values without a common letter in their superscript are significantly different.

TABLE 4

Level of liver glycogen in chicks fed diets with and without carbohydrate

Treatment		Liver glycogen		
Source of energy	Glycerol	Exp. 1	Exp. 3	Exp. 4
	g/g SFA [1]	*% wet wt*	*% wet wt*	*% wet wt*
SFA	—	0.10 [2]	0.13 [3]	0.13 [4]
SFA	0.108	0.41	—	—
Soybean oil	—	0.93	0.69	0.48
Glucose	—	2.28	3.46	3.31

[1] Soybean fatty acids.
[2] See table 1, footnote 2.
[3] See table 1, footnote 4.
[4] Values are averages of duplicate determinations on 10 chicks (5/replicate group). Chicks fed experimental diets from 4 to 28 days of age.

fatty acids was as effective in preventing ketosis as the addition of three or six times this amount. Analysis of variance and application of Duncan's multiple range test (12) showed that the addition of protein in amounts to supply 13.2 and 11.0 kcal metabolizable energy/g protein, reduced levels of blood ketones to levels not significantly different from those of chicks fed the diet in which non-protein calories were supplied by glucose. In contrast, Brambila and Hill (3) showed that chicks fed "carbohydrate- and glycerol-free" diets containing 13.5 kcal metabolizable energy/g protein had markedly elevated levels of blood ketone bodies (41.9 mg/100 ml) at 2 weeks of age.

For comparative purposes fasting levels of blood ketone bodies were determined in fifteen 4-week-old chicks that had been raised with a "carbohydrate-free" diet in which non-protein calories were supplied by soybean oil. Values for total ketone bodies obtained after fasting for zero, 24 and 48 hours were 7.8, 10.8 and 12.0 mg/100 ml blood, respectively. Analysis of variance (12) showed that fasting for 24 or 48 hours significantly increased level of blood ketone bodies ($P < 0.05$). Results showed, however, that the increase in blood ketones brought about by fasting 24 or 48 hours was smaller in magnitude than the increase brought about by feeding a "carbohydrate- and glycerol-free" diet.

Results of the present studies indicate the average level of blood ketone bodies in chicks fed diets in which non-protein calories were supplied by glucose or soybean oil to be 5.5 and 10.0 mg/100 ml, respectively. Values of 4.0 and 14.5 mg/100 ml, respectively, were obtained by Brambila and Hill (3) when chicks were fed diets with and without carbohydrate. Comparable values for rats fed diets in which non-protein energy is supplied by either carbohydrate or triglyceride have been reported by Roberts and co-workers (18) to be < 1.0 and 6.1 to 9.4 mg/100 ml, respectively, and by Tepperman and Tepperman (19) to be 1 and 3 mg/100 ml, respectively. Azar and Bloom (15) report values of 0.47 and 3.0 mg/100 ml, respectively, for humans fed diets with and without carbohydrate. These results suggest that levels of blood ketone bodies in chicks fed diets containing carbohydrate are higher than for rats and humans receiving dietary carbohydrate and are approximately equal to values obtained for these species when "carbohydrate-free" diets are fed.

Levels of glycogen in the livers of chicks fed diets in which non-protein energy was supplied by glucose, soybean oil and soybean fatty acids are summarized in table 4. Analysis of variance and application of Duncan's multiple range test (12) to the data in the 3 experiments showed that level of liver glycogen is reduced significantly ($P < 0.05$) when calories from glucose are replaced by calories from soybean oil. The results also showed that level of liver glycogen is reduced still further ($P < 0.05$) when glycerol is deleted from the diet by substituting soybean fatty acids for soybean oil. These results are in agreement with the results reported by Brambila and Hill (3). The results show that in the chick gluconeogenesis is not of sufficient magnitude to maintain level of liver glycogen when a "carbohydrate-free" diet containing soybean oil is fed. Carr and Krantz (20) and Mayes (16) have reported simi-

TABLE 5

Level of muscle glycogen in chicks fed diets with and without carbohydrate

Source of non-protein energy	Muscle glycogen	
	Exp. 4	Exp. 5
	% wet wt	*% wet wt*
Glucose	0.22 [1]	0.38 [2]
Soybean oil	0.30	0.68
Soybean fatty acids	0.29	0.45

[1] See table 4, footnote 4.
[2] Values are averages of duplicate determinations on 10 chicks (5/replicate group). Chicks fed experimental diets from 7 to 28 days of age.

TABLE 6

Level of liver fat in chicks fed diets with and without carbohydrate

Source of non-protein energy	Liver fat	
	Exp. 3	Exp. 4
	% wet wt	*% wet wt*
Glucose	7.04 [1]	7.32 [2]
Soybean oil	7.56	7.32
Soybean fatty acids	6.23	5.91

[1] See table 1, footnote 4.
[2] See table 4, footnote 4.

lar results in rats fed high fat, low carbohydrate diets.

In contrast with the liver, the glycogen content of breast muscle (table 5) is not altered significantly by replacing dietary glucose by soybean oil or soybean fatty acids. These results indicate that in the chick, as in other species (21) muscle glycogen is not significantly depleted as a result of a demand for carbohydrate elsewhere in the body.

Summarized in table 6 are data showing the lipid content of livers of chicks fed carbohydrate-containing, "carbohydrate-free" and "carbohydrate- and glycerol-free" diets. Analysis of variance and application of Duncan's multiple range test (12) to the data showed that deletion of carbohydrate from the diet by substituting soybean oil for glucose did not alter the level of liver lipid; however, feeding a "carbohydrate- and glycerol-free" diet resulted in a significant reduction ($P < 0.05$).

The finding that the chick does not develop a fatty liver when fed high fat, "carbohydrate-free" diets is in contrast with results reported for other species (22). Feigenbaum and Fisher (11) have observed that unlike rats, chicks do not develop fatty livers during starvation.

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β-Carotene vs. Retinyl Acetate for the Baby Pig and the Effect upon Ergocalciferol Requirement [1]

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ABSTRACT Twenty-four pigs were used in a study to compare the utilization of dietary fermentation β-carotene with retinyl acetate by the baby pig and to determine whether β-carotene has a rachitogenic effect which may increase the vitamin D requirement. Purified isolated-soy diets containing 2.5, 6.25 or 12.5 μg of ergocalciferol/kg and containing either 8 mg/kg of fermentation β-carotene or 688 μg/kg of retinyl acetate were used in a 5-week feeding period. Total liver vitamin A storage of pigs receiving preformed vitamin A was about twice that of pigs receiving β-carotene (2.4 mg vs. 1.2 mg). Neither bone composition nor strength were greatly affected by source of vitamin A activity. In this experiment β-carotene did not increase the need for ergocalciferol above that normally required by baby pigs fed a soy protein diet containing retinyl acetate.

Early studies by Tabor et al. (1) showed that a majority of rats receiving diets supplemented with 90 IU of vitamin A/g of food manifested more marked rickets than animals receiving lower levels. Following these studies there were several reports of rickets in sheep fed green feed (2–5). It was observed that cod liver oil but not bone flour would alleviate this problem. Further studies culminated in the conclusion in 1953 by Grant that the rachitogenic factor was carotene (6–9). Studies in 1954 by Weits (10) confirmed Grant's observations. Later work by Weits (11) indicated that both carotene and vitamin A diminished the degree of healing of rickets that occurred when vitamin D alone was given to rats. Grant and O'Hara (12) concluded from data obtained on sheep that the rachitogenic factor was probably vitamin A itself since equivalent amounts of vitamin A and carotene had the same rachitogenic potency.

The present studies were conducted to compare the utilization of dietary fermentation β-carotene with retinyl acetate by the baby pig and to determine whether carotene has a rachitogenic effect which may increase the vitamin D requirement of the baby pig. Criteria used were growth and feed economy; levels of serum inorganic P, Ca, Mg, alkaline phosphatase and vitamin A; liver storage of vitamin A; percentage of humeral ash, Ca, P and Mg and measures of femur density and strength. Calcium and phosphorus balance trials were also conducted to determine where the antagonism occurs between the source of vitamin A activity and vitamin D, if it does, in fact, occur at all in the baby pig.

MATERIALS AND METHODS

A trial was conducted using 24 Yorkshire-Hampshire crossbred pigs of either sex. Pigs were taken from their dam at 3 to 5 days of age and reared in metal cages with wire-mesh bottoms. Room temperature was maintained at 27° and infra-red heat lamps were used to maintain a cage temperature of 30° during the first 2 weeks of the trial. All windows in the room were painted to eliminate the entrance of sunlight. The purified diets and methods of adjustment were similar to those described by Miller et al. (13). After 5 days the animals became well-adjusted to the diet and were assigned at random to experimental lots balancing for sex, litter and weight. Table 1 shows the experimental diet used. Irradiated ergosterol [4] was diluted in corn

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[4] Viosterol, Nutritional Biochemicals Corporation, Cleveland.

TABLE 1

Composition of purified diets

	%	%
Isolated soybean protein [1]	29.7	(19.7) [2]
DL-Methionine	0.3	
Lard	5.0	
α-Cellulose [3]	5.0	
Retinyl acetate or β-carotene premix [4]	5.0	
Mineral [5]	6.0	
Glucose [6]	48.0	(58.0) [2]
Corn oil [7]	1.0	
Vitamin mixture [5]	+	

[1] ADM C-1 Assay protein, Archer-Daniels-Midland Company, Minneapolis.
[2] Changed to this value during the final 2 weeks of trial.
[3] Solka Floc, Brown Company, Chicago.
[4] 13.8 μg retinyl acetate or 160 μg fermentation β-carotene (USDA Northern Regional Research Laboratory) per g in a glucose carrier.
[5] As in Miller et al. (13) with retinyl palmitate omitted.
[6] Cerelose, Corn Products Company, Argo, Illinois.
[7] Mazola, Corn Products Company.

oil and supplied ergocalciferol levels of 2.5, 6.25 and 12.5 μg/kg of diet. Retinyl acetate [5] and fermentation β-carotene (FBC) [6] were made into premixes with glucose to supply 688 μg retinyl acetate or 8 mg FBC/kg of diet (14, 15). Constant dietary levels of Ca (0.8%) and P (0.6%) were maintained throughout the 5-week experiment by alteration of the mineral mixture when protein level was changed after the third week of the trial. With the exception of the time during which mineral balance studies were conducted, food and tap water were available for ad libitum consumption.

Blood was drawn from the anterior vena cava on the first and fifth weeks of the trial for determination of levels of serum Ca, inorganic P, Mg and alkaline phosphatase activity. The methods of Mori (16), Gomorri (17), Orange and Rhein (18) and Bessey et al. (19) were used for the respective analyses. Determination of levels of serum vitamin A were made from serum drawn on the fifth week using the antimony trichloride method of Embree (20). Upon autopsy a section was removed from the lower one-half of the left central lobe of the liver for analysis according to the method of Gallup and Hoefer (21) for liver vitamin A concentration. A Bausch and Lomb Spectronic 20 spectrophotometer rather than an Evelyn colorimeter was used in this determination. Bone composition and strength data were obtained by methods described by Miller et al. (22).

Immediately following the fifth week of the experiment all pigs were caged separately and 3 times daily were fed an amount of food and water which they would consume in a 10-minute period. After an adjustment period of 3 days, separate 72-hour collections of urine and feces were carried out with each pig receiving a constant amount of food and water. Food, water, feces and urine were analyzed for Ca and P concentration (16, 17). 1, 2-Cyclohexylenedinitrilotetraacetic acid (CDTA) served as a titrant in the Ca determination.

Data were examined by analysis of variance with statistical significance of treatment differences being determined by the multiple range test of Duncan (23).

RESULTS AND DISCUSSION

The results of growth, serum analyses, liver vitamin A analyses, mineral balance and skeletal determinations are presented in table 2, 3 and 4. There were no significant treatment differences in rate of gain, food consumption or the efficiency of food utilization. Baby pigs receiving carotene in the diet (8 mg FBC/kg) gained as rapidly and efficiently as those receiving retinyl acetate (688 μg/kg). There was a trend for pigs receiving higher levels of ergocalciferol to gain more rapidly (daily gains of 0.20, 0.22 and 0.23 kg for pigs receiving 2.5, 6.25 and 12.5 μg of ergocalciferol/kg, respectively). This trend agrees with that obtained in earlier studies (24) of isolated soybean protein diets with concentrations of ergocalciferol from 1.25 to 12.5 μg/kg. Efficiency of food utilization for growth followed a similar pattern, with pigs receiving the higher levels of ergocalciferol gaining more efficiently (0.55, 0.59 and 0.61 gain/food, respectively). Some of the pigs receiving 2.5 or 6.25 μg ergocalciferol and either retinyl acetate or FBC exhibited some bowing of the front legs at about the fourth week of the trial. At no time, however, was lameness observed.

[5] Crystalets, crystalline retinyl acetate stabilized with gelatin and sugar, Chas. Pfizer, New York.
[6] Dry fermentation solids containing 94 to 96% all-*trans*-β-carotene, USDA Northern Regional Research Laboratory, Peoria, Illinois.

TABLE 2

Growth, serum analyses, and liver analysis of baby pigs fed different levels of ergocalciferol and different sources of vitamin A activity [1]

Dietary vitamin A source and level	688 μg/kg retinyl acetate			8 mg fermentation β-carotene/kg		
Dietary ergocalciferol, μg/kg	2.5	6.25	12.5	2.5	6.25	12.5
Initial wt, kg	2.6 ±0.2 [2]	2.6 ±0.3	2.6 ±0.1	2.6 ±0.2	2.6 ±0.2	2.6 ±0.2
Daily gain, kg	0.21 ±0.01	0.22 ±0.02	0.23 ±0.01	0.20 ±0.02	0.22 ±0.02	0.23 ±0.00
Daily food intake, kg	0.37	0.39	0.39	0.37	0.35	0.37
Gain/food	0.56	0.56	0.59	0.54	0.63	0.63
Serum Ca, mg/100 ml						
Initial	10.6 ±0.4	12.1 ±0.5	12.3 ±0.3	11.9 ±0.2	12.8 ±1.0	10.6 ±0.5
5-week	10.9 ±0.1	10.8 ±0.1	11.4 ±0.1	10.9 ±0.3	11.2 ±0.1	11.1 ±0.4
Serum inorganic P, mg/100 ml						
Initial	4.3 ±0.2	5.2 ±0.3	4.7 ±0.1	4.8 ±0.1	4.6 ±0.3	3.7 ±0.2
5-week	4.4 ±0.2	5.0 ±0.2	4.6 ±0.2	4.4 ±0.1	4.9 ±0.1	4.7 ±0.3
Serum Mg, mg/100 ml						
Initial	2.7 ±0.2	2.8 ±0.1	3.2 ±0.3	3.0 ±0.2	2.6 ±0.2	2.7 ±0.2
5-week	2.9 ±0.2	2.8 ±0.1	2.7 ±0.2	2.9 ±0.1	2.9 ±0.1	2.9 ±0.1
Serum alkaline phosphatase, Bessey-Lowry units						
Initial	16.1 ±1.5	13.9 ±1.1	16.1 ±2.8	17.2 ±2.7	16.3 ±2.5	17.9 ±1.8
5-week	5.3 ±0.5	4.9 ±0.6	5.6 ±0.5	6.1 ±0.8	5.8 ±0.4	5.2 ±0.8
Serum vitamin A, μg/100 ml						
5-week	17.1 ±0.9 [aa]	15.0 ±1.5 [a]	21.5 ±0.5 [d]	12.0 ±0.9	15.3 ±0.9 [a]	15.1 ±0.8 [a]
Liver vitamin A, dry basis, μg/g	31 ±6 [bb,c]	31 ±4 [bb,c]	29 ±2 [aa,b]	19 ±2	14 ±4	10 ±2
Total liver vitamin A, mg	2.3 ±0.3 [b]	2.5 ±0.3 [aa,b]	2.5 ±0.3 [aa,b]	1.4 ±0.3	1.2 ±0.4	0.9 ±0.3

[1] Four pigs/group.
[2] Mean ± SE.
[a] Significantly greater than least value ($P < 0.05$); [aa] $P < 0.01$.
[b] Significantly greater than 2 least values ($P < 0.05$); [bb] $P < 0.01$.
[c] Significantly greater than 3 least values ($P < 0.05$).
[d] Significantly greater than all other values ($P < 0.01$).

TABLE 3

Daily Ca and P excretion and retention as affected by dietary ergocalciferol and different sources of vitamin A activity[1]

Dietary vitamin A source and level	688 μg/kg retinyl acetate			8 mg fermentation β-carotene/kg		
Dietary ergocalciferol, μg/kg	2.5	6.25	12.5	2.5	6.25	12.5
Daily food intake, g	412 ± 22 [2]	442 ± 8	450 ± 0	450 ± 0	450 ± 0	431 ± 19
Ca balance						
Daily Ca intake, g	3.30 ± 0.17	3.53 ± 0.07	3.60 ± 0.00	3.60 ± 0.00	3.60 ± 0.00	3.45 ± 0.15
Daily fecal Ca, g	0.69 ± 0.21	1.25 ± 0.22	1.27 ± 0.26	1.02 ± 0.17	1.20 ± 0.20	1.43 ± 0.10
Daily urinary Ca, g	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.03 ± 0.00 [a]	0.03 ± 0.00 [a]
Daily Ca retention, g	2.60 ± 0.32	2.27 ± 0.19	2.32 ± 0.26	2.56 ± 0.17	2.37 ± 0.19	1.99 ± 0.22
Ca retention, %	78 ± 7	65 ± 6	64 ± 7	71 ± 5	65 ± 6	56 ± 4
P balance						
Daily P intake, g	2.50 ± 0.24	2.65 ± 0.05	2.70 ± 0.00	2.70 ± 0.00	2.70 ± 0.00	2.59 ± 0.11
Daily fecal P, g	0.26 ± 0.10	0.42 ± 0.07	0.46 ± 0.09	0.40 ± 0.08	0.54 ± 0.10	0.53 ± 0.07
Daily urinary P, g	0.11 ± 0.02	0.09 ± 0.02	0.14 ± 0.02	0.11 ± 0.02	0.15 ± 0.02	0.12 ± 0.03
Daily P retention, g	2.13 ± 0.19	2.14 ± 0.04	2.10 ± 0.07	2.19 ± 0.06	2.01 ± 0.10	1.94 ± 0.15
P retention, %	84 ± 4	81 ± 2	78 ± 3	81 ± 2	74 ± 4	75 ± 3

[1] Four collections/dietary ergocalciferol group.
[2] Mean ± SE.
[a] Significantly greater than 3 least values ($P < 0.05$).

Serum Ca, P and Mg levels were similar in all treatments. The level of serum Ca concentration tended to increase with increasing dietary ergocalciferol level (10.9, 11.0 and 11.2 mg/100 ml in lots receiving 2.5, 6.25 and 12.5 μg/kg of ergocalciferol, respectively). Animals fed diets containing 2.5 μg of ergocalciferol/kg tended to have higher serum alkaline phosphatase levels than pigs receiving either 6.25 or 12.5 μg/kg (5.7, 5.4 and 5.4 Bessey units, respectively). Serum alkaline phosphatase level in pigs receiving carotene was somewhat higher than in pigs receiving retinyl acetate (5.7 vs. 5.3 Bessey units). Pigs receiving higher levels of ergocalciferol also showed a significant increase ($P < 0.01$) in serum vitamin A levels (14.5, 15.2 and 18.4 μg/100 ml). Ullrey et al. (14) observed that FBC or carotene isomers will not maintain levels of serum or liver vitamin A that can be maintained by feeding retinyl acetate or palmitate when carotene was fed to equal 500 IU vitamin A/kg. A highly significant interaction was observed between source of vitamin A activity and level of ergocalciferol. Retinyl acetate and 12.5 μg of ergocalciferol/kg supported the highest level of serum vitamin A (table 2).

Pigs receiving FBC stored less vitamin A in the liver as the level of ergocalciferol in the diet increased. This was not the case, however, with pigs fed preformed vitamin A. In this case all lots, regardless of ergocalciferol level, had the same level of liver storage. All pigs receiving preformed vitamin A had greater concentrations of vitamin A in the liver and more total liver storage than pigs receiving β-carotene. Using total liver vitamin A storage as the criterion, the relative biopotency of 1 mg of fermentation β-carotene was 152 IU, 120 IU and 90 IU of vitamin A activity in diets containing 2.5, 6.25 and 12.5 μg of ergocalciferol/kg for the 10 kg baby pig. This is much lower than the value allowed by the National Research Council (15) and somewhat lower than that obtained by Ullrey et al. (14) who found in work with 50- to 100-kg pigs that 1 mg FBC was equivalent to 192 IU from all-*trans* retinyl palmitate for the support of liver storage in the depleted pig.

TABLE 4

Skeletal development of baby pigs fed different levels of ergocalciferol and different sources of vitamin A activity [1]

Dietary vitamin A source and level	688 μg/kg retinyl acetate			8 mg fermentation β-carotene/kg		
Dietary ergocalciferol, μg/kg	2.5	6.25	12.5	2.5	6.25	12.5
Humeral analysis (dry, fat-free basis)						
Ash, %	47.2 ±1.0 [2]	48.5 ±1.3	49.4 ±0.6	47.5 ±0.7	48.5 ±1.0	48.8 ±0.5
Ca, %	16.7 ±0.4	17.2 ±0.3	17.6 ±0.3	16.8 ±0.3	17.2 ±0.4	17.1 ±0.3
P, %	9.0 ±0.2	9.3 ±0.3	9.4 ±0.1	9.1 ±0.1	9.3 ±0.2	9.4 ±0.1
Mg, %	0.41 ± 0.01	0.42 ± 0.02	0.49 ± 0.02 [a]	0.49 ± 0.03 [a]	0.56 ± 0.04 [bb]	0.52 ± 0.02 [b]
Specific gravity						
Femur	1.18 ± 0.01	1.19 ± 0.00	1.20 ± 0.01	1.19 ± 0.00	1.19 ± 0.01	1.18 ± 0.01
8th rib	1.22 ± 0.02	1.32 ± 0.06	1.35 ± 0.07	1.28 ± 0.04	1.31 ± 0.06	1.26 ± 0.04
Weight, g						
Femur	55.9 ±1.0	60.7 ±3.1	62.6 ±2.0	56.0 ±1.8	60.1 ±1.3	60.3 ±3.0
8th rib	6.2 ±0.9	6.0 ±0.4	5.7 ±0.4	5.6 ±0.3	6.1 ±0.6	5.3 ±0.3
Femur strength [3]						
Breaking load, kg	132 ± 11	137 ± 15	168 ± 11	158 ± 17	131 ± 8	155 ± 10
Bending moment, kg-cm	241 ± 22	266 ± 31	322 ± 28	289 ± 30	279 ± 32	291 ± 28
Moment of inertia, cm^4	0.14 ± 0.01	0.16 ± 0.01	0.15 ± 0.01	0.13 ± 0.01	0.16 ± 0.01	0.14 ± 0.02
Breaking stress, kg/cm^2	1173 ± 55	1184 ± 193	1514 ± 133	1499 ± 142	1293 ± 144	1549 ± 186

[1] Four pigs/dietary ergocalciferol group.
[2] Mean ± SE.
[3] For formulas, see Miller et al. (22).
[a] Significantly greater than least values ($P < 0.05$).
[b] Significantly greater than 2 least values ($P < 0.05$); [bb] ($P < 0.01$).

Calcium and phosphorus retention were not significantly affected by level of ergocalciferol or form of dietary vitamin A activity. Retention of Ca and P tended to decrease with increasing ergocalciferol levels from 2.5 to 12.5 μg/kg and preformed vitamin A tended to provide for greater Ca and P retention than did FBC in the diet. The only statistically significant difference observed in the mineral balance data was a greater excretion of urinary Ca when FBC was the source of vitamin A activity. Urinary Ca, however, was such a small portion of the total Ca excretion ($< 3\%$) that it had little effect on Ca balance.

Bone ash, Ca and P, expressed as percentage of the dry, fat-free weight, increased with increasing levels of dietary ergocalciferol as observed earlier (24) with no effect of dietary vitamin A source. Bone Mg deposition was affected by both vitamin A source and level of ergocalciferol in the diet. As dietary ergocalciferol level was increased there was a marked increase of humeral Mg. Pigs receiving FBC had more bone Mg deposition than those receiving retinyl acetate.

Bone weight and specific gravity measurements did not reflect any treatment differences.

Under the conditions of this experiment the relative biopotency on a mole/mole basis of fermentation β-carotene was 9.1, 7.2 and 5.4% of retinyl acetate when incorporated in diets containing 2.5, 6.25 and 12.5 μg ergocalciferol/kg of feed, respectively. Total liver storage of vitamin A was used as the basis of comparison. Ullrey et al. (14) using the same basis of comparison found FBC to have 11.4% of the biopotency of retinyl palmitate when fed to 50 to 100 kg pigs.

Bone composition and strength tests and serum alkaline phosphatase levels, which are the usual criteria used to determine a rachitic condition, show no consistent trends indicating that with the dietary levels of retinyl acetate and fermentation β-carotene used in this experiment FBC is no more rachitogenic than the preformed source of vitamin A.

Mineral balance trials indicate that dietary β-carotene may not result in as much Ca and P retention as that of pigs receiving retinyl acetate. If this were true over the entire experimental period it should be reflected in the humeral analyses; however, there were no differences in bone Ca or P deposition due to treatment. Bone mineral deposition therefore appears to be a more sensitive criterion of Ca and P utilization over the experimental period than the 72-hour balance trial used in this study.

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Alleviation of Methionine and Homocystine Toxicity in the Rat [1]

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ABSTRACT Alleviation of methionine and homocystine toxicity by various dietary supplements was examined. Rats fed a diet containing 10% casein and 3% of L-methionine gain only 2 to 3 g per week; this is significantly less than the growth of rats fed an equivalent amount (moles of sulfur/kg of diet) of DL-homocystine. Glycine and serine both alleviate, but do not prevent, the growth depressions caused by methionine and homocystine. Glycine appears to be more effective than serine in alleviating methionine toxicity but serine is more effective in alleviating homocystine toxicity. Glycine and serine appear to be quite specific in alleviating the toxicity because several other amino acids in equivalent amounts were ineffective. Rats appear to undergo some form of metabolic adaptation to high intakes of methionine or homocystine because the beneficial effects of glycine and serine supplements were observed only after the animals had been eating the diets for several days.

Growth rate and voluntary food intake of the young rat fed a low protein diet are usually depressed if a disproportionate amount of one of the indispensable amino acids is included in the diet (1, 2). If these effects are equated with toxicity (1–3) then on a weight basis or as a percentage of the requirement, methionine is the most toxic of the nutritionally important amino acids (4–6). Increasing the methionine content of a low protein diet much above 1.5%, or about 3 times the requirement, depresses growth rate and food intake (4–18); the growth depression is greater than that observed for pair-fed controls (8, 10, 15, 17, 18). Effects of methionine toxicity are not restricted to depressions in growth rate and food intake; pathological lesions of the spleen, pancreas, liver, small intestine and kidney have also been observed in rats fed high methionine diets (7, 17).

Although the basis for the adverse effects of a dietary excess of methionine has not been established, these effects are alleviated by supplements of certain individual amino acids or mixtures of them. Arginine is somewhat effective and glycine alone or in combination with arginine is much more effective in reducing the toxicity of methionine (8, 10). Originally it was thought, since arginine and glycine are precursors of guanidinoacetic acid (19), which is converted to creatine on being methylated by S-adenosylmethionine, that increased guanidinoacetic acid formed from these amino acids facilitated methionine degradation by accepting the methyl group from S-adenosylmethionine. This explanation is untenable because rats supplemented in this way do not excrete enough extra creatinine to explain the beneficial effects observed (8, 20) and also because methionine toxicity is not alleviated by including guanidinoacetic acid directly in the diet (6, 13, 14, 22) nor by feeding another methyl acceptor, nicotinamide (16, 23). However, still another methyl acceptor, ethanolamine, has been reported to improve the growth of animals receiving high methionine diets (13).

Growth rate and food intake of rats are depressed also by a high homocystine intake (13, 18). Cohen et al. (13) concluded from experiments on the effects of excessive intakes of methionine and homocystine that the homocysteine portion of the methionine molecule was responsible for the growth-depressing effect of excess me-

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thionine. Supplements of glycine or serine increased the growth rate of rats fed a high homocystine diet (13), supporting the suggestion of Roth et al. (20) that alleviation of methionine toxicity by glycine is probably effected through conversion of glycine to serine which then combines with the extra homocysteine to form cystathionine (24), an important intermediate in methionine degradation.

Nevertheless, the specific metabolic role of glycine in alleviating methionine toxicity is far from clear. Addition of glycine to high methionine diets results in a slight increase in urinary sulfur excretion (21), but does not completely prevent the histological lesions observed (17). Serine, on the other hand, is reported to be of no benefit in preventing the growth depression (13) nor the lesions caused by a high methionine intake (18). It is possible that optimal levels of glycine or serine were not used in these experiments because Klain et al. (16) have shown that increasing the amount of glycine from 2 to 4% nearly doubled the growth rate of rats fed a diet containing 4% of methionine. Studies of the effectiveness of various levels of serine on methionine toxicity have not been reported.

Ethionine toxicity has recently been attributed to the accumulation of S-adenosylethionine leading to depletion of adenine for ATP formation (25). In view of the possibility that a large excess of methionine might similarly deplete the body of adenine even though S-adenosylmethionine is a normal metabolic intermediate, and in view of the generally unsatisfactory state of knowledge concerning methionine toxicity, we are reinvestigating this problem. The objective of the experiments reported below on methionine, homocystine, glycine and serine interrelationships is to provide a sound nutritional base for subsequent metabolic studies of this problem.

METHODS

Male rats of the Holtzman and Sprague-Dawley strains weighing from 60 to 90 g, were used in these investigations. They were kept in individual, suspended, wire-bottom cages at 23° in a room with a 12-hour light-12 hour-dark cycle. After being fed an 18% casein diet for 3 to 5 days, the animals were separated into groups with the same average weight, and then assigned to treatment groups. The basal diet used in these experiments consisted of vitamin-test casein,[2] 10.0; L-methionine, 0.3; vitamin mix (26), 0.5; salt mix (26), 5.0; choline chloride, 0.2; glucose monohydrate,[3] 38.5; starch,[4] 38.5; and corn oil,[5] 5.0. Except where noted the diets were prepared as agar gels containing 50% water (26). Dry food consumption was calculated daily and the animals were weighed each morning just before being fed.

RESULTS

The results from a number of experiments on the effects of increasing increment of DL-methionine on weight gain have been compiled and are presented in figure 1. The growth-stimulating effect of a small supplement of methionine was very marked. Increasing the methionine content of the diet to 1.4%, or a total of about 1.7%, depressed growth rate slightly but not significantly. As the increment of methionine in the diet was increased growth rate fell steadily, until, with levels of 2.5 to 3.0%, growth rate was below that of the unsupplemented control.

In the initial experiments rats fed diets containing 2.5% of DL-methionine gained weight during the second week of the trial; therefore 3.0% of L-methionine was used routinely in the later experiments. Figure 2 shows that rats fed a diet containing 3% of L-methionine did not gain

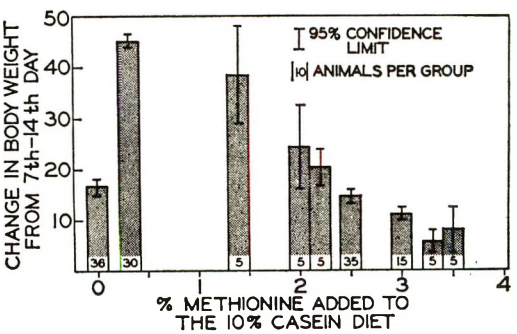

Fig. 1 Effect of increasing levels of methionine on growth of rats fed a 10% casein diet in dry form.

[2] General Biochemicals, Chagrin Falls, Ohio.
[3] Cerelose 2001, Corn Products Company, New York.
[4] Cornstarch, Corn Products Company.
[5] Mazola oil, Corn Products Company.

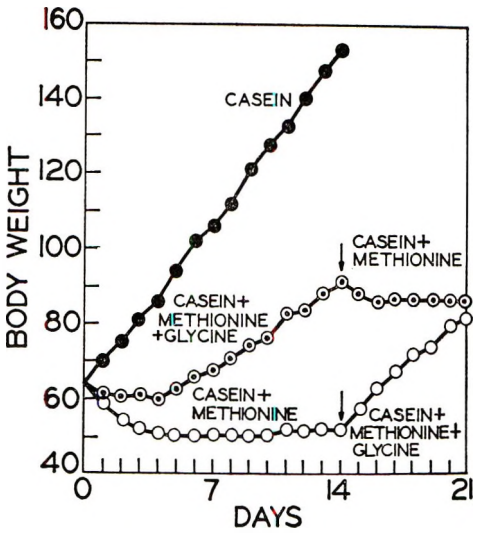

Fig. 2 Growth rates of rats (6/group) fed 10% casein diets with 3% L-methionine or 3% L-methionine + 3% glycine as agar gels. Diets changed on day 14 ↓ .

weight over a 2-week period. Weight changes from the seventh to the fourteenth days of the trial have been used for comparisons of the effects of various supplements; growth was fairly uniform over this time period (fig. 2) and is thought to be more truly representative of the effect of the dietary supplement than the value for the entire 2-week period because rats appear to have become adapted to different diets at different rates during the first week.

Since the growth of rats fed a 10% casein diet containing 3.0% of L-methionine (0.2 moles/kg) was nearly completely inhibited (figs. 1 and 2), effects of including 0.2 moles/kg of each of several other amino acids in a similar but somewhat more adequate diet were compared with that of methionine (table 1). These amounts represent fivefold the requirement for methionine, about 27-fold the requirements for tryptophan and histidine, and about fourfold the requirements for phenylalanine and threonine. Methionine and tryptophan both depressed growth rate, methionine more than tryptophan. Histidine, tryrosine and phenylalanine did not retard growth in this experiment, but these amounts do retard the growth of rats fed a diet in which the protein is less adequate (27).

Adenine was ineffective in alleviating the growth retardation due to 2.5% DL-methionine (table 2). Guanidinoacetic acid was not only ineffective, but 1% in the diet reduced growth rate (table 2).

The effects of supplementing the high methionine diet with glycine or serine to provide the same amount (mole/kg of diet) as for methionine or 2 or 3 times (serine) or 2 to 5 times that amount (glycine) are shown in table 3. Growth of rats fed the glycine-supplemented control diets was impaired by the higher levels of glycine. Growth of the rats fed the high methionine diet supplemented with glycine was highest when the glycine level was 4.5% and then declined. Growth rates of rats fed the serine-supplemented control diets were depressed but each of the serine supplements improved the growth of rats fed the high methionine diet. The protective effect of serine is not as great as that of glycine if the unsupplemented control is used as a basis for the comparison. However, if the group fed the control diet supplemented with the same amount of glycine or serine as was added to the high methionine diet is used as the basis for comparison, then the 2 amino acids appear to be equally effective at the two lower levels in alleviating the toxicity, but glycine was still slightly superior at the highest level.

The results presented in table 4 show that alanine, aspartic acid and glutamic acid were ineffective in alleviating the growth depression caused by methionine.

TABLE 1

Effects on growth of amino acids included in excess in a 10% casein diet supplemented with 0.3% L-cystine and 0.2% L-threonine

Amino acid in excess [1]	Wt change (7–14 days)
	g
None	40.2 ± 8.5 [2]
3.0% L-methionine	5.4 ± 0.9
4.1% L-tryptophan	26.4 ± 3.9
4.2% L-histidine	45.6 ± 4.2
3.6% L-tyrosine	42.6 ± 2.5
3.3% L-phenylalanine	47.8 ± 3.3
2.4% L-threonine	42.0 ± 3.8

[1] Amino acids added to equal 0.2 moles/kg diet; diet was in dry form.
[2] SE of mean for 5 rats.

TABLE 2

Effect of adenine and guanidinoacetic acid (GAA) on growth of rats fed a high methionine diet containing 10% of casein [1]

Supplement	No. of animals	Wt change (7–14 days)
		g
None	11	17.8 ± 1.0 [2]
0.5% adenine	5	20.8 ± 1.9
1.0% adenine	6	24.5 ± 1.9
1.0% GAA	5	9.0 ± 0.9
0.5% adenine + 1.0% GAA	5	3.2 ± 1.3
2.5% DL-methionine	10	10.1 ± 0.8
2.5% DL-methionine + 0.5% adenine	11	11.8 ± 0.7
2.5% DL-methionine + 1.0% adenine	6	12.6 ± 1.0
2.5% DL-methionine + 1.0% GAA	5	−3.0 ± 0.4
2.5% DL-methionine + 0.5% adenine + 1% GAA	5	−1.6 ± 0.7

[1] Diet was in dry form.
[2] SE of mean.

TABLE 3

Effect of increasing levels of glycine or serine on weight gain of rats fed a high methionine diet [1]

Diet		Wt gain (7–14 days)	Diet		Wt gain (7–14 days)
L-methionine [2]	Glycine [2]		L-methionine	L-serine [2]	
%	%		%	%	*g*
0.3	—	46.6 ± 2.0 [3]	0.3	—	45.0 ± 4.3
0.3	1.5	49.4 ± 1.3	0.3	2.1	42.5 ± 2.8
0.3	3.0	46.0 ± 1.7	0.3	4.2	33.6 ± 2.4
0.3	4.5	41.3 ± 1.3	0.3	6.3	32.0 ± 0.7
0.3	6.0	38.1 ± 3.2	—	—	—
0.3	7.5	30.5 ± 4.1	—	—	—
3.0	—	2.0 ± 2.1	3.0	—	2.3 ± 1.0
3.0	1.5	14.8 ± 1.8 *	3.0	2.1	12.8 ± 1.6
3.0	3.0	21.3 ± 1.2 *	3.0	4.2	15.8 ± 1.3
3.0	4.5	27.3 ± 1.7 *	3.0	6.3	17.5 ± 1.0
3.0	6.0	21.1 ± 0.9	—	—	—
3.0	7.5	24.3 ± 1.6	—	—	—

[1] Six animals/group except for * which have 16 animals/group.
[2] Three percent methionine equals 0.2 moles/kg diet and 1.5, 3.0, 4.5% glycine and 2.1, 4.2, 6.3% L-serine equals 0.2, 0.4, 0.6 moles/kg diet, and 6.0 and 7.5% glycine equal 0.8 and 1.0 moles/kg diet.
[3] SE of mean.

TABLE 4

Effects of glycine, alanine, aspartic acid and glutamic acid (0.4 moles/kg of diet) on growth of rats fed a high methionine diet containing 10% of casein

Additions to diet		No. of animals	Wt change (7–14 days)
L-methionine	Other amino acids		
%			*g*
0.3	—	6	47.6 ± 3.4 [1]
3.0	—	6	2.8 ± 1.2
3.0	3.0% glycine	6	24.1 ± 0.8
3.0	3.6% L-alanine	7	7.0 ± 1.2
3.0	5.3% L-aspartic acid	6	5.0 ± 0.9
3.0	5.9% L-glutamic acid	7	6.6 ± 0.4

[1] SE of mean.

In another experiment, not reported, proline was also ineffective.

The results presented in table 5, compiled from several experiments in which the control group gained close to the same amount of weight, provided a basis for comparison of the effectiveness of glycine and L-serine in equimolar amounts in preventing growth depressions due to excess L-methionine or DL-homocystine. Both glycine and serine improved the growth rate of rats fed the 3% L-methionine diet, glycine somewhat more than serine. The two together in equimolar amounts in the high methionine diet were no more effec-

TABLE 5

Effects of glycine and serine on growth of rats fed a diet containing 10% casein and excess methionine or homocystine

Additions to diet		Wt changes (7–14 days)			
Glycine [1]	L-serine [1]	No L-met	0.3% L-met	3.0% L-met [1]	0.3% L-met + 2.4% DL-homocystine [1]
%	%	g			
—	—	28.6 ± 2.1 [2] (5)	48.6 ± 2.0 (17)	3.0 ± 0.7 (22)	14.5 ± 2.3 (11)
3.0	—	—	44.9 ± 2.0 (10)	23.4 ± 1.0 (39)	33.6 ± 2.7 (10)
—	4.2	—	28.4 ± 1.9 (10)	17.4 ± 1.2 (27)	34.8 ± 2.4 (5)
1.5	2.1	—	42.1 ± 2.4 (6)	22.3 ± 1.1 (12)	—

[1] Three percent L-methionine = 0.2 moles/kg diet; 2.4% DL-homocystine = 0.18 moles of homocysteine/kg diet; 3% glycine = 0.4 moles/kg diet; 4.2% L-serine = 0.4 moles/kg diet.
[2] SE of mean. Numbers in parentheses indicate number of animals.

tive than glycine alone. DL-Homocystine caused less growth retardation than L-methionine when the two were fed in amounts that provided the same amount of sulfur. Glycine and serine partially prevented the growth retardation caused by homocystine but neither restored growth to that of the group fed the control diet containing 0.3% of L-methionine. If the control group supplemented with the same level of glycine or serine as was present in the high homocystine diet is used as the basis for comparison, then serine is about one and one-half times more effective than glycine in alleviating homocystine toxicity.

Curves representing the time course of weight gain for the control, high methionine, and high methionine plus glycine groups are shown in figure 2. Two important points are demonstrated in this figure. First the protective effect of supplemental glycine was not apparent until after the third or fourth day of the experiment. Second, supplementation of the high methionine diet with glycine on the fourteenth day of the trial resulted in an immediate resumption in growth.

The curves in figure 3 show that supplementation of the high methionine diet with glycine or removal of glycine from the high methionine diet on day 14 resulted in an immediate alteration in food intake.

DISCUSSION

In interpreting the results of investigations of methionine toxicity and its alleviation, careful attention must be paid not only to methionine content and the nutritional adequacy of the basal diet (6, 27, 28) but also to the particular control groups used as the basis for comparisons. The growth depression caused by a given level of methionine decreases as the amount of the protein in the diet is increased (6, 28) and may be affected by relatively small changes in dietary protein content. In experiments in which the basal diet contained only 8% of casein (9) the growth of rats fed 1.5% of methionine was less than that of an unsupplemented control group, whereas 1.4% of methionine did not cause a significant depression in the growth of rats fed a 10% casein diet in our experiments (fig. 1). However, the growth of rats fed a diet containing as much as 40% of casein is depressed by 3% of L-methionine (6). Also a small

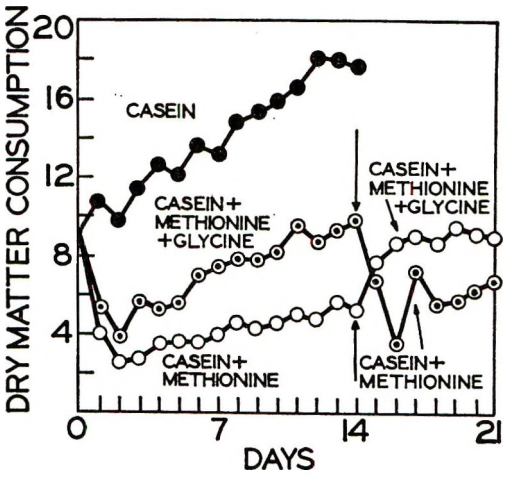

Fig. 3 Dry matter consumption of rats (6/group) fed 10% casein diets with 3% L-methionine or 3% L-methionine + 3% glycine as agar gels. Diets changed on day 14 ↓ .

amount of methionine depresses the growth of rats fed a vitamin B_6-deficient diet, but a large surplus of the vitamin does not alleviate the growth depression caused by 2.5% or more of methionine (23).

Reports concerning the effectiveness of glycine in alleviating methionine toxicity are not consistent. Klain et al. (16) observed that the growth of rats fed a 15% casein diet containing 4% of methionine and 4% of glycine was 90% of that of control animals. However, the growth rate of their control group was low, probably because their basal diet was not supplemented with methionine. If the control group not receiving supplemental methionine is used as the base for comparisons in our experiments glycine would appear to completely prevent the growth depression due to 3% of L-methionine (table 5). Similarly, if the value for the control group receiving 7.5% of glycine (table 3) is used to indicate the effectiveness of glycine in preventing methionine toxicity then glycine would appear to restore growth to 80% of the control value. However, the growth of both of these controls is low, one because of the lack of a methionine supplement, the other because of the excess of glycine. Obviously conclusions regarding the effectiveness of glycine or serine supplements are greatly influenced by the selection of the control.

Such conclusions are similarly influenced by the relative proportions of methionine and protein in the diets. Cohen et al. (13) observed that the growth of rats fed a 12% casein diet containing 2.4% of methionine and 1.8% glycine was 77% of that of control animals. However, their high methionine group gained 40 to 50% as much as the control group compared with 10% for the high methionine group in our experiments. Thus it is not possible to generalize concerning the effectiveness of glycine in alleviating methionine toxicity.

Another complication is that growth rates of rats fed the high methionine diet in dry or in gel form are not comparable; rats fed the 10% casein control diet in dry form gained only 58% as much as those fed the same diet in gel form (fig. 1 and table 5). This difference in growth rate is a reflection of a difference in food intake (26). Conversely, rats fed the high methionine diet in the dry form tend to gain more rapidly than those fed this diet as a gel (table 1 vs. tables 3, 4, 5). Also, in earlier experiments in which dry diets were used, groups fed the high methionine diet supplemented with glycine or serine at 1, 2, or 3 times the level of methionine grew at the same rate, but when gel diets were used definite increases in growth rate were observed when the level of glycine or serine was increased from 1 to 2 or 3 times that of methionine (table 3). These differences also appear to be due to the effect of the form of the diet on voluntary food intake.

Although Roth et al. (20) postulated that glycine alleviates methionine toxicity through being converted to serine and although Cohen et al. (13) found that serine alleviates the growth depression caused by a high homocystine intake, there is little evidence that serine alleviates methionine toxicity (13, 18, 23). Cohen et al. (13) used DL- rather than L-serine in their study and then at a level only three-fourths that of methionine, which may have been too low to cause a significant response. In our experiments a growth response to serine of rats fed the high methionine diet was quite clearly demonstrated but it is difficult to assess the true effectiveness of serine because, in the amounts used, it depressed the growth rate of the control group (tables 3 and 5). As indicated in the results section, whether serine is considered to be less effective or as effective as glycine depends upon which group is assumed to be the appropriate control.

The growth-depressing effects of 4.2 or 6.3% L-serine (tables 3 and 5) are unexpected. In other trials high dietary levels of serine have resulted in slight (6) or no depression in growth (29, 30, 31) but severe toxic signs were observed when B-vitamins were not supplied with the diet (32). Marginal vitamin intake would not appear to be a factor in our experiment because a tenfold increase in vitamin B_6 and 100-fold increases in folic acid and vitamin B_{12} were not effective in overcoming the growth-depressing effects of serine nor in increasing the effectiveness of glycine or serine in alleviating

methionine toxicity. The results of the experiments on homocystine (table 5) suggest that part of the growth depression due to a high serine intake is alleviated by homocystine because the growth rates of groups fed excess homocystine with glycine and serine, expressed as a percentage of that of similarly supplemented controls, are 76 and 124%, respectively.

Rats fed the high methionine diet supplemented with glycine appear to undergo some form of metabolic adaptation because the glycine supplement is not effective for 3 to 4 days (fig. 2). Glycine is not required for the adaptation because animals fed the high methionine diet for 14 days respond immediately to a supplement of glycine. The adaptation does not appear to involve reactions related to the interconversion of glycine and serine because groups fed glycine and serine together did not grow more rapidly than those receiving glycine alone. Those receiving serine alone required 1 to 2 days longer to become adapted. Rats fed a diet high in homocystine undergo a similar period of adaptation after the diet is supplemented with glycine or serine. It is possible that certain enzymes, such as cystathionine synthetase or glycine methyl transferase (33), are limiting initially and that the toxicity is alleviated only after their activity increases.

The relative involvement of the methyl and homocysteine portions of the methionine molecule in causing adverse effects has not been adequately assessed. An excess of homocystine in the diet (table 5) did not depress growth as much as an equivalent amount of methionine, but the effects of homocystine and methionine cannot be compared directly because the homocystine was in the DL-form while the methionine was in the L-form. Under the conditions of these experiments L- and DL-methionine appear to be equally toxic; however, homocystine has to undergo reduction to homocysteine to be metabolically active. Therefore, if absorption and reduction of homocystine were not complete, the metabolic load of homocysteine would not be as great as that of methionine. Also, if the D-enantiomorph of homocysteine is metabolized differently from the L-enantiomorph, the effective level of homocysteine would be lower than that of methionine and thus should result in less toxicity. The relative toxicities of L-methionine and L-homocysteine remain to be studied.

Experiments using methyl acceptors do not clarify the picture because guanidinoacetic acid and nicotinic acid, two methyl acceptors, do not reduce the toxicity of methionine (6, 13, 14, 16, 22, 23), while another methyl acceptor, ethanolamine, appears to be as effective as glycine in alleviation of the toxicity (13). One probably cannot assume that all methyl acceptors are equally effective (34). Nor is information from experiments on effects of equimolar amounts of methyl donors other than methionine particularly helpful. Choline chloride and betaine·HCl do not depress growth (13) but dimethylthetin, another methyl donor (34–36), depresses growth far more than methionine (13). At this stage it is not possible to conclude that one portion of the methionine molecule is more important than the other in methionine toxicity.

The specificity of glycine and serine in alleviating methionine toxicity (tables 3 and 4) may be explained by the ready metabolic interconversion of these 2 molecules and by the need for an adequate supply of serine for conversion of the homocysteine derived from methionine to cystathionine. If serine were more effective than glycine in preventing growth depressions due to excess homocystine and methionine then this explanation would appear to be adequate, but since glycine is superior to serine in protecting against methionine toxicity some further explanation may be required. If both the methyl and the homocysteine moieties of methionine, rather than the homocysteine portion alone, are involved in methionine toxicity, glycine would appear to serve some function in relation to methyl metabolism more effectively than serine. Studies of CO_2 production from the methyl groups of methionine (37) and choline (36) which indicate that methionine methyl groups are converted to CO_2 more rapidly than choline methyl groups, do not support the idea that the methyl group of methionine is metabolized via choline (38, 39). The glycine methyl transferase

reaction (33), in which S-adenosylmethionine and glycine react to yield S-adenosylhomocysteine and sarcosine, with the latter being rapidly converted to CO_2 (40, 41), should be examined in relation to the beneficial effect of glycine in methionine toxicity. The importance of the increased cysteine and sulfur loads also remains to be investigated.

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Effect of Protein Intake on the Development of Abnormal Tryptophan Metabolism by Men during Vitamin B_6 Depletion [1]

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ABSTRACT The effect of the level of dietary protein in the development of abnormal tryptophan metabolism in man during vitamin B_6 depletion was studied. Subjects fed a diet containing 54 g protein and 0.16 mg vitamin B_6 daily developed abnormal tryptophan metabolism very slowly; after 40 days of vitamin B_6 depletion individual subjects excreted from zero to 29% of a 2-g loading dose of L-tryptophan as kynurenine, hydroxykynurenine, xanthurenic acid, kynurenic acid and acetylkynurenine. In contrast, subjects fed a diet containing 150 g of protein and 0.16 mg of vitamin B_6 daily excreted 29% of a 2-g loading dose of tryptophan as the 5 metabolites after 14 days of vitamin B_6 depletion. The post-tryptophan amounts of the tryptophan metabolites excreted by subjects given daily supplements of 1.5 mg pyridoxine were normal whether the protein intake was 54 or 150 g.

The appearance of abnormally high amounts of xanthurenic acid in the urine after a loading dose of tryptophan has been generally accepted as a reliable indioation of vitamin B_6 deficiency in man and in experimental animals. Price et al. (1) and Coursin (2) recommend, however, that quantitative measurement of several tryptophan metabolites following a test dose of L-tryptophan might provide a more definitive test for the evaluation of vitamin B_6 deficiency. Yess et al. (3) measured the excretion of several tryptophan metabolites by young men fed a diet containing 100 g of protein and 0.16 mg of vitamin B_6 daily. The amounts of individual metabolites excreted during the early days of vitamin B_6 deprivation varied from subject to subject, but eventually hydroxykynurenine was excreted in the greatest amount by all subjects, followed by kynurenine and xanthurenic acid. As vitamin B_6 deficiency developed, urinary excretion of hydroxykynurenine, kynurenine, xanthurenic acid, acetylkynurenine and kynurenic acid by the subjects increased significantly in response to a 2-g dose of L-tryptophan.

The requirement for vitamin B_6 by rats and mice has been shown to be related to the protein content of the diet (4–8). Baker et al. (9) reported that men fed daily a diet containing 33 g of protein required 6 weeks of vitamin B_6 deprivation to excrete the same amounts of hydroxykynurenine and xanthurenic acid following tryptophan loading that were excreted after 3 weeks by men consuming 100 g of protein daily.

In the present paper, the effect of the level of dietary protein on the development of abnormal tryptophan metabolism during vitamin B_6 deficiency is reported. Men were fed a diet deficient in vitamin B_6 which contained either 54 or 150 g of protein daily. Tryptophan metabolites measured were: acetylkynurenine, kynurenine, hydroxykynurenine, xanthurenic acid and kynurenic acid.

EXPERIMENTAL

Diets. Two different human metabolic studies were carried out. The diets fed were essentially the same as the one described by Swan et al. (10) with the exceptions that the amounts of protein and the amounts of methionine were different. The daily diet fed during study 1 con-

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TABLE 1

Vital statistics and caloric intake of the subjects

Subject	Age	Weight		Height	Calories/day
		Initial	Final		
	years	*kg*	*kg*	*cm*	*kcal*
		Study 1			
1	21	66.1	64.8	173	3000
2	21	68.0	66.6	175	2800
3	21	64.3	65.0	168	3000
4	25	70.7	72.0	191	3700
5	20	72.9	69.8	174	3400
		Study 2			
6	19	70.9	69.5	179	3600
7	22	69.8	71.8	188	3200
8	21	66.8	66.8	175	3000
9	18	68.4	69.1	180	3100
10	24	62.7	63.2	178	2800
11	31	67.3	68.2	173	3100

tained 9.0 g of nitrogen or approximately 54 g of protein. Vitamin-free casein contributed 5.0 g of nitrogen and gelatin, 2.5 g. The subjects participating in study 2 were given 24.0 g of nitrogen daily or aproximately 150 g of protein; 15.0 g of the nitrogen was supplied by vitamin-free casein and 7.5 from gelatin. A supplement of 0.83 g of L-methionine was given during study 1 and 2.50 g, during study 2. Rice, rice cereal, and several selected fruits and vegetables furnished the remainder of the nitrogen of the diets. Both diets supplied approximately 0.16 mg of vitamin B_6 per day. A mixture of animal and vegetable fats supplied 40% of the total caloric intake of the subjects of both studies. Carbohydrate provided from 52 to 54% of the caloric intake of the subjects ingesting the low protein diet and from 39 to 43% of that of subjects ingesting the high protein diet. Essential minerals and vitamins, with the exception of vitamin B_6, were given.

Subjects. The young men who served as subjects in both studies were students at the University and each followed his regular schedule of activities during the experiment. The vital statistics of the subjects are presented in table 1. Subject 2 of study 1 was subject 8 of study 2. All subjects were free from any known disease or metabolic disorder. A physician at Student Health Service examined the subjects at regular intervals throughout each study. Weekly electroencephalographs were obtained for each subject who participated in study 2.

Procedures. Before the experimental periods, the subjects were studied while they were consuming self-selected diets. When the diet containing 54 g protein was fed, the subjects were given supplements of 1.5 mg of pyridoxine for the first 6 days; then the supplement of pyridoxine was withdrawn and the subjects were fed the vitamin B_6-deficient diet, subjects 1 and 3 for 33 days and subjects 2, 4 and 5 for 40 days. Following the period of vitamin B_6 depletion, subjects 2, 4, and 5 were given a supplement of 0.6 mg of pyridoxine daily for 7 days.

During study 2 when the high protein diet was fed, subjects were given a daily supplement of 1.5 mg of pyridoxine for the first 18 days. Then, for 16 days, they were depleted of vitamin B_6. Following the period of vitamin B_6 depletion, the subjects were given daily supplements of 0.6 mg of pyridoxine for 16 days. For the remaining 2 days, the subjects were given 50 mg of pyridoxine per day.

Fig. 1 Effect of vitamin B_6 depletion of the excretion of hydroxykynurenine (HK), kynurenine (KYN), xanthurenic acid (XA), kynurenic acid (KA) and acetylkynurenine (ACK) after a 2-g (9800 μmoles) load dose of L-tryptophan by subjects fed the low protein diet. The vertical lines indicate the days on which the feeding of the different levels of vitamin B_6 were started. The vitamin B_6 intake includes the 0.16 mg present in the diet. SS indicates self-selected diet of unknown vitamin B_6 content.

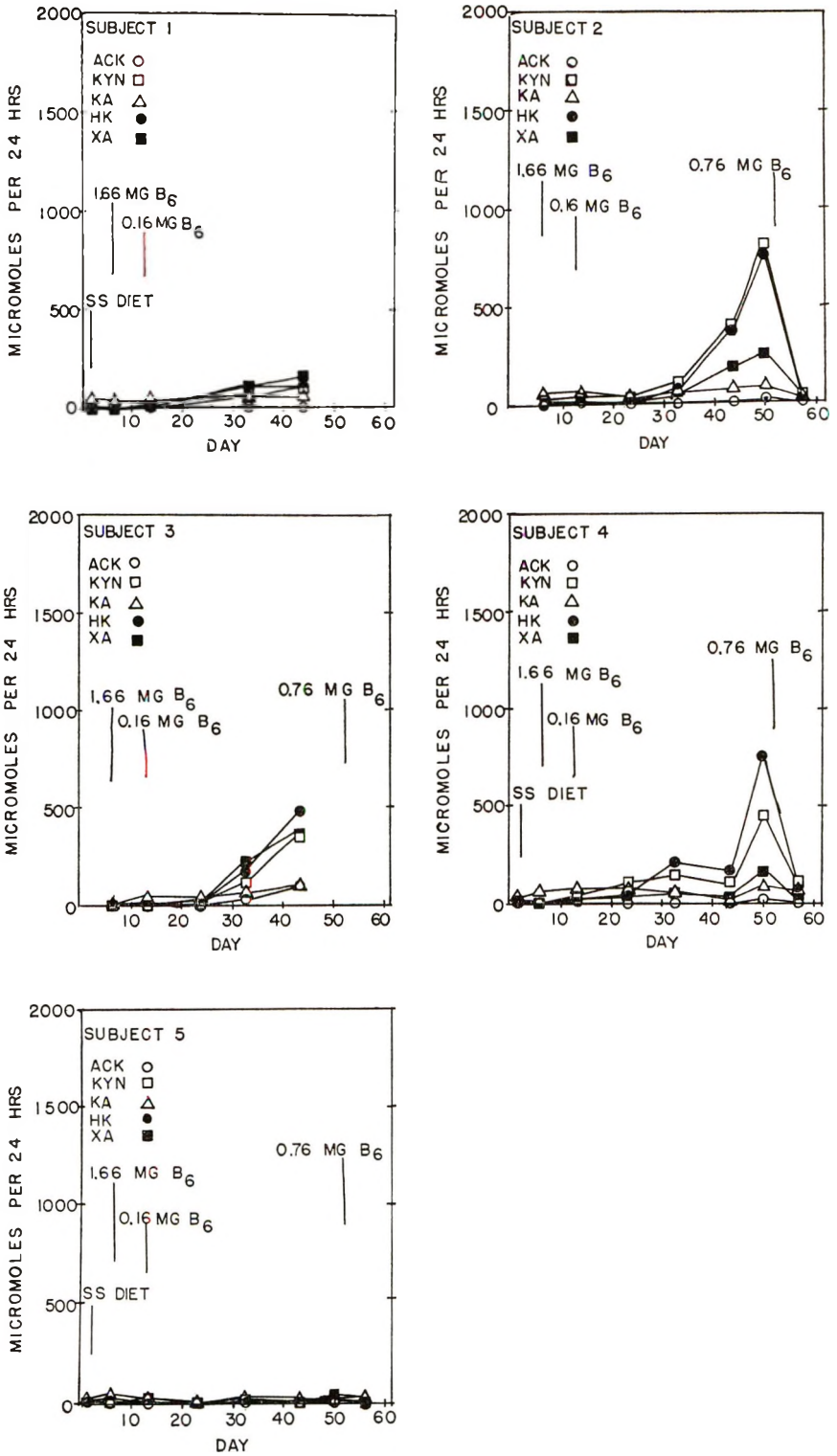
SUBJECT 1
ACK
KYN
KA
HK
XA
1.66 MG B6
0.16 MG B6
SS DIET
SUBJECT 2
0.76 MG B6
SUBJECT 3
SUBJECT 4
SUBJECT 5
MICROMOLES PER 24 HRS
DAY
2000
1500
1000
500
0
10
20
30
40
50
60

Figure 1

Methods. The tryptophan metabolism of each subject was studied at regular intervals. The subjects collected a 24-hour urine sample (basal or pre-tryptophan). Then 2 g of L-tryptophan were administered and the subjects collected a second 24-hour urine sample, the post-tryptophan sample. The tryptophan metabolites were determined according to the following methods: acetylkynurenine and kynurenine by the method of Brown et al. (11); hydroxykynurenine by the method of Brown (12); and xanthurenic acid and kynurenic acid by the method of Satoh and Price (13).

RESULTS

The pre-tryptophan (basal) amounts of hydroxykynurenine, kynurenine, xanthurenic acid, acetylkynurenine, and kynurenic acid excreted by the subjects were considered to be within normal limits, both when the subjects were adequately nourished with vitamin B_6 and when they were depleted of the vitamin. The values were similar to those reported by Price et al. (1) for male subjects free of any known disease. The mean basal amounts of the tryptophan metabolites excreted per 24 hours by the subjects during all periods of both studies ranged in micromoles from 6 to 16 for acetylkynurenine, 9 to 24 for kynurenine, 7 to 19 for kynurenic acid, 9 to 18 for hydroxykynurenine and 6 to 14 for xanthurenic acid. The post-tryptophan amounts of hydroxykynurenine, kynurenine, xanthurenic acid, kynurenic acid and acetylkynurenine excreted by subjects supplemented daily with 1.5 mg of pyridoxine were normal whether the protein intake was 54 or 150 g daily (figs. 1 and 2). When the subjects were deprived of vitamin B_6, however, they excreted elevated quantities of the tryptophan metabolites following loading with 2 g (9800 μmoles) of L-tryptophan. The level of dietary protein markedly affected the rate at which abnormal tryptophan metabolism developed.

Subjects fed the low protein diet developed abnormal tryptophan metabolism slowly (fig. 1). One subject excreted slightly elevated amounts of kynurenine and hydroxykynurenine following tryptophan loading after 13 days of depletion, but the other subjects excreted normal amounts of all the tryptophan metabolites (fig. 1). All subjects depleted of vitamin B_6 for 23 days, with the exception of subject 5, excreted abnormal quantities of kynurenine, hydroxykynurenine and xanthurenic acid; the 4 subjects exhibiting abnormal tryptophan metabolism excreted an average of 2.4% of the loading dose as kynurenine, 3.1% as hydroxykynurenine, 1.9% as xanthurenic acid, 0.2% as acetylkynurenine and 0.5% as kynurenic acid. At no time did subject 5 excrete an abnormal quantity of any of the tryptophan metabolites measured, even though he was depleted of vitamin B_6 for 40 days. However, subjects 2 and 4, who were also depleted for 40 days, excreted 19.8 and 25.5%, respectively, of the 2-g dose of tryptophan as the 5 metabolites.

In contrast, after only 6 days of vitamin B_6 deprivation, subjects fed the diet containing 150 g of protein excreted an average of 7.3% of the loading dose of tryptophan as kynurenine, hydroxykynurenine, xanthurenic acid, kynurenic acid and acetylkynurenine (fig. 2). The amount of tryptophan metabolites excreted continued to increase as time progressed and, after 14 days of vitamin B_6 deprivation, the subjects excreted 29.4% of the loading dose as the 5 metabolites. Hydroxykynurenine and kynurenine were excreted in the largest amounts and as the subjects become more depleted the urinary excretion of these 2 metabolites increased at a faster rate than those of the other three. Subjects depleted of vitamin B_6 for 6 days excreted an average of 2.0% of the loading dose as hydroxykynurenine and 2.9% as kynurenine; after 14 days of depletion, hydroxykynurenine accounted for 11.3% of the administered tryptophan, and kynurenine for 9.8%. The amount of xanthurenic acid excreted by the subjects after 6 days of vit-

Fig. 2 Effect of vitamin B_6 depletion on the excretion of hydroxykynurenine (HK), kynurenine (KYN), xanthurenic acid (XA), acetylkynurenine (ACK) and kynurenic acid (KA) after a 2-g (9800 μmoles) load dose of L-tryptophan by subjects fed the high protein diet. The vertical lines indicate the days on which the feeding of the different levels of vitamin B_6 were started. The vitamin B_6 intake includes the 0.16 mg present in the diet. SS indicates self-selected diet of unknown vitamin B_6 content.

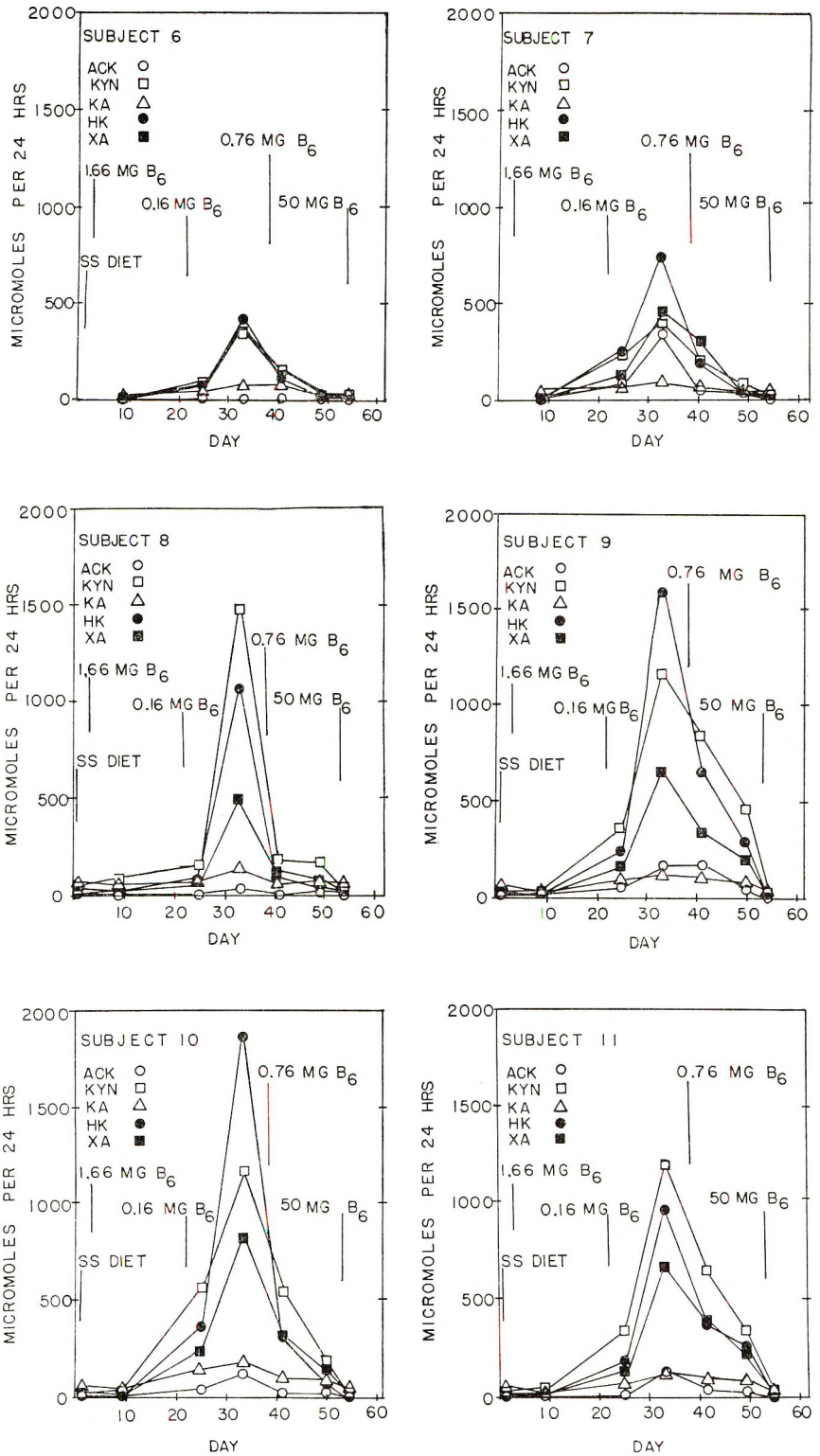
SUBJECT 6
SUBJECT 7
SUBJECT 8
SUBJECT 9
SUBJECT 10
SUBJECT 11
ACK
KYN
KA
HK
XA
1.66 MG B6
0.16 MG B6
0.76 MG B6
50 MG B6
SS DIET
MICROMOLES PER 24 HRS
DAY
0
500
1000
1500
2000
10
20
30
40
50
60

Figure 2

amin B_6 depletion was approximately two-thirds that of hydroxykynurenine, and after 14 days of depletion, approximately one-half that of hydroxykynurenine. Even though the amount of kynurenine excreted increased markedly during the deficiency of vitamin B_6, the amount of kynurenic acid increased but slightly. Xanthurenic acid, kynurenic acid and acetylkynurenine accounted for 1.4, 0.8, and 0.3%, respectively, of the tryptophan load dose after 6 days of vitamin B_6 depletion, and 5.9, 1.2 and 1.3%, respectively, after 14 days.

Daily supplements of 0.6 mg of pyridoxine to the subjects following the period of depletion caused a marked decrease in the amount of the tryptophan metabolites excreted. However, after 13 days of vitamin B_6 supplementation, most of the subjects continued to excrete abnormal amounts of hydroxykynurenine, kynurenine and xanthurenic acid in response to tryptophan loading. The pattern of excretion of tryptophan metabolites by subjects partially repleted with vitamin B_6 was different from that observed during the time they were most depleted. Kynurenine was the metabolite excreted in the greatest quantity by subjects during repletion, and xanthurenic acid was excreted in an amount that was greater than or equal to that of hydroxykynurenine.

A daily supplement of 50 mg of pyridoxine for 2 days to the subjects consuming 150 g of protein per day resulted in normal tryptophan metabolism.

Physical examinations by Student Health physicians failed to detect any clinical evidence of vitamin B_6 deficiency in any of the subjects. Electroencephalographs obtained for subjects of study 2 revealed no abnormalities when they were deficient in vitamin B_6.

DISCUSSION

When the subjects of the present study became sufficiently depleted of vitamin B_6, they excreted abnormally high quantities of some tryptophan metabolites following loading with 2 g of L-tryptophan. However, normal amounts of the metabolites were excreted on days when tryptophan loads were not given. Vitamin B_6-deficient rats (5, 14–16) and mice (5) excreted elevated quantities of xanthurenic acid without the stress of tryptophan loading. The rat (5, 14, 16) and the mouse (5) deficient in vitamin B_6 converted dietary tryptophan to xanthurenic acid. The fact that abnormal tryptophan metabolism developed much more rapidly in men given the high protein intake than in those given the low protein intake following withdrawal of vitamin B_6 from the diets suggests that the requirement for the vitamin is dependent upon the protein content of the diet. The carbohydrate content of the 2 diets also varied, but the relative change was much less for carbohydrate than for protein. Protein furnished 7 and 19%, respectively, of the calories of the low protein and high protein diets, whereas carbohydrate furnished 53 and 41%. Also it is well-known that vitamin B_6 coenzymes participate in the synthesis and catabolism of all naturally occurring amino acids; this subject has been reviewed recently (17).

Supplementation with 0.6 mg pyridoxine daily to the vitamin B_6-deficient men fed 54 g protein caused a prompt decrease in the amount of the tryptophan metabolites excreted to normal values. Although the same amount of pyridoxine supplement to the vitamin B_6-deficient men fed 150 g protein resulted in a marked decrease in the excretion of those tryptophan metabolites which had been elevated so markedly during the period of depletion, abnormally high amounts of hydroxykynurenine, kynurenine and xanthurenic acid continued to be excreted even after the subjects had been supplemented with the vitamin for 13 days.

Other evidence exists which supports the view that increasing the protein content of the diet increases the requirement for vitamin B_6 by man. Additional dietary casein aggravated the central nervous system manifestations of vitamin B_6 deficiency in infants, whereas a high carbohydrate diet alleviated these symptoms (18). In a dietary-induced deficiency of vitamin B_6, subjects fed a diet containing 100 g of protein per day required 1.5 mg of pyridoxine daily to reduce the excretion of xanthurenic acid to normal levels, whereas subjects fed a similar diet containing 33 g of protein required 1.25 mg.

The fact that after 40 days of vitamin B_6 deprivation one of the subjects failed to excrete abnormal amounts of tryptophan metabolites in response to tryptophan loading may indicate that this subject has a lower requirement for vitamin B_6 than the others. However, the negative response of this subject to tryptophan loading does not preclude the possibility of abnormalities in other vitamin B_6-dependent systems which are affected during the early stages of a vitamin B_6 deficiency. In the rat most of the activity of cysteinesulfinic acid decarboxylase is lost after a few days of vitamin B_6 deprivation (19); and in the mouse, cysteine desulfhydrase is also very sensitive to vitamin B_6 deficiency (20).

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Serum Selenium and Related Parameters of Naturally and Experimentally Fed Horses [1]

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ABSTRACT A survey was made of serum selenium levels and some related parameters of naturally and experimentally fed horses as part of a study to determine the significance of selenium in light-horse nutrition. Serum selenium levels of suckling foals and naturally fed weanlings, yearlings, trainees, adult mares and stallions were 7.00, 14.7, 13.1, 12.4, 12.7 and 12.1 μg Se/100 ml serum, respectively. Orphaned foals fed a commercial milk replacer had 16.25 μg Se/100 ml. Foals fed a selenium, vitamin A- and vitamin E-deficient semipurified ration containing 33% Torula yeast had serum selenium values (μg/100 ml) of 3.68 in unbedded concrete floor stalls, 7.40 in straw-bedded stalls, and 17.33 in tobacco stem-bedded stalls. Foals fed the semipurified ration supplemented with 0.5, 1 and 2 ppm selenium (as sodium selenite) had serum selenium values of 14.68, 15.30 and 16.67 μg/100 ml, respectively. By selenium repletion-depletion techniques using parenterally administered selenium, an equine selenium requirement was estimated at 2.4 μg/kg body weight/day. The SGOT reducing effects of a single intramuscular injection of selenium occurred over a 45-day period.

Selenium was first unfavorably associated with equine nutrition in 1935 when Franke and Painter (1) identified selenium as the toxic property of certain roughages associated with the incidence of alkali disease and blind staggers in livestock. The nutritional importance of selenium was disclosed by Schwarz and Foltz (2) who demonstrated that selenium was the active ingredient in a "Factor 3" which prevented hepatic necrosis in vitamin E-deficient chicks and rats. The metabolic significance of selenium and its relationship to tocopherol and myopathies have since become a topic of considerable interest. The present report, part of a study relative A, vitamin E and selenium in light-horse nutrition, presents the serum selenium status and some related parameters of naturally and experimentally fed horses.

EXPERIMENTAL

Selenium analyses were made on 542 serum samples from (a) naturally fed suckling, weanling, yearling, and adult Standardbreds and Throughbreds on central Kentucky farms, (b) Thoroughbreds in training at Aqueduct Raceway, Long Island, New York, and Keeneland Race Track, Lexington, Kentucky, and (c) experimentally fed horses at the Department of Veterinary Science, University of Kentucky, Lexington, Kentucky.

The rations of the naturally fed horses included (a) the predominantly mare's milk diet of the suckling foal; (b) a predominantly oat concentrate with good quality mixed legume and grass hay or bluegrass pasture for weanlings, yearlings and adults; and (c) the predominantly oat concentrate-limited roughage ration for horses in training.

The experimentally fed horses were obtained as orphaned foals, 3 to 14 days of age and fed initially a commercial milk replacer. Subsequently, while the animals were being depleted of vitamin A, tocopherol and selenium they were fed the pelleted semipurified ration presented in table 1. These horses were confined in concrete block stalls with unbedded concrete floors except where indicated in the text.

The effect of selenium supplementation upon the serum selenium levels of previously selenium-depleted horses being fed the vitamin A- and vitamin E-deficient

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TABLE 1

Composition of semipurified equine ration

	%
Torula yeast, feed grade	33.0
Cornstarch	51.35
α-Cellulose [1]	13.00
Limestone	1.65
Trace mineral salt [2]	1.00
	IU/kg
Vitamin D_2	500

[1] BW 20 Solka Floc, Brown Company, Boston.
[2] Contained not less than: (in per cent) NaCl, 96; Mn (manganous oxide), 0.25; Fe (ferrous carbonate), 0.1; S (sodium sulfate), 0.05; Cu (copper oxide), 0.003; Cu (cobalt carbonate), 0.015; Zn (zinc oxide), 0.008; I (calcium iodate), 0.007; and MgO, 0.58 (Sterling Blue Salt, International Salt Company, Clark Summit, Pennsylvania).

semipurified ration was determined by selenium supplementation of the semipurified ration at 0.5, 1.0 and 2.0 ppm selenium as sodium selenite for a 60-day period.

Twelve selenium-deficient foals being depleted of vitamins A and E and which had been fed the semipurified ration for 4 months were used to evaluate the effect of selenium upon growth rate before clinical evidence of either vitamin deficiency occurred. During a 52-day period, the growth rate of 6 foals fed the semipurified ration at 17.6 g/kg body weight was compared with the growth rate of the other 6 foals fed at the same rate the semipurified ration supplemented with 2 ppm selenium as sodium selenite. The foals were weighed 3 times a week and their feed intake was adjusted according to gains at each weighing.

The rate of utilization of parenterally administered selenium was estimated in 2 vitamin E-deficient, vitamin A-supplemented horses previously selenium-depleted with the semipurified ration and having high serum glutamic oxalacetic transaminase (SGOT) values. Each horse was given a single intramuscular injection of 0.11 μg selenium (as sodium selenite)/kg body weight. Serum selenium and SGOT determinations were made daily for the first 5 days post-injection and weekly thereafter for 7 weeks.

The selenium assay procedure used was a modification of a procedure described by Taussky et al.,[2] making use of the Schöniger combustion flask for ashing followed by the fluorometric determination of the selenium diamino-benzidine complex described by Cousins (3).

Serum glutamic oxaloacetic transaminase determinations were made as a clinical index of myopathy according to an industrial method.[3]

RESULTS AND DISCUSSION

The serum selenium data obtained from several age groups of horses under natural feeding regimens are presented in table 2. The most important observation therein is the low selenium status of suckling foals. Although the adult mares had serum selenium levels averaging 12.78 μg/100 ml and, according to Smith et al. (4), selenium is present in the milk of domestic animals, the serum selenium levels of suckling foals appear to remain low even at an age when the suckling foal is consuming considerable quantities of grain and roughage. This observation suggests the possibility of an absorption inhibiting effect of mare's milk upon dietary selenium or a selenium-dependent intestinal microflora of the suckling foal.

The central Kentucky area has been identified by Bruins et al.[4] as a white muscle disease area and selenium-responsive nutritional myopathies of sheep are frequently observed here. It therefore appears reasonable to conclude from the low serum selenium values of suckling foals that subclinical selenium-responsive nutritional myopathy of foals in the central Kentucky area exists although gross myopathy is rarely observed in foals necropsied there. A number of foals sampled had serum selenium values approximating the 1.4 μg/100 ml level reported by Oldfield et al. (5) to be associated with ovine nutritional myopathies.

There was a conspicuous tendency for the Standardbreds to have higher serum selenium values than Thoroughbreds of a comparable classification. While this ap-

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TABLE 2

Serum selenium content of naturally fed Standardbreds and Thoroughbreds of several age groups

Group	Breed	Selenium		No. of assays
		μg/100 ml serum		
Sucklings	Standardbred	7.88 ± 3.41 [1]	(2.73–14.60) [2]	28
	Thoroughbred	6.12 ± 1.42	(3.30– 8.67)	30
Weanlings	Standardbred	16.21 ± 1.77	(12.00–18.00)	10
	Thoroughbred	13.18 ± 3.19	(7.67–21.67)	51
Yearlings	Standardbred	13.73 ± 3.42	(5.91–23.00)	43
	Thoroughbred	12.41 ± 2.90	(7.50–18.33)	25
Trainees [3]				
Aqueduct	Thoroughbred	14.34 ± 2.67	(9.63–19.17)	29
Keeneland	Thoroughbred	10.47 ± 1.63	(7.43–13.20)	18
Adult mares	Standardbred	14.07 ± 2.80	(6.13–21.50)	34
	Thoroughbred	11.49 ± 2.47	(5.53–20.27)	58
Stallions [4]	Thoroughbred	12.08 ± 1.18	(9.53–15.17)	28

[1] SD of mean.
[2] Range.
[3] Two and three years old.
[4] Five to 22 years old.

TABLE 3

Serum selenium content of horses fed experimental rations in unbedded and bedded concrete floor stalls

Age group	Ration	Bedding	Selenium		No. of assays
			μg/100 ml serum		
Suckling foals	Commercial milk replacer [1]	none	16.25 ± 2.20 [2]	(13.26–20.67) [3]	12
Weanling-yearling	Semipurified ration [4]	none	3.68 ± 1.31	(1.60– 7.30)	35
Weanling-yearling	Semipurified ration	wheat straw	7.40 ± 2.00	(3.30– 9.30)	8
Yearling	Semipurified ration	tobacco stems	17.33 ± 3.83	(9.60–25.03)	40
Yearling	Semipurified ration	none [5]	9.30 ± 1.66	(6.00–11.67)	10

[1] Land O'Lakes Creameries, Inc., Minneapolis 55413.
[2] SD of mean.
[3] Range.
[4] See table 1.
[5] Seven days after stopping tobacco stem bedding.

peared to be a clinical difference between the 2 breeds, it is now realized that the Standardbred serum was obtained only from farms using tobacco stem bedding, which, as noted later, can affect serum selenium levels.

The serum selenium data from the experimentally fed horses in variously bedded cement block stalls are presented in table 3 and several factors appear important. The selenium levels of orphaned foals fed commercial milk replacers are high in comparison with their nursing counterparts. These commercial milk replacers contain no supplemental selenium, indicating either a higher or more readily available selenium content of the milk replacer than a mare's milk.

Very low serum selenium values were obtained in horses fed the semipurified (Torula yeast) ration indicating its suitability to the experimental production of hyposelenosis of horses.

The high serum selenium values of experimentally fed foals bedded with tobacco stems, a common horse stall bedding in areas adjacent to tobacco redrying plants, are of special interest. The stem bedding was used in these experiments when cold weather prevented washing the unbedded stall floors and because the stems were considered inedible, that is, normally fed horses are reported not to eat stem bedding. Although the experimentally fed foals were seldom observed eating the stems, serum selenium levels above those caused by selenium supplementation of the semipuri-

fied ration at 2 ppm were obtained after the tobacco stem bedding was started.

In the absence of satisfactory selenium analyses of tobacco stems, their effect upon serum selenium cannot be definitely attributed to the selenium content of the stems. Martin and Trelease (6), however, have alluded to the selenium accumulative property of tobacco.

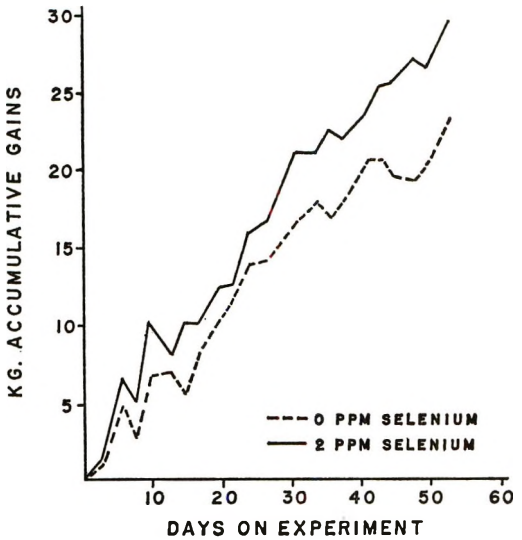

Fig. 1 Comparison of the mean accumulative gains of 6 selenium-deficient weanling foals with the gains of 6 selenium-supplemented foals fed semipurified diets.

The data comparing the accumulative gains of selenium-deficient foals with the accumulative gains of selenium-supplemented foals are presented in figure 1. At the onset, the selenium-deficient group averaged 216 ± 59 (SD) kg and the selenium-supplemented group averaged 229 ± 62 (SD) kg. There was a tendency ($P < 0.1$) for the selenium-supplemented foals to gain more rapidly than the selenium-deficient foals. These data lend support to the report of Hartley and Grant (7) who indicated lambs and calves show growth responses to selenium supplementation.

The serum responses of selenium-deficient horses to selenium supplementation of the semipurified ration are presented in table 4. It appears that selenium supplementation at 0.5 ppm permits maintenance of serum selenium levels twice as high as found in suckling foals and comparable to the general average of the naturally fed non-suckling horse serum assayed. The moderate increases in serum selenium re-

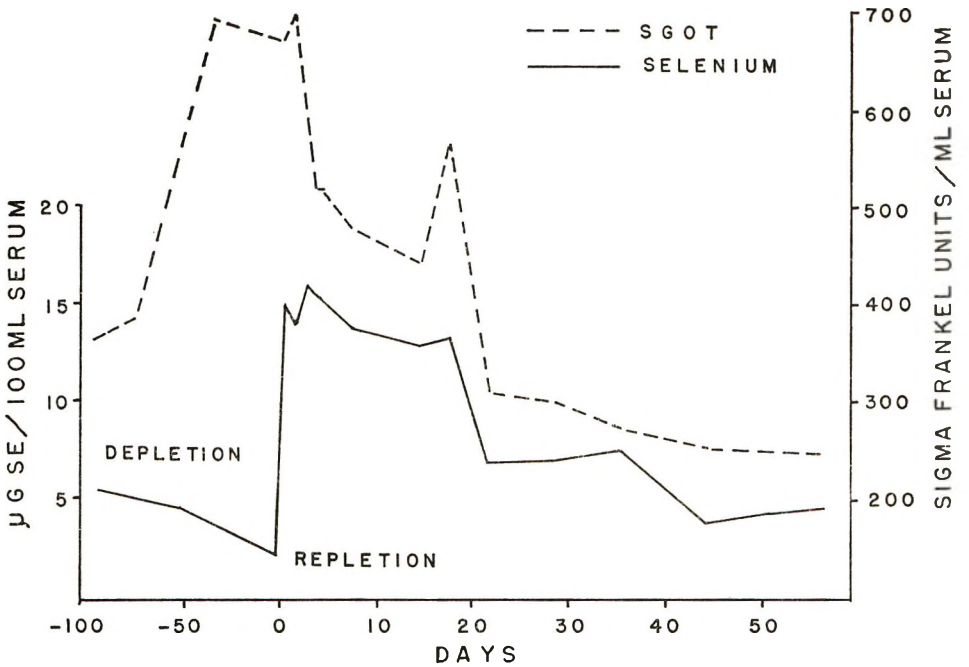

Fig. 2 Mean equine serum selenium and GOT values before and after a single intramuscular injection of 0.11 mg selenium/kg body weight of foals fed semipurified ration.

TABLE 4

Effects of selenium supplementation upon serum selenium levels of foals fed semipurified Torula yeast ration

Supplemental selenium	Selenium	No. of assays
ppm	*μg/100 ml serum*	
—	3.68 ± 1.31 [1]	35
0.5	14.20 ± 1.24	18
1.0	15.30 ± 1.43	18
2.0	16.67 ± 1.95	15

[1] SD of mean.

sulting from the 1 and 2 ppm supplemental selenium, respectively, are believed to reflect a urinary selenium threshold mechanism promoting selenium excretion when serum selenium levels approach 14-16 μg/100 ml.

The mean serum selenium and SGOT levels before and after a single intramuscular administration of 0.11 mg selenium/kg body weight of 2 selenium-deficient, hypertransaminasemic yearling foals fed the semipurified ration at 12 g/kg body weight (maintenance ration) are illustrated in figure 2. The data illustrate, first, an unexpectedly slow SGOT response to a single intramuscular injection in hyposelenotic-hypertransaminasemic horses. This should be considered when anticipating responses to selenium therapy for the tying-up (transient myotonia) syndrome (8) and other hypertransaminasemias of horses (9); that is, 45 to 60 days may be required for optimal physiological effects from selenium supplementation. Second, the selenium repletion-depletion study indicated 0.11 mg selenium/kg body weight was utilized in approximately 45 days. The equine selenium requirement, then, under the experimental conditions, was estimated to be 2.4 μg/kg body weight per day.

The relative efficacy of oral as compared with parenteral selenium supplements may be also indicated by this study. If equine rations must in fact contain 0.5 ppm selenium to maintain optimal serum selenium levels and a horse consumes 20 g of the 0.5 ppm selenium ration per kg body weight/day, a horse's daily oral selenium intake would approximate 10 μg Se/kg body weight. Thus one part parenteral selenium would be comparable to 10/2.4 or 4.15 parts oral selenium. This relationship is probably dependent upon such factors as dietary sulfur which has been shown by Schubert et al. (10) to have an antagonistic effect upon the biological availability of dietary selenium.

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Effect of Various Sugars and Sugar Substitutes on Dental Caries in Hamsters and Rats

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ABSTRACT Two series of experiments, one in hamsters the other in rats, were conducted to compare the dental caries conduciveness of sucrose with that of several other sugars and carbohydrates. Hamsters and albino rats fed a diet containing 56% sucrose in the form of confectionery sugar developed highly active carious lesions in their molar teeth. The lesions were located on "smooth surfaces" as well as in crevicular areas. Fructose, dextrose, maltose, hydrogenated starch, sorbitol, and mixtures of starch were used as substitutes for the sucrose product. In hamsters, which are more prone to experience smooth surface caries, the substitutes used were followed by a reduction in plaque accumulations, less active progression of lesions, and little or no new lesion incipience. In the rats, which are susceptible to a wider variety of lesions, almost all the sucrose-free substitutes were associated with less active cavitation in crevices and on buccal, lingual, and proximal surfaces. However, only the hydrogenated starch product, which is fermented more slowly than the sucrose, was not conducive to circumferential lesions, but it was associated with a low grade form of activity in crevicular areas (sulci).

To account for the several types of carious lesions which are found in humans and which can now be induced in hamsters and rats, one needs to explore the supposition of Cox (1) that carious processes in one affected site may differ etiologically from carious processes in another site. Cavitation in crevices or sulci ("pit and fissure" cavities), on smooth surfaces (buccal, lingual, and proximal lesions), and on root surfaces does not always develop with equal severity. This phenomenon can be explained with the hypothesis that these lesions may differ with respect to the microorganisms involved and the type of carbohydrate consumed. In fact, there appears to be evidence available to support this postulation (2–4). New experimental procedures and deeper insight into processes associated with dental caries now permit further testing of the hypothesis by providing methods to study some of the interactions between various carbohydrates, bacterial flora, plaque formation, and variations in cavitation in the aforementioned areas.

Although several workers (5–7) have reported that the extent of cavitation in experimental animals may vary with the types of carbohydrate in the diet, generally speaking little distinction has been made between various lesions, apparently because only one kind has been found or studied. The present paper describes several exepriments with various dietary sugars and sugar substitutes which were undertaken to explore interactions between these ingredients and odontopathic bacteria and patterns of carious lesions in the molar teeth of hamsters and rats.

EXPERIMENTAL METHODS

A better appreciation of the interactions between dietary components, bacteria, and dental caries can be attained by using both hamsters and rats whenever possible. Hamsters are well-suited for studies of bacterial colonization on the teeth, smooth surface cavitation, and periodontal lesions (8–10), since these phenomena can be easily followed by examining the mouths of living unanesthetized animals. Rats are also satisfactory for studies of smooth-surface lesions, and their teeth are more suitable for studies on proximal and crevicular lesions (11). However, in unanesthetized rats it is not possible to examine the molar denti-

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tion and to evaluate lesions in recessed areas. Moreover, methods for assessing populations of caries-conducive bacteria in rats have been more difficult than in hamsters.

Diet formula no. 2000 (3) which is highly conducive to dental caries was used as a control ration in the series of assays to be described. It contains: (in percent) confectioner's sugar, 56; skim milk power, 28; whole wheat flour, 6; alfalfa powder, 3; brewer's yeast, 4; liver powder, 1; and sodium chloride, 2.[2] The effect of the various carbohydrates was compared by using the following substitutions for the sucrose fraction in diet no. 2000: fructose; dextrose; fructose (28%) and dextrose (28%); maltose; hydrogenated potato starch,[3] that is, product 6563 (12); hydrogenated potato starch with calcium carbonate, 0.5%; potato starch; potato starch (28%) and sucrose (28%). All of the sugars were powdered and analytically pure except sucrose and dextrose [4] which were commercial products.

Hamster experiments. Diet no. 2000 was fed ad libitum to control animals during the entire assay periods (exps. HA, HB, HC, and HD). In the experimental groups this diet was fed during a preassay induction period of 10 days, and then the sucrose was replaced by either fructose (exps. HA and HB), dextrose (exps. HB and HC), equal parts of dextrose and fructose (exps. HB and HC), maltose (exp. HC) and hydrogenated potato starch (exps. HB, HC, and HD).

Weanling golden or albino hamsters between 19 and 24 days of age weighed approximately 30 g when they were started on the various experiments. All animals were inoculated intraorally 3 times, at intervals of one week, with 0.1 ml of an 18-hour culture of caries-conducive streptococci and given about 5 ml of the culture in 200 ml of drinking water for 24-hour periods. The streptococcal strain used was a streptomycin-resistant mutant (E- 49) described previously (13, 14). After 1, 2, and 5 weeks, samples of plaques and saliva were taken by means of a cotton-tipped swab and were transferred to 1 ml of a 0.05% yeast extract water. The swabs were shaken in the yeast extract water and were then streaked on fuchsin-azide blood agar plates containing 200 μg/ml of streptomycin. The plates were inoculated for 48 hours at 37° in 95% nitrogen plus 5% carbon dioxide. The number of microorganisms recovered on the plates was expressed as follows: over 1000 colonies per swab = 4+; 1000–500 = 3+; 500–250 = 2+; 250–50 = 1+; < 50 = ±; – means no recovery (13, 14). The development of bacterial plaques and coronal cavitation was followed in the live animals by periodic examination of their teeth and gingivae with a dissecting microscope (magnification, × 15). The mouths of representative animals were stained with eosin and then photographed (15). At the end of the experimental interval, the extent of cavitation was evaluated in living animals or in defleshed jaws (9, 16).

Osborne-Mendel rats experiments. The rats used in this series of studies were obtained from the NIH stock colony. This is a Cesarian-derived, relatively pathogen-free colony maintained in a controlled environment, semi-barrier system.[5] Females were used throughout. At the start of an experiment they were approximately 21 days of age and had an average weight of 45 g. They were housed in metal cages in groups of two or three, and provided with their respective diets and water ad libitum. On the first day of an experiment each animal was infected with feces containing bacteria conducive to both crevice and smooth surface lesions. A few drops of softened mixed feces from caries-active rats were placed in the mouth of each animal, and a drinking solution of 15 fresh fecal pellets per liter of distilled water was prepared daily and provided for the first 5 days of the experiment. Distilled water was used thereafter. The animals were

[2] The hamsters received a weekly supplement of a puree of equal parts of apple, carrot, and kale. Each animal received about 10 g of the blend. No supplement was given to the rats.

[3] Hydrogenated potato starch (product 6563) is manufactured by the Lyckeby Starch Refining Company, Lyckeby, Sweden. It contains sorbitol and hydrogenated dextrins with molecular weights corresponding to the equivalent of 2–10 glucose units, with a mean of 4 glucose units. Each dextrin has a sorbitol residue at one end of the chain.

[4] Cerelose, Corn Products Company, Argo, Illinois.

[5] These rats are not specific pathogen-free animals. However, they do not harbour a highly caries-conducive microflora and develop very low levels of caries activity unless they are infected with appropriate microorganisms.

weighed again at termination of the 56-day experimental period.

Four experiments were set up at different times (exps. RA, RB, RC and RD). A control group which received diet 2000 was included in each experiment and each of the sucrose substitutes was assessed at least twice: fructose (exps. RA and RD), dextrose (exps. RA and RD), equal parts of fructose and dextrose (exps. RA and RD), maltose (exps. RA and RB), hydrogenated potato starch (exps. RA, RC, and RD), hydrogenated potato starch with calcium carbonate (0.5%) (exps. RA, RC, and RD), potato starch (exps. RB and RD), equal parts of potato starch and sucrose (exps. RA, RB, and RC), and sorbitol and starch (exps. RB, RC, and RD).

At the end of the experimental period the animals were killed and plaque accumulation was studied in animals selected at random in each of the dietary groups. The examinations were made at 25 × magnification immediately after death, after the teeth and gingivae were flooded with an alcoholic solution of green stain (FD&C green no. 3, fast green F.C.F.). Findings were noted as follows: negligible with traces in isolated areas in pits and fissures; continuous deposits along the gingival margin; and gross amounts on the surfaces.

The jaws and teeth were cleaned of soft tissues by dermestid beetles and odontal cavitation was evaluated by a method which included staining of lesions and the sectioning of molars so that sulcal and proximal areas could be examined as readily as the buccal and lingual surfaces (17).

RESULTS

Data obtained from studies of hamsters in experiments HA through HD are tabulated in table 1. Data derived from the examination of the teeth of rats in experiments RA through RD are tabulated in table 2. Figure 1 is a graphic presentation of average scores for rats in all experiments on each of the dietary variations studied. The average initial and final weights of the rats have been listed in table 3.

TABLE 1

Summary of observations in hamsters fed a diet containing sucrose for 10 days and then fed a sucrose substitute for the remainder of the experimental period

Principal carbohydrate	No. of animals	Preassay-assay period [1]	Streptococcal recovery	Coronal plaque	Avg caries score [2]	Final wt
						g
			Experiment HA			
Sucrose	6	10+40	++++	extensive	37	77
Fructose	6	10 / 40	++ to ±	sparse	7	68
			Experiment HB			
Sucrose	4	10+45	++++	extensive	83	80
Fructose	4	10 / 45	+++ to +++++	sparse	5	73
Dextrose	4	10 / 45	++ to +++++	sparse	2	85
Fructose-dextrose	5	10 / 45	++ to +++++	sparse	10	75
Hydrogenated starch (6563)	4	10 / 45	+	sparse	<1	84
			Experiment HC			
Sucrose	5	10+73	++++	extensive	169	92
Dextrose	6	10 / 73	+ to −	sparse	4	98
Fructose-dextrose	6	10 / 73	± to −	sparse	10	89
Maltose	6	10 / 73	± to −	moderate	22	97
			Experiment HD			
Sucrose	6	10+50	+ to +++++ [3]	extensive	139/40	86
Hydrogenated starch (6563)	5	10 / 50	± to +	sparse	56/0	63

[1] The number to the left of the virgule refers to the number of days the animals were fed sucrose (diet 2000); that to the right the days fed the test diet.

[2] The average values listed are for the entire dentition. In experiment HD the number to left of virgule is average score for dentition as a whole; that to the right is for third molars only; P. H. Keyes (9).

[3] No organisms were isolated from one animal.

TABLE 2

Comparison of various carbohydrates in caries test diets fed to Osborne-Mendel rats in experiments RA, RB, RC, and RD, showing the number of rats in each group and the number of areas of carious enamel on the bucco-lingual (BL), proximal (P), and sulcal (S) surfaces

Carbohydrate in diet	No. rats/ group	Exp. RA			No. rats/ group	Exp. RB			No. rats/ group	Exp. RC			No. rats/ group	Exp. RD		
		BL	P	S		BL	P	S		BL	P	S		BL	P	S
Sucrose	5	26.5	13.8	49.2	5	23.6	9.6	45.8	7	14.3	5.3	44.4	6	11.5	9.5	41.5
Dextrose	4	12.5	6.3	32.0									11	10.7	9.0	21.7
Fructose	4	6.0	7.5	28.3									4	9.8	11.5	22.1
Dextrose-fructose	2	8.5	5.0	20.0									12	8.6	8.0	24.6
Maltose	2	8.0	1.0	20.0	4	2.3	2.0	29.1								
Sucrose-potato starch	2	16.0	9.0	31.0	4	11.5	1.0	28.0	6	21.3	6.0	45.5				
Sorbitol starch					2	7.0	0.0	15.0	4	10.0	1.0	42.3	6	3.5	1.3	29.6
Potato starch					5	7.4	0.6	18.2					6	5.5	0.8	14.7
Hydrogenated starch (6563)	5	0.0	1.0	21.2 [1]					6	0.2	0.0	16.8	6	0.0	0.0	11.0
Hydrogenated starch + $CaCO_3$	5	0.2	0.0	15.8 [1]					6	0.0	0.0	23.6	6	0.0	0.0	6.0

[1] On experiment 63 days; all other groups assessed for 55 or 56 days.

Fig. 1 Bar graph showing variations in the pattern of smooth-surface and sulcal (fissure) lesions which developed in rats fed various sugars and sugar substitutes.

Findings in animals fed 56% sucrose. In 21 hamsters which served as controls, dietary sucrose was consistently associated with extensive growth of bacterial plaque on the coronal surfaces of the teeth and in the gingival crevices. The caries-conducive streptococcus (E-49) was generally isolated from the teeth of all animals, except for 3 animals in experiment HD, in sufficient numbers to produce moderate-to-confluent growth on the culture plates. Cavitation of the smooth coronal surfaces was highly active and caused massive destruction, especially in the second and third molars (fig. 2). The severity of cavitation was greater in animals maintained for longer experimental periods. The terminal weights of animals was typical for this diet and the experimental conditions used.

In 23 rats the feeding of sucrose was also conducive to the formation of bacterial deposit along the buccal surfaces of the teeth and in the occlusal crevices. However, the deposits were not so extensive as those noted in hamsters. In all 4 groups buccal-lingual (BL) and proximal (P) lesions were found repeatedly, and sulcal lesions were highly prevalent. Areas of carious enamel from individual animals ranged from 3 to 45 (average, 27.3) on the circumferential surfaces (buccal, lingual and proximal) and 21 to 49 (average, 45) in the sulci. The typical appearance of teeth from an animal fed sucrose is illustrated in figure 2. The growth of rats on this regimen was typical of ani-

TABLE 3
Average initial and final weights and weight gain of rats fed various sugars and sucrose substitutes

Carbohydrate in diet	No. of animals	Avg initial wt	Avg final wt	Avg wt gain
		g	*g*	*g*
Sucrose	24	44.1	172.3	128.2
Dextrose	17	46.5	186.0	139.5
Fructose	10	45.8	188.4	142.6
Fructose-dextrose	18	46.1	174.7	128.6
Maltose	6	42.7	167.3	124.6
Sucrose-starch	12	39.0	161.3	122.3
Sorbitol-starch	12	47.4	115.9	68.5
Starch	11	47.2	160.4	113.2
Hydrogenated starch	18	42.2	157.7	115.5
Hydrogenated starch + $CaCO_3$	18	44.3	143.2	98.9

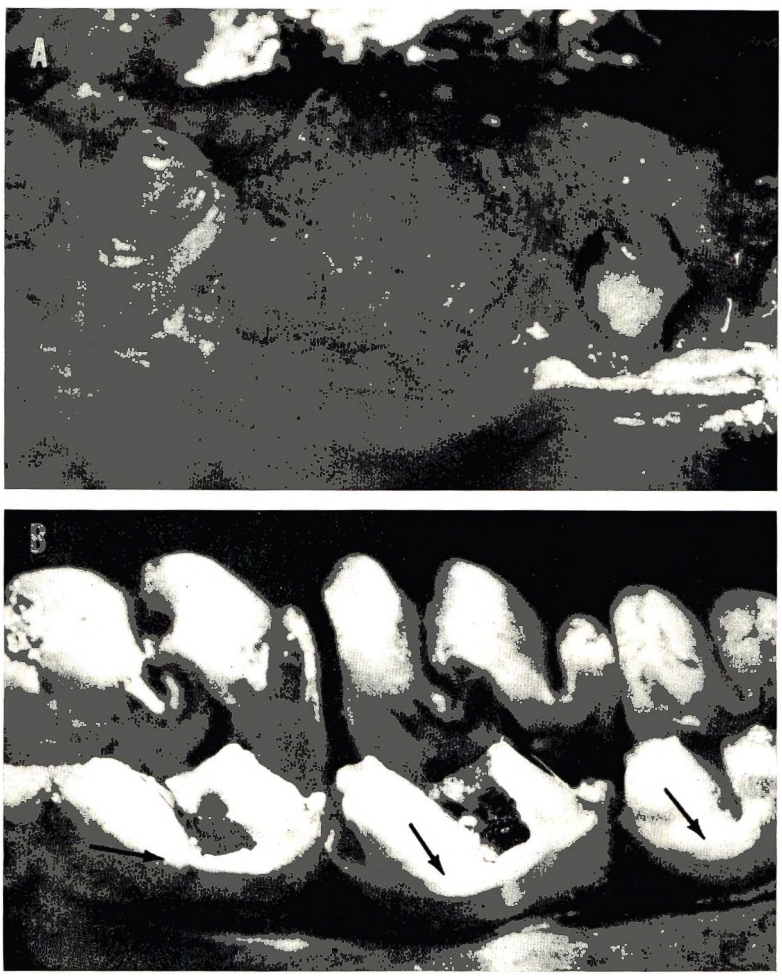

Fig. 2 Typical cavitation found in hamsters and rats fed a diet composed largely of sucrose and skim milk powder. (*A*) Photograph of maxillary molars of living hamsters shows extensive plaque and almost total destruction of second and third molars. (*B*) Sectioned mandibular molars of rat with lesions in all sulcal areas and smooth-surface lesions on the buccal surfaces of each tooth (arrows).

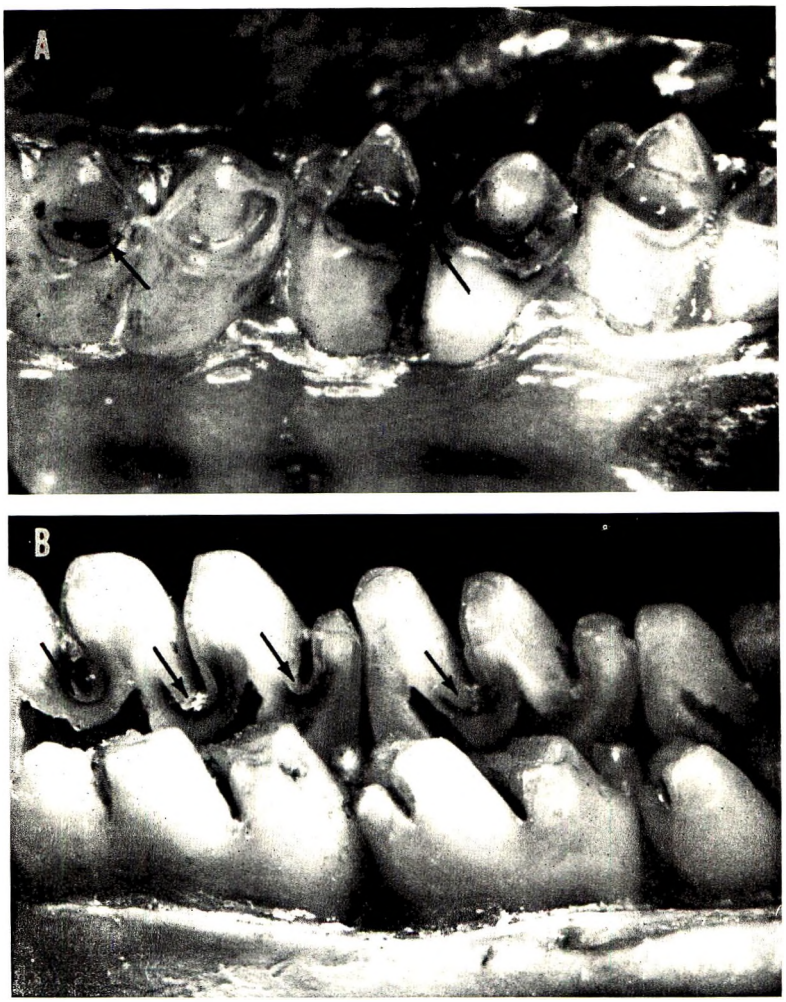

Fig. 3 Appearance of hamster and rat molars in animals fed sucrose substitutes. (*A*) The substitution of fructose, glucose, and hydrogenated starch was associated with a marked reduction in plaque deposits and less active cavitation shown in this photograph. Arrows point to relatively inactive lesions in maxillary first and second molars which started before the replacement of sucrose by fructose. (*B*) Teeth of rat fed hydrogenated starch (product 6563). No smooth decay is apparent, but small lesions are present in the occlusal sulci (fissures).

mals subjected to the conditions used (see table 3).

Findings in animals fed fructose as a replacement of sucrose. In hamsters (10 animals), plaque deposits which had commenced with the sucrose diet largely disappeared after fructose was substituted, and very little further accumulation occurred. When swabs of the oral cavities of these animals were plated out for recovery of streptomycin-resistant streptococci, plates in experiment HA tended to range between < 50 to 250–500 colonies per plate. In experiment HB, however, the recoveries were higher and ranged between 500–1000 colonies and confluent growth per plate. Carious lesions which had started with sucrose remained small, and no smooth-surface decay was detected on the third molars which erupted after the change in diet. Figure 3 shows a representative photograph taken of the maxillary teeth in living animals during the experimental period.

In rats (10 animals), the activity of lesions on the circumferential surfaces (buccal-proximal) was less than in sucrose-fed animals in experiments RA and RB, but it was not different in assay RD. The range in individual animals was from 3 to 31 (average, 17.4). Activity was consistently less in the occlusal crevices of animals fed fructose than in those continued with the sucrose diet, range 2 to 48 (average, 24.7).

Findings in animals fed dextrose as a sucrose substitute. Hamsters (10 animals) responded to dietary dextrose in much the same manner as those fed fructose. Again, plaque deposits which started to accumulate with sucrose became scanty, and only traces could be found on the third molars. Recoveries of the "labeled" streptococcus were variable. In experiment HB the microorganisms ranged between 250 colonies per plate and confluent growth, whereas in experiment HC the counts were lower, ranging between 50 to 500 colonies per plate. Carious lesions which had their inception with sucrose did not progress rapidly, and no smooth-surface activity was noted in third molars. The appearance of the mouths did not differ from that described previously for fructose and illustrated in figure 3. The growth of the animals and the terminal weights did not differ from those noted in animals fed sucrose.

In rats fed dextrose, the findings with respect to plaque deposits and cavitation appeared similar to those noted in animals fed fructose. Lesions occurred on all surfaces of the teeth, although on the basis of areas involved they had progressed somewhat less actively than those under the influence of sucrose.

Findings in animals fed equal parts of fructose and dextrose as a replacement for sucrose. In 11 hamsters (exps. HB and HC) fed the fructose-dextrose combination the results were similar to those when the simple sugars were fed alone. Neither plaque formation nor lesion formation (cavitation) was highly active. The streptomycin-resistant streptococcus was recovered in higher numbers from the mouths of animals assessed in experiment HB than from those tested in experiment HC. Terminal weights of animals did not appear importantly different from animals fed sucrose.

In 18 rats fed the combination of fructose and dextrose, plaque deposits and carious lesions did not differ in quantity and distribution from those found in animals fed fructose or dextrose alone.

Findings in animals fed maltose as a replacement of sucrose. In 6 hamsters (exp. HC) the consumption of maltose was associated with more noticeable plaque deposits although bacterial counts were low. Carious lesions progressed more actively than in animals fed either dextrose or the fructose-dextrose combination.

In 6 rats the consumption of maltose was associated with smooth-surface caries which were less active than those enhanced by sucrose. Activity on the smooth surfaces was somewhat lower for rats fed maltose than for those receiving dextrose, fructose, or the dextrose-fructose combination, but cavitation in the sulci was essentially the same for rats whether fed maltose, dextrose, fructose or dextrose-fructose (fig. 1).

Findings in animals fed hydrogenated potato starch (product 6563). The results were the same for the animals fed the hydrogenated potato starch with or without the addition of calcium carbonate. Animals of both species tended to develop diarrhea soon after they started to consume it, and the fecal deposits remained soft during the entire experimental period.

The growth and development of hamsters in experiment HB was satisfactory. However, all the animals in experiment HC developed diarrhea and died; none of the findings of this group are reported. Five of the six hamsters in experiment HD survived, although they did not grow well.

The teeth and gingival tissues of hamsters fed hydrogenated potato starch did not appear different from those in animals fed the other sucrose-free diets. Gross deposits of plaque could not be found in the teeth nor along the gingival crest. Very few odontopathic streptococci were recovered from the mouths of animals consuming this carbohydrate. In most instances fewer than 50 colonies per plate were noted. In experiment HB the pre-assay lesion did not progress rapidly, and new lesions did not form on the smooth surfaces of third

molars. As a result the overall level of activity was low and the teeth and gingivae resembled that depicted in figure 3. In experiment HD, cavitation in second molars progressed slowly but the final average score for the entire dentition was 56. However, smooth surface lesions did not form in third molars which had never been exposed to sucrose.

A total of 34 rats was fed one form or another of this product. Only negligible levels of plaque were noted along the buccal-lingual surfaces of the molars. However, deposits of debris and plaque were found in the occlusal sulci. The level of caries activity was lower on all surfaces for animals consuming this diet than for those fed any other carbohydrate studied. Smooth-surface activity was negligible, with the most active animal developing only 3 small lesions. A number of small lesions developed in the sulci with an average number of carious areas approximately one-third that of the sucrose fed animals (fig. 3).

Findings in rats fed potato starch alone or in combination with sucrose. The diet containing 56% potato starch was associated with more smooth-surface cavitation than the hydrogenated product, but it was not conducive to more active lesions in the crevicular areas (sulci). Rats fed equal parts of potato starch and sucrose experienced as much plaque and lesion formation as controls in experiment RC. In the other 2 trials, RA and RB, cavity formation was somewhat more active in the groups fed sucrose.

Findings in rats fed a sorbitol-starch combination. Rats (12 animals) fed a diet containing the sorbitol-starch combination developed essentially the same low level of smooth surface lesions as those with potato starch as the only source of carbohydrate. However, the level of sulcal activity with the sorbitol-starch diet was essentially the same as for those fed the sucrose-starch diet, and in experiment RC it was comparable to activity in rats fed 56% sucrose.

DISCUSSION

Dental caries usually has been considered a nonspecific bacterial disease associated with many types of acidogenic microorganisms, and usually little distinction has been made between lesions originating in crevices, on smooth surfaces, or on root surfaces. Some investigators, assuming dental caries to be microbiologically nonspecific, have postulated that experimental animals were resistant to either crevice or smooth-surface cavitation if lesions did not form while the animals were fed a certain type of test diet (18, 19).

The possibility that etiologic factors (bacterial and carbohydrate) may differ for the various lesions has received more attention since the observation that hamsters and rats thought to be resistant to various forms of dental caries become susceptible when exposed to an appropriate bacterial-dietary challenge (20, 21). In order for lesions to form on smooth surfaces, acidogenic microorganisms must adhere to the enamel surfaces in tenacious plaque deposits. In crevices this phenomenon does not appear to be essential, as bacteria and food residues may be retained by wedging action and impaction. It is now evident that investigations of dental caries become more meaningful when the different types of lesions are considered separately, and when etiologic factors are related to the potential of various microorganisms to colonize, to the potential of various dietary components to influence such colonization, and to the by-products produced by the interaction between bacteria and dietary substrates. Since more than one type of bacterial infection may be involved, this possibility needs to be considered in the interpretation of results in animals and in the extrapolation of results to humans.

In hamsters it was possible to see that the bacterial plaques which had formed in animals fed sucrose for 10 days disappeared after the sucrose had been replaced by fructose, dextrose, and hydrogenated starch. Since it was not possible to assess plaque deposits in live rats, the jaws of the animal were examined after the animal had been killed. Gross deposits were noted in animals fed sucrose, whereas only small amounts or traces were noted with other sugars, and negligible amounts were present with hydrogenated starch. The marked difference in plaque

accumulations seen in the mouths of living hamsters (figs. 2 vs. 3) was a more sensitive indication of a reduction in bacterial population on the teeth than the bacterial counts. The cultural methods used herein are useful to reveal the presence of caries-conducive streptococci but are apparently not suited to quantify amounts of plaque in a mouth. Large numbers of the E-49 type streptococcus were not recovered from one control hamster, although it developed extensive plaque and cavitation. This result may have been due to a technical error or to "competitive" exclusion (14).

The present experiments demonstrate that a diet which contains sucrose may have a caries-conducive potential which differs from one that contains other sugars and carbohydrates. Even the combination of fructose and glucose in amounts equal to those present in sucrose did not have the pathogenic potential of the disaccharide. Some of the explanation of these results may be found in the work of Gibbons and co-workers (22) who have isolated strains of streptococci from humans which are conducive to dental caries in rats and appear to be biochemically and serologically comparable to the hamster strains, for example, E-49 used in this experiment. These workers also reported that some strains form large quantities of extracellular non-dialyzable carbohydrate from sucrose and not from glucose. Wood and Critchley (23) observed that the extracellular polysaccharide synthesized primarily from sucrose is a dextran-like polymer and these investigators have postulated that extracellular polysaccharide synthesis by microorganisms contributes to the formation of dental plaque.

Fitzgerald and Keyes (13, 24) found that hamsters fed a diet containing 56% sucrose, as confectioner's sugar, developed abundant accumulations of coronal plaque and highly active cavitation only after they had been infected with a specific acidogenic streptococcus. Other equally acidogenic streptococci did not colonize on the teeth and form coronal plaques; nor were they associated with enamel demineralization and cavitation. The initial biochemical and fermentation tests did not disclose striking differences between the streptococcal strains which were associated with smooth surface caries and the types which were not. Quantitative determinations of the acid production rate of washed suspensions of caries-conducive and non-conducive streptococci did not reveal any significant differences, but the filament-forming bacterium *Odontomyces viscosus* (25), associated with plaque in the gingival crevice and root caries in hamsters (10), produced about one-fifth the acid released by the streptococci.[6]

Using a streptomycin-resistant mutant strain of caries-conducive streptococci, Krasse (7, 26) showed that sucrose in the diet favored implantation and cavitation in hamsters whereas dextrose did not; lactose also appeared to favor implantation of these microorganisms. Zinner et al. (27), Krasse (28), Gibbons and co-workers (22), and workers [7] at the National Institute of Dental Research (unpublished data) have found that human strains of streptococci which produce extracellular polysaccharides in the presence of sucrose may induce highly active caries in hamsters and gnotobiotic rats fed diets containing this sugar.

Jordan and Keyes (29) reported that microorganisms that are conducive to caries in hamsters and rats will colonize on surfaces of glass, plastic, steel wire, wax, and extracted teeth when they are periodically exposed to an environment containing sucrose and mucin. They did not colonize when sucrose was replaced with glucose and if mucin were not available. Microorganisms which have not been associated with plaque formation and smooth-surface lesions have not colonized in adherent masses under the conditions mentioned above.

Carlsson and Egelberg (30) have described the formation of large gelatinous plaques in human volunteers who did not brush their teeth, who consumed a basal diet of meat and eggs, and who sucked cubes of sucrose at regular intervals during the day. Neither fructose nor glucose consumed in the same manner was conducive to the formation of such deposits. Further

[6] Frostell, G., and O. Larje. Acid production from different carbohydrates in suspensions of rodent oral microorganisms associated with dental caries and periodontal disease (manuscript in preparation).

[7] Robert J. Fitzgerald, Harold V. Jordan and Paul H. Keyes.

work disclosed that the predominant organisms in the plaque deposits which formed on sucrose were streptococci (31).

Hydrogenated potato starch (product 6563) is made of potato starch by partial hydrolysis and hydrogenation at increased pressure and temperature and contains hydrogenated dextrins and disaccharides, and sorbitol. In Sweden this product is being used in the manufacture of low-caloric candies which are claimed to be less conducive to dental caries, or "non-cariogenic." Frostell (32, 33) has reported that the interaction between this product and bacterial plaques from human teeth does not produce as rapid a fall in pH as the interaction between sucrose and similar bacterial deposits.

Hydrogenated starch was not conducive to plaque formation nor to smooth-surface caries in hamsters of rats. However, small lesions had formed in the sulci of the rat molars (fig. 3). This product contains sorbitol which can be slowly fermented by the streptococci associated with dental caries (13). One should hesitate to conclude that this product, potato starch, or sorbitol and starch are "non-cariogenic" carbohydrates.

Since all of the diets used in these experiments contained 28% skim milk powder, they contained approximately 14% lactose. The investigations of Krasse (26) suggest that lactose favors the implantation of caries-associated streptococci. However, the caries-conducive potential of lactose has been difficult to assess, because animals do not remain reasonably healthy when fed diets containing large amounts of this sugar (6, 34). The finding that plaque disappeared from the smooth surfaces of hamster molars when sucrose was replaced and from rat molars when hydrogenated starch was fed suggest that lactose played no more than a supplemental role in smooth surface activity under these conditions. Lactose is not an essential factor for caries activity in either rats or hamsters, as infected animals have developed lesions when casein rather than milk powders has been used as source of protein (6, 35).

The results obtained in hamsters in this study do not appear to be in accord with the findings described by Gustafson et al. (6), who reported cavitation in hamsters fed glucose, fructose, lactose, maltose, and sucrose. But in all of their assays the experimental periods ranged between 70 and 150 days. Moreover, sucrose and fructose were equally conducive to cavity formation and favored more active lesions than the other sugars. There are obviously many differences between the present study and that of Gustafson and co-workers. Our diets were different and the animals harbored plaque-forming streptococci. Under these conditions active smooth-surface lesions and massive coalescence destroyed major portions of the crowns in about 50 days. The relatively low "caries scores" reported by Gustafson et al. (6) suggest that the lesions observed may have been more of the crevicular type. If this were so, their findings would be in accord with the pathosis in albino rats described herein.

In an unusually well-managed study in rats Guggenheim and co-workers (36) evaluated the effect of sucrose substitutes in a diet which contained 25% skim milk powder, 43% wheat flour, and 7% other supplements. The rats had been treated with erythromycin and maintained on a low level of this antibiotic throughout the experiment. At the beginning of the study they were infected with erythromycin-resistant caries-conducive streptococci which produce extracellular polysaccharide, and they were maintained on the experimental regimen for 23 days. In general the findings of Guggenheim et al. (36) coincide with ours. Sucrose was conducive to implantation of the streptococci, to the formation of plaque-like aggregates beneath which demineralization occurred, and to highly active caries both on bucco-lingual surfaces and in the occlusal sulci. Animals that consumed the sucrose substitutes harbored fewer streptococci and developed less plaque, enamel decalcification, and cavitation. The more striking reductions occurred in animals fed fructose, maltose, and starch. Guggenheim and co-workers (36) concluded that on smooth surfaces the aggregation of caries-inducing plaque depends upon the establishment of microorganism producing dextran-type polysaccharide; whereas in the deep narrow fissures the formation of plaque is not essen-

tial for aggregation of microorganisms and the accumulation of acids.

Thus evidence from various sources strengthens the premise that the anatomical locus of carious lesions is related in part to the chemical structure of the carbohydrate retained in the mouth and to the specific type of bacteria present. Unquestionably, sucrose provides a substrate which is especially conducive to plaque formation and smooth surface decay if polysaccharide-producing microorganisms are present. Lesions originating in crevices (pits, fissures, sulci) apparently depend on the interaction between a wider variety of carbohydrates and a broader sprectrum of acidogenic bacteria.

Until recent years few researchers seriously considered Cox's postulation that carious lesions may differ etiologically (1), partly because his suggestion has been difficult to assess experimentally. However, some workers have advocated the induction and evaluation of all types of carious lesions in experimental animals since assay procedures which affect one type of cavity may not affect another (2, 21, 37, 38). It would be unusual to have oral conditions which would favor smooth-surface cavitation and negligible crevice decay, although such findings have been reported in rats (39). However, the teeth of the animals described were not sectioned to reveal sulcal lesions, and in at least one re-examination of such teeth after sectioning, both crevice and smooth surface lesions were equally prevalent (40).

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Toxicity of Red Kidney Beans (*Phaseolus vulgaris*) in the Rat [1]

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ABSTRACT A series of experiments was conducted to obtain information on the etiology of "kidney bean toxicosis." Some of the deaths observed among weanling rats fed high levels of raw kidney beans were attributed to hypoglycemia. Low blood glucose values and brain lesions comparable to those of hypoglycemia as reported from other causes were observed in rats fed diets containing 74% kidney beans. It is suggested the hypoglycemia resulted because raw beans contain factors such as hemagglutinin and trypsin inhibitor that decrease feed intake and feed utilization. Force-feeding of diets containing raw beans to increase intake increased survival time over that of rats fed the diets ad libitum. Limiting the intake of rats fed a control diet to that consumed by rats fed 74% bean diet resulted in low blood glucose values in the control animals. Older rats survived longer than young rats when fed diets containing 74% beans. Autoclaving or soaking the beans in water for 72 hours eliminated the toxic effect.

Diets containing high levels of raw kidney beans (*Phaseolus vulgaris*) are toxic to several species of animals (6, 8, 12, 13, 24). Hemagglutinins toxic to rats when fed at levels as low as 0.5% of the diet have been isolated from kidney beans by Jaffé (14) and Honavar et al. (11). However, the mechanism by which the hemagglutinins produce the toxicosis has not been established. Other factors are present in beans, such as trypsin inhibitors (19), which also may be involved in the toxicosis. Kakade and Evans (16) concluded that additional factors were present in navy beans (also *Phaseolus vulgaris*) that were related to toxicosis observed after rats were fed raw beans.

The studies reported in the present paper were made to obtain further information on the etiology of "kidney bean toxicosis." The effect of the dietary level of raw beans, the treatment of the beans and the age of rats on resistance to kidney bean toxicosis and the effect of kidney bean diets on blood glucose, hemoglobin and brain histology were studied. Digestibility studies also were included.

EXPERIMENTAL AND RESULTS

Experiment 1. Groups of 11 male rats of the Holtzman strain 26 days of age, were assigned at random to one of 4 diets containing zero, 18.5, 37.0, or 74.0% raw kidney beans. The levels of casein, lard and cellulose were adjusted so that all diets were isonitrogenous (26% crude protein), isocaloric (4.3 kcal/g) and contained the same level of crude fiber (3.7%). The compositions of the diets are given in table 1.

TABLE 1

Composition of diets (exp. 1)

	1	2	3	4
	%	%	%	%
Kidney beans	—	18.5	37.0	74.0
Casein	29.0	24.2	19.4	10.0
Sucrose	57.6	45.0	32.4	7.0
Mineral mixture [1]	4.0	4.0	4.0	4.0
Cellulose [2]	3.7	2.8	1.9	—
Vitamin mixture [3]	2.0	2.0	2.0	2.0
Lard	3.7	3.5	3.3	3.0

[1] The mineral mixture contained: (in percent) dipotassium phosphate, 32.2; calcium carbonate, 30.0; sodium chloride, 16.7; magnesium sulfate (hydrate), 10.2; calcium phosphate ($CaHPO_4 \cdot 2H_2O$), 7.5; ferric citrate, 2.75; manganese sulfate, 0.51; potassium iodide, 0.08; copper sulfate, 0.03; zinc chloride, 0.025; and cobalt chloride, 0.005.

[2] Solka Floc, Brown Company, Berlin, New Hampshire.

[3] Vitamin mixture supplied the following/100 g of diet: vitamin A, 400 IU; vitamin D, 44 IU; and (in milligrams) α-tocopherol, 9.0; inositol, 2.2; choline chloride, 75; menadione, 1.0; niacin, 2.0; riboflavin, 0.4; pyridoxine·HCl, 0.2; thiamine·HCl, 0.4; Ca pantothenate, 1.2; and (in μg) biotin, 9; folic acid, 40; and vitamin B_{12}, 0.6.

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TABLE 2

Effect of feeding raw, water-soaked or autoclaved beans on weight gain, diet intake and survival time of rats

Diet no.	Treatment	No. of rats	Avg daily gain [1]	Avg daily diet intake	Avg survival time [1,2]
			g	*g*	*days*
		Experiment 1			
1	0% raw beans	11	3.40 [a]	15.1 [a]	77 [a]
2	18.5% raw beans	11	2.90 [b]	14.5 [a]	77 [a]
3	37% raw beans	11	2.28 [c]	12.6 [b]	64.5 [b]
4	74% raw beans	11	−1.01 [d]	4.6 [c]	9.9 [c]
		Experiment 2			
5	74% water-soaked beans (24 hr)	10	−0.59 [c]	4.0 [b]	9.8 [b]
6	74% water-soaked beans (72 hr)	10	0.31 [b]	6.2 [b]	70 [a]
7	74% water-soaked beans (72 hr) + trypsin	10	0.37 [b]	7.0 [b]	70 [a]
8	74% autoclaved beans [3]	10	2.25 [a]	11.4 [a]	70 [a]

[1] Means in comparable columns with unlike superscripts are significantly different ($P < 0.01$).
[2] Experiment 1 was conducted for 77 days, experiment 2 for 70 days.
[3] At a pressure of 1 kg/cm² for 30 minutes.

All animals were housed individually in wire cages and fed ad libitum. Weight gain, feed intake and survival time of each animal were recorded over an 11-week period.

Rats fed the diet containing no kidney beans gained significantly more weight ($P < 0.01$) than rats in any other group. Also, they consumed significantly more feed ($P < 0.01$) than any other group except rats fed the 18.5% diet (table 2). Rats fed this latter diet consumed more feed and gained significantly more weight ($P < 0.01$) than those fed 37% beans who, in turn, had significantly greater weight gains and feed intake ($P < 0.01$) than the rats fed the 74% bean diet.

Two rats fed the 37% bean diet died on the eighth day, but the other nine lived to the end of the 11-week period. The weight gains (fig. 1) in this group indicate that the rats that survived through 8 days may have become adjusted to the diet, as they gained at an increased rate after day 14. The increased weight gains corresponded to increases in feed intake. All rats receiving the 74% diet died within 14 days; average survival time was 9.9 days. Rats fed this diet developed severe diarrhea a day or two before death and were cyanotic. Several had convulsions immediately before death. At necropsy, no abnormalities other than a general emaciation were observed; the liver, kidney, intestines, adrenal glands and heart were histologically normal.

Experiment 2. Groups of 10 female weanling rats were housed as in experiment 1 and assigned at random to one of four diets. The diets were similar in composition to the 74% bean diet in trial 1 (diet 4, table 1) except for the treatment of the beans. The beans were autoclaved

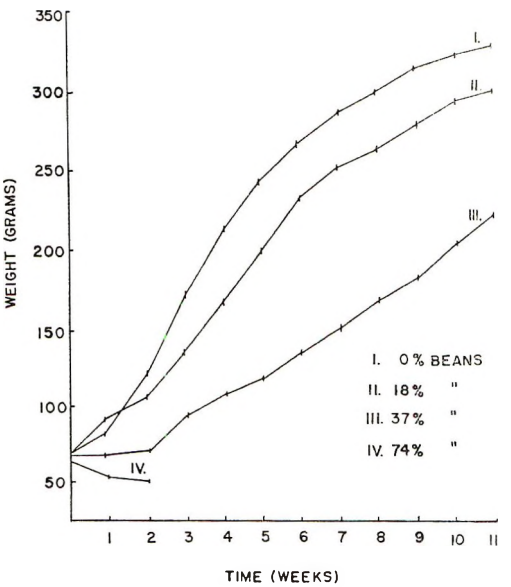

Fig. 1 Effect of level of raw kidney beans (*Phaseolus vulgaris*) in the diet on growth rate of weanling rats.

for 30 minutes at a pressure of 1 kg/cm² (120°) or soaked for 24 or 72 hours. Also, the effect of trypsin supplementation was tested by replacing with trypsin (4 × USP pancreatin) [3] 5% of the sucrose in the diet containing 74% beans soaked for 72 hours. The trial extended for 10 weeks. Weight gain, diet intakes and survival times were recorded.

The weight gains of rats fed autoclaved beans were similar to the expected gains of Holtzman female rats (25). Rats fed diets containing beans soaked in water 24 hours died within 14 days. The rats fed beans soaked in water 72 hours lived, but gained only an average of 22 g in the 10-week period. The addition of trypsin did not significantly affect weight gains. Every rat fed beans soaked 72 hours had a hypertrophic pancreas. The changes in the pancreas were not observed in rats on the other treatments and presumably were a response to trypsin inhibitors (4). Autoclaving destroyed the inhibitor and apparently rats fed beans soaked for only 24 hours did not survive long enough to develop the condition.

Experiment 3. Male rats 26, 36, 46 or 106 days of age were individually fed a diet containing 74% raw beans (diet 4, table 1) ad libitum. The number of animals in each group is shown in table 3.

The 106-day-old rats had a significantly longer survival time ($P < 0.01$) than the younger rats and the 46-day-old rats had a significantly longer survival time than the 36- or 26-day-old rats (table 3).

Experiment 4. Digestibility of the diets fed in trial 1 and of the diets containing 74% autoclaved beans or beans soaked in water for 72 hours with or without trypsin, was determined with adult male rats. Digestibility of autoclaved beans fed alone was also determined. A 5-day preliminary period and a 10-day collection period were used in conventional digestion trial techniques.

The results are summarized in table 4. Increasing the percentage of beans in the diet, soaking the beans in water, or the addition of trypsin did not affect the digestibility of kidney bean protein as calculated by difference. The average digestibility of kidney bean protein was 28.6% for these treatments. Autoclaving the beans increased the digestibility to 72.7%. There was no difference in bean protein digestibility when the diet contained 74% autoclaved beans and 10% casein and when the diet contained 100% autoclaved beans.

Experiment 5. Forty-eight weanling male rats of the Holtzman strain were divided into 8 groups, each group containing 6 rats of almost equal weight (range 2 g). The 6 rats within each weight group were assigned at random to diets as follows: 1) zero percent raw beans, fed ad libitum; 2) 37% raw beans, fed ad libitum; 3) 74% raw beans, fed ad libitum; 4) zero percent raw beans, trio-fed; 5) 37% raw beans, trio-fed; or 6) 74% raw beans, trio-fed. Trio-fed indicates that the amount of feed offered was equivalent to the lowest intake by one of the 3 rats within a weight group assigned to diets 4, 5 or 6. The diets were of the same composition as this in experiment 1 (table 1).

The animals were caged individually, with free access to water and were fed daily. Two animals from each treatment were killed with ether on the third, seventh and tenth day of the trial, except for those receiving the 74% bean diet as none of

[3] Obtained from Nutritional Biochemicals Corporation, Cleveland.

TABLE 3

Effect of age of rats on survival time when fed a diet containing 74% kidney beans [1] *(exp. 3)*

Age of rats, days	26	36	46	106
No. of rats	11	12	13	18
Avg initial wt, g [2]	63.8 [a]	75.0 [b]	128.1 [c]	278.8 [d]
Avg daily diet consumption, g [2]	4.6 [a]	4.2 [a]	7.6 [b]	6.5 [b]
Avg survival time, days [2]	9.2 [a]	9.8 [a]	16.4 [b]	32.0 [c]

[1] Diet 4, table 1.
[2] Means in same line with unlike superscripts are significantly different ($P < 0.01$).

the animals fed the 74% bean diet were alive on the tenth day. Two rats fed each of the zero percent and 37% bean diets were killed after 30 days on trial. Blood samples were taken from the posterior vena cava. Blood glucose was determined by the method described by Somogyi (22) and hemoglobin by the method described by Sanford and Sheard (21). Liver, pancreas, duodenum, kidney, adrenal glands and myocardium were fixed in Bouin's solution and brain in 10% buffered formalin. Paraffin embedding was used and sections of 6 μ were stained with hematoxylin and eosin. Brain sections were stained with cresyl echt violet and with Bodian's protargol, the latter on 20-μ sections.

Blood glucose and hemoglobin levels are shown in table 5. Data from the ad libitum and trio-fed groups receiving the same diets were combined because these were no differences between the groups. Thus the values are averages of 4 samples. The values were tested for significance by the Tukey *hsd* procedure (7).

Hemoglobin levels were not affected by the percentage of beans in the diet, but the beans did cause a significant decrease in blood glucose after 3 days if fed at the

TABLE 4

Digestibility of kidney bean diets by adult rats (exp. 4)

Diet [2]		No. of rats	Apparent digestion coefficients [1]		
			Dry matter	Total protein	Bean protein [3]
			%	%	%
1	0% raw beans	5	91.7 [a]	92.9 [a]	—
2	18.5% raw beans	5	88.2 [a]	79.4 [b]	26.5 [a]
3	37% raw beans	5	82.1 [b]	68.8 [c]	26.0 [a]
4	74% raw beans	11	74.2 [c]	51.8 [d]	32.0 [a]
6	74% water-extracted beans (72 hr)	4	64.5 [d]	48.1 [d]	26.2 [a]
7	74% water-extracted beans (72 hr) + 5% trypsin [4]	4	64.5 [d]	48.8 [d]	27.5 [a]
8	74% autoclaved beans	4	83.6 [b]	82.8 [b]	72.7 [b]
9	100% autoclaved beans	4	81.9 [b]	75.2 [b,c]	75.2 [b]

[1] Means in the same column with unlike superscripts are significantly different ($P < 0.05$).
[2] Diets 1, 2, 3 and 4 from experiment 1, diets 6, 7 and 8 from experiment 2.
[3] Calculated by difference using protein digestion coefficient obtained with diet 1.
[4] Trypsin assumed to be of the same digestibility as casein.

TABLE 5

Effect of raw kidney beans on the blood glucose and hemoglobin levels of rats (exp. 5)

Treatment	Glucose		Hemoglobin, avg
	Avg [1]	Range	
	mg/100 ml serum		*g/100 ml serum*
Rats killed on day 3 of trial (4 samples each)			
0% beans	108 [a]	(91–125)	11.1
37% beans	72 [b]	(64–80)	12.1
74% beans	68 [b]	(55–85)	13.5
Rats killed on day 7 of trial (4 samples each)			
0% beans	97 [a]	(90–115)	12.1
37% beans	78 [b]	(70–89)	11.8
74% beans	48 [c]	(35–76)	11.4
Rats killed on day 10 of trial (4 samples each)			
0% beans	102	(98–108)	14.5
37% beans	105	(98–109)	11.5
Rats killed on day 30 of trial (2 samples each)			
0% beans	125	(114–136)	13.6
37% beans	111	(108–114)	12.4

[1] Values with unlike superscripts are significantly different ($P < 0.01$).

37% or 74% level. By the seventh day, the rats fed 74% beans had significantly lower blood glucose values than those fed the 37% bean diet.

On the tenth day, none of the rats fed the 74% bean diet were alive. There were no differences in blood glucose between the rats fed the 37% diet and those fed the basal diet, although those trio-fed the 37% bean diet did not increase in weight until the fourteenth day.

Feed consumption and changes in body weight are shown in table 6. The addition of beans to the diet resulted in a large decrease in diet consumption and weight gain in the ad libitum-fed group and decreased weight gains when the trio-fed animals were compared.

Histological examination. With the exception of the brain, all organs were histologically normal. Brain changes were observed only in rats fed the 74% bean diet for 7 days.

Telencephalon. The most striking changes were observed in the cortex, notably the layer of large pyramidal cells. Frequent examples of ischemic changes were recorded. Such neurons were markedly shrunken. They stained deep blue with cresyl echt violet and had a triangle nucleus with an eccentrically located, sometimes swollen nucleolus (fig. 2). Cellular response to this change was minimal, although occasional lipid phagocytes were present in immediate association with neurons. Clear-cut examples of detached *boutons terminaux*, the so-called incrustation of the Golgi network, were not seen. Other pyramidal cells had a marked loss of RNA, and gave a diffuse, rather than granular, blue reaction with cresyl echt violet. The only change in the neurons of the basal ganglia was a moderate loss of RNA.

Diencephalon. The changes were similar to those of the basal ganglia.

Mesencephalon. In addition to a diffuse loss of Nissl substance, Nissl's acute cell disease was oberved; many neurons, especially those of nucleus tractus mesencephalici n. trigemini. Such neurons were swollen, with poor affinity for cresyl echt violet. Both the nucleus and nucleolus were eccentric. Neurons of nucleus motorius n. trigemini contained numerous examples of vacuolization of the cytoplasm (fig. 3), a change which usually was combined with a distorted, sometimes triangular, nucleus with an eccentric nucleolus. In other neurons there was central chromatolysis, with the Nissl substance clumped in the periphery. Neuronophagia occurred but rarely.

Metencephalon. Severe, diffuse changes were present in practically all Purkinje cells. Some of these cells had undergone central or peripheral chromatolysis with little nuclear change, but the majority of the Purkinje cells were in an advanced state of degeneration. The cytoplasm was vacuolated or had undergone lysis. The nuclei had decreased staining properties and were swollen or fragmented. Marked neuronophagia was present (fig. 4). There were

TABLE 6

Effect of raw kidney beans on feed consumption and body weight changes of rats (exp. 5)

Treatment	Day killed					
	3		7		10	
	Avg daily intake	Avg daily gain	Avg daily intake	Avg daily gain	Avg daily intake	Avg daily gain
	g	*g*	*g*	*g*	*g*	*g*
Ad libitum						
0% beans	11.3	6.8	13.1	6.1	11.8	6.3
37% beans	7.5	2.8	7.4	3.4	7.2	3.5
74% beans	4.3	−1.3	6.0	−0.6	—	—
Trio-fed						
0% beans	5.0	2.3	5.7	2.1	5.3	1.8
37% beans	5.3	1.2	5.7	1.0	4.8	1.8
74% beans	4.1	−2.0	4.8	−1.5	—	—

changes in the roof nuclei and the nuclei pons. Nissl's acute cell disease was common, especially in pars gigantocellularis of substantia reticularis. The nucleus pontis contained frequent examples of central and peripheral chromatolysis (fig. 5). In other pontile nuclei the neurons had only a decreased affinity for cresyl echt violet, as compared with the controls.

Experiment 6. Twenty-four male weanling rats weighing 45 to 54 g were fed diets containing zero, 37 or 74% beans plus the same components as in experiment 1 (table 1). Four of the 8 rats on each treatment were fed ad libitum. The remaining 4 rats receiving the 74% bean diet were fed via stomach tube to increase intake. The intake of the remaining 4 rats on each of the other treatments was limited to that of the rats fed the 74% bean ration ad libitum. The results are summarized in table 7. The experiment was stopped after

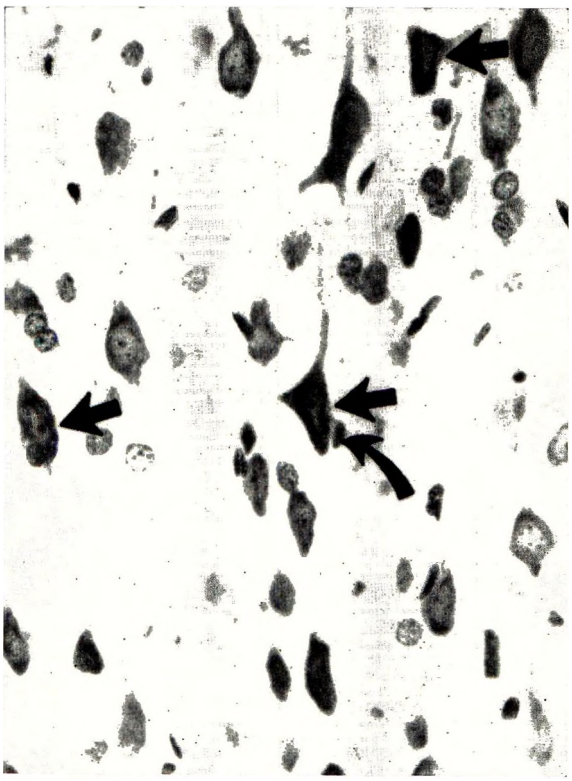

Fig. 2 Brain section from rat fed 74% kidney bean diet. Parietal cortex, layer of large pyramidal cells. Ischemic cell change in neurons at straight arrows. Lipid phagocyte at curved arrow. Cresyl echt violet. × 450.

TABLE 7

Effect of kidney beans on survival time of trio-fed and ad libitum fed rats (exp. 6)

Treatment	Avg daily intake	Avg survival time	Avg daily gain	Mortality
	g	*days*	*g*	*%*
0% beans, ad libitum	11.0	15 [1]	3.57	0
0% beans, limited	2.1	5.6	−1.25	100
37% beans, ad libitum	7.3	14.6	0.07	25
37% beans, limited	2.0	6.7	−1.70	100
74% beans, force-fed	4.8	9.3	−1.46	100
74% beans, ad libitum	2.0	4.2	−2.27	100

[1]Experiment stopped after 15 days.

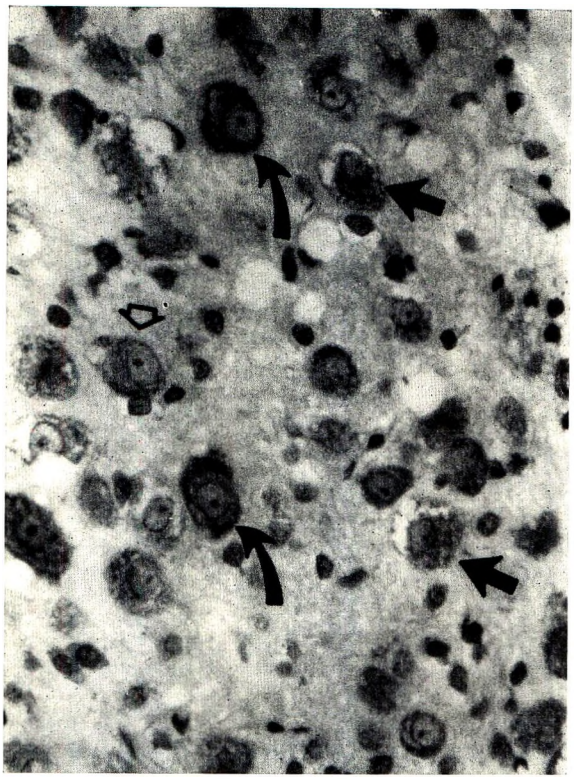

Fig. 3 Brain section from rat fed 74% kidney bean diet. Nucl. motorium n. trigemini. At straight arrows, vacuolization of neuronal cytoplasm, eccentrically located nucleoli; at curved arrows, central chromatolysis; at unfilled arrow, neuronophagia. Cresyl echt violet. × 450.

15 days because all the rats fed the 74% diet were dead.

When the rats fed the zero and 37% bean diets were limited to the same daily intake as the rats fed the 74% bean diets ad libitum, mortality was 100% by 15 days. All rats fed the zero percent bean diet ad libitum survived and gained weight. Three of the 4 rats fed the 37% bean diets ad libitum survived, but only one gained weight. Force-feeding to increase average daily intake from 2.0 to 4.8 g significantly increased average survival time ($P < 0.05$) of rats fed the 74% bean diet, but did not prevent mortality or weight loss. Blood samples were taken via heart puncture from rats that appeared about to die. Samples were obtained from 2 rats fed the zero percent diet (limited intake), 2 rats fed the 37% diet (limited intake), 3 rats fed the 74% diet (ad libitum), and one rat fed the 74% diet (force-fed). Blood samples also were taken at 15 days from the rats fed zero percent and 37% bean ad libitum diets. All samples from rats that appeared about to die contained low levels of blood glucose regardless of dietary treatment. The values ranged from 7 to 11 mg glucose/100 ml blood serum. The average blood glucose value of the 4 rats fed the zero percent bean diet ad libitum was 88 mg/100 ml serum. The average glucose value of the 3 rats fed the 37% bean diet ad libitum was 59 mg/100 ml serum.

DISCUSSION

Several factors may be related to the poor growth and high mortality rate observed when rats were fed diets containing high levels of kidney beans. For example, beans are low in methionine (3) and the methionine present may not be efficiently

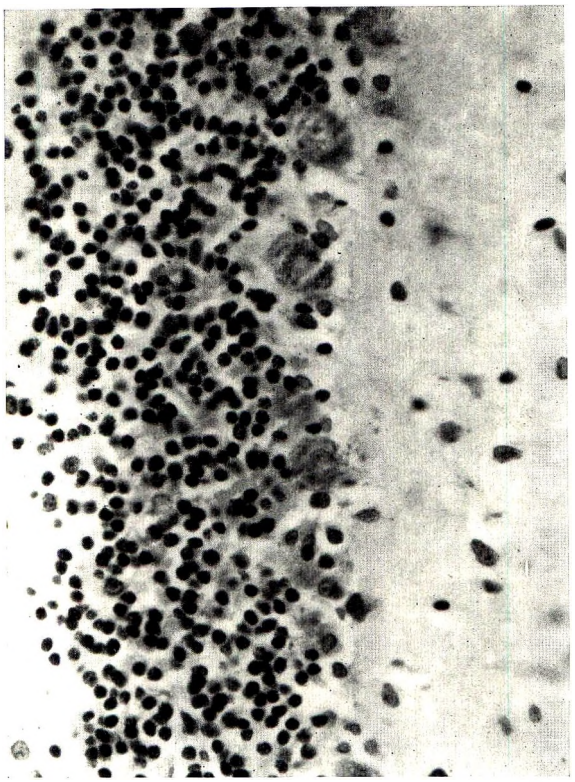

Fig. 4 Brain section from rat fed 74% kidney bean diet. Cerebellum, Purkinje cell layer. Advanced nuclear and cytoplasmic chromatolysis of Purkinje cells; neuronophagia. Cresyl echt violet. × 450.

utilized because of trypsin inhibitors (2). However, Kakade and Evans (15) reported that supplementation of methionine to raw beans did not prevent mortality.

Raw kidney beans contain factor(s) that increase the amount of vitamin E required to prevent muscular dystrophy in chicks (9) but the addition of α-tocopheryl acetate at levels up to 2500 ppm did not increase survival time of rats fed a 74% bean diet.[4] Trypsin inhibitors decrease weight gains, but neither Borchers and Ackerson (5) nor Jaffé (12) found any obvious correlation between trypsin inhibitor content of beans and their toxic effects.

Our results indicate that at least some of the toxicosis observed in rats fed beans was caused by hypoglycemia. Low blood glucose levels were noted in several rats before death. Brain lesions in rats fed 74% kidney bean diet were comparable to those associated with hypoglycemia reported in other species from other causes, including spontaneous or experimental hyperinsulinism, accidental hyperinsulinism, schizophrenia and morphinism, hepatic disease and lesions in the brain stem and hypothalamus. Reviews on this subject have been published by Baker (1), Hoff et al. (10) and Meyer (20). It is generally agreed that in hypoglycemia the predominating neuronal lesion is a homogenizing change. The cortex, corpus striatum, and hippocampus are considered sites of predilection, although, as has been pointed out by Lawrence et al. (18), very few nerve cells are entirely normal. In the present study the homogenizing change of neurons was rare; the predominate changes were the presence of Nissl's acute cell disease and the ischemic change. This may be related to the acute course. Terplan (23) reported

[4] Hintz, H. F., and D. E. Hogue, unpublished data, Cornell University, 1964.

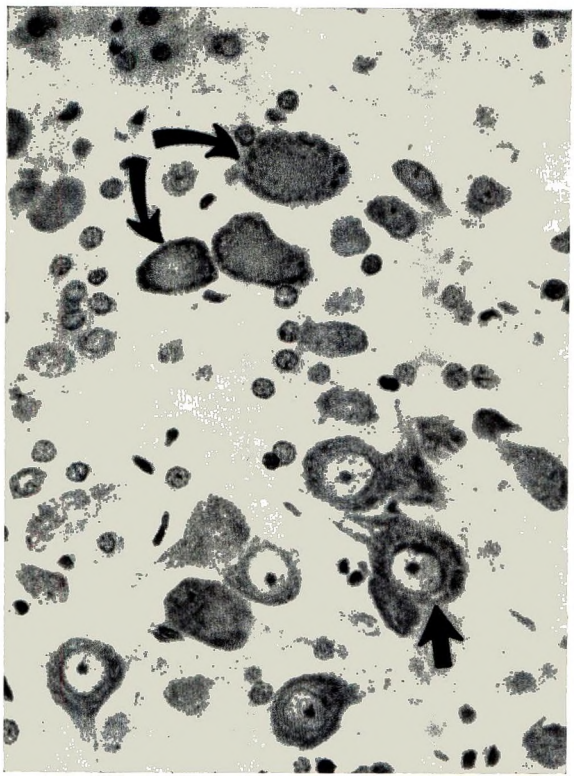

Fig. 5 Brain section from rat fed 74% kidney bean diet. Nucl. pontis. At curved arrows, central chromatolysis; at straight arrow, peripheral chromatolysis. Cresyl echt violet. × 400.

on the neuropathology of a young diabetic boy who died from hypoglycemia 3 days after an overdose of insulin. Nissl's acute cell disease was common and the homogenizing cell change was not described. Further, in dogs with metastasizing insular carcinoma and in which clinical symptoms had been present for several months, Krook and Kenney (17) noted the ischemic change to be more common than the homogenizing one. In agreement with the standard patterns of hypoglycemia, in our work the cortex had extensive lesions. In contrast, corpus striatum and hippocampus, had a diffuse loss of Nissl substance.

It is suggested that the hypoglycemia resulted because of decreased food intake and decreased utilization of nutrients. The addition of raw beans at levels greater than 18.5% decreased intake. Adult rats digested only 29% of the bean protein and young rats probably digested less, as they often developed diarrhea. Jaffé (14) reported the hemagglutinin fraction of raw kidney beans combined with the mucosa of the intestinal wall and interfered with nutrient absorption.

In experiment 5 the intake of rats fed diets containing zero or low levels of beans was limited approximately to that consumed by rats fed diets containing 74% beans. Hypoglycemia was observed in rats fed the 74% bean diet, but not in the other rats. However, the actual amount of energy absorbed by the rats fed the lower-level bean diets probably was considerably higher than that for the rats fed 74% beans even though equalization was attempted. The control rats consumed 1 to 1.5 g more feed per day and the digestibility of the low-bean diets was higher than that of high-bean diets. The control rats (trio-fed) gained weight whereas the rats fed kidney beans lost weight.

In experiment 6, hypoglycemia was observed in rats fed the control diet but lim-

ited in intake to that of rats fed the diet containing 74% beans. Force-feeding the 74% bean diet increased intake and survival time.

The longer survival time of adult rats fed kidney beans observed in experiment 3 could have been the result of greater energy reserves rather than increased resistance to toxicity.

It is interesting that the concentration of beans in the diet was more important in the development of toxicosis than total bean intake. In experiment 1, rats fed a diet containing 37% beans consumed 4.7 g of beans per day and the mortality was 18%. But rats fed a diet containing 74% beans consumed only 3.5 g beans per day and the mortality was 100%.

Honavar et al. (13) and Jaffé (12) reported that soaking kidney beans in water before autoclaving was necessary to destroy the growth-depressing factors. In contrast, autoclaving alone appeared to be satisfactory in the present studies. Kakade and Evans (16) concluded that soaking before heating is not necessary to eliminate the toxicity of navy beans and suggested that the difference between their results and those of Honavar et al. (13) and Jaffé (12) may have been due to differences in bean varieties.

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Relative Magnesium Deficiency in the Rat

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ABSTRACT In experiments originally designed to study vitamin E deficiency, pathological lesions consistent with magnesium deficiency were produced in male Fisher 344 rats and these were subsequently studied. The diet used contained, by chemical analysis, 1.16% calcium, 0.95% phosphorus, and 0.019% magnesium. Although the magnesium-deficient rats were asymptomatic and had normal weight gains, early in the experiment renal and cardiac calcification were produced. When the diet was supplemented to a magnesium level of 0.064%, no cardiac or renal lesions were found. The renal lesions, although comparable to previously described lesions of magnesium deficiency, had a more intense inflammatory reaction and abundant foreign body giant cells. Prominent noninflammatory proliferating lesions of the lining cells unaccompanied by inflammation were seen in the collecting tubules. The myocardial calcification was only rarely accompanied by an inflammatory reaction. No difference was seen between the cardiac and renal lesions found in magnesium-deficient and magnesium-deficient, vitamin E-deficient rats. The muscular degeneration of vitamin E deficiency was apparently intensified by magnesium deficiency. Depigmentation of the lower incisors beginning after 6 weeks occurred regularly in the vitamin E-deficient, magnesium-deficient animals.

The pathological lesions of magnesium deficiency in the rat have been described extensively (1–7). In most instances these experiments were performed using diets containing 0.2 to 0.6% calcium, 0.2 to 0.5% phosphorus and less than 0.002% magnesium. Since the dietary requirements of these 3 elements are interrelated, it is possible to produce magnesium deficiency with higher levels of dietary magnesium (8–11). Magnesium deficiency, with 0.019% magnesium in the diet, occurred during an experiment originally designed to study the effect of vitamin E deficiency on testicular chromosome morphology. The renal and cardiac lesions produced differed from those usually described for magnesium deficiency and were subsequently studied. These lesions, as well as the lesions of combined magnesium and vitamin E deficiencies, are the subjects of the present report.

EXPERIMENTAL

One hundred and twenty male weanling Fisher 344 rats,[1] initial weight 50 to 75 g, were divided into 3 groups. Group 1 (20 animals) was fed a standard laboratory diet,[2] group 2 (20 animals) was fed a low magnesium diet, and group 3 (80 animals) was fed a low magnesium, vitamin E-deficient diet (table 1). The animals were kept two in a cage in an air conditioned animal room, temperature 22°, and fed pelleted forms of the diet and tap water ad libitum. Periodically, after intervals ranging up to 7 months, animals were killed by cervical dislocation. The tissues were fixed in Bouin's solution or in 10% formalin and were routinely stained with hematoxylin and eosin and in selected cases with Von Kossa, Alizarin red or periodic acid Schiff stains.

In a second experiment 30 male Fisher weanling rats, and a small group of female Fisher rats, were fed the low magnesium, normal vitamin E diet and were killed at intervals ranging from 18 hours to 33 days to observe the histogenesis of the renal lesions.

Before the etiology of the lesions was established there was a question of possible strain specificity of the lesions; therefore, the low magnesium diet was given to small numbers of male rats of the following strains: NIH Sprague-Dawley, NIH Osborne-Mendel, and Holtzman.[3]

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[1] Microbiological Associates, Walkersville, Maryland.
[2] Purina Laboratory Chow, Ralston Purina Company, St. Louis.
[3] Holtzman Company, Madison, Wisconsin.

TABLE 1

Composition of basic diet [1]

	%
Vitamin-free casein [2]	22.0
Stripped lard [3]	5.0
Sucrose	66.0
Salts [4]	6.0
Vitamin mixture [5]	1.0

[1] The diet, as formulated, successfully produced vitamin E deficiency in the experiments of Bieri and Prival (12). The diet was compounded and pelleted by the Nutritional Biochemicals Corporation, Cleveland.

[2] Nutritional Biochemicals Corporation.

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

[4] Fox, M. R. S., and G. M. Briggs 1960 Salt mixtures for purified-type diets. III. An improved salt mixture for chickens. J. Nutr., 72: 243; obtained from Nutritional Biochemicals Corporation; stated composition: (in %) $CaHPO_4$, 2.84; $CaCO_3$, 1.0; $NaHPO_4$, 0.7; NaCl, 0.4; KCl, 0.7; $MgSO_4$, 0.3; $FeC_6H_5O_7$, 0.02; $MnSO_4$, 0.025; KIO_3, 0.001; $ZnCO_3$, 0.013; $CuSO_4$, 0.001.

[5] Contained per 45.4 kg diet: (in grams) ascorbic acid, 45.0; inositol, 5.0; choline chloride, 75.0; menadione, 2.25; *p*-aminobenzoic acid, 5.0; niacin, 4.5; riboflavin, 1.0; pyridoxine·HCl, 1.0; thiamine·HCl, 1.0; Ca pantothenate, 3.0; (in milligrams) vitamin A palmitate (gelatin coated), 22.8; biotin, 20.0; folic acid, 90.0; vitamin B_{12}, 1.35; and vitamin D, 100,000 IU/45.4 kg diet. In the non-vitamin E-deficient animals: α-tocopherol, 5.0 g/45.4 kg diet (Vitamin Diet Fortification Mixture, Nutritional Biochemicals Corporation).

Since the renal lesions which were produced occurred in all animals after a week of the low magnesium diet, a final set of experiments was performed with 4 to 5 Fisher rats in each group. The diet groups included (a) the magnesium-deficient diet in a powdered form (basic diet), (b) the basic diet with the substitution of regular lard for stripped lard, (c) the basic diet using Fox-Briggs salt mix at a 4% level instead of at a 6% level with the difference made up as sucrose, and (d) the basic diet with the addition of anhydrous magnesium sulfate to make a final magnesium concentration of 0.064% in one group of rats, and of 0.109% in another group of rats.

Samples from the 8 different lots of the diets used in the 4 sets of experiments were taken at random from the individual drums of the diet mix and portions of these specimens were ashed and analyzed from calcium, phosphorus and magnesium (13).[4] Dialuric acid hemolysis tests (14) were performed to evaluate vitamin E deficiency. The vitamin D level of the diets was determined [5] by bioassay in the rat.

Muscle lesions were scored in the manner of West and Mason (15) after a 1-cm^2 area of psoas muscle was examined. Degeneration and regeneration were scored on a scale of one to four plus. A rating of four plus was given to those sections showing the most severe lesions while one plus expressed at least one area of degeneration or regeneration. The score for each muscle expressed a subjective average for the entire section.

RESULTS

Diet analysis. The results of the diet analysis (table 2) indicated that the amount of magnesium in the diet was considerably less than that specified for the diet. The reason for this is uncertain. The vitamin D level was measured in 2 lots of the diet and was within the specified limits.

Symptomatology and growth. There was no definite symptomatology such as peripheral vasodilatation, excitability, convulsions, or diarrhea although a rare animal developed thinning of the nuchal hair for a 2-week period near the beginning of the experiment. For the first 3 months there was no difference in the weight gain between the animals in groups 1, 2, and 3. During the initial 3 weeks of the experiment the Fisher rats gained on the average 5.1 g a day, whereas the larger Sprague-Dawley rats gained on the average 5.9 g while fed the low magnesium diet. After

[4] Bio-Science Laboratories, Van Nuys, California.

[5] Wisconsin Alumni Research Foundation, Madison, Wisconsin.

TABLE 2

Mineral composition of diets with relative magnesium deficiency

	Calcium	Phosphorus	Magnesium
	g	*g*	*mg*
Observed level/100 g diet	1.16 ± 0.03 [1]	0.95 ± 0.03	19 ± 0.7
95% confidence limits of observed level	1.09 to 1.23	0.88 to 1.02	17.3 to 20.7
Expected level/100 g diet [2]	1.24	0.80	60

[1] Each value represents the mean ± SE of mean of the analysis of samples from 8 different lots of the diets.

[2] Calculated values from the diet as formulated and calculated by Fox, M. R. S., and G. M. Briggs 1960 Salt mixtures for purified-type diets. III. An improved salt mixture for chickens. J. Nutr., 72: 243.

several months some animals in groups 2 and 3 had marked weight loss and when killed these animals invariably had marked renal changes.

Renal pathology. Medullary calcification was noted in the kidneys of all of the animals fed magnesium-deficient diets. There was no significant difference between the lesions produced with the vitamin E-deficient, low magnesium diet or the low magnesium diet alone, in animals receiving regular lard instead of stripped lard, in animals fed the diets containing the salt mix at the 4% level instead of at the 6% level, among the different strains of rats tested, or among littermates fed the diet for the same length of time. The lesions were not present in the stock-fed animals or in the animals whose basic diet was supplemented with magnesium.

The renal lesion seen in the rats varied slightly from the renal pathology usually described in magnesium deficiency. After 6 days calcifications were distributed throughout the inner stripe of the medulla with a preponderance near the corticomedullary junction. Lesions in various stages of development were present after 8 days and commonly foreign body giant cells surrounded by intense fibrotic reactions were present (figs. 1–3). After 2 weeks the inner medullary zone contained many collecting tubules with calcium and most of these had no, or at most a minimal, inflammatory reaction. Corticomedullary calcification was especially prominent at 3 weeks (figs. 4 and 5).

After one month the kidneys appeared enlarged, grey-brown, nodular, and contained small cortical cysts. Microscopically, the inner stripe and the lower portion of the outer stripe contained confluent inflammatory masses. The changes progressed slowly after that time (fig. 6).

In the portions of the collecting tubules within the inner stripe, an unusual collection of cells was seen after one month. These areas consisted of groupings resem-

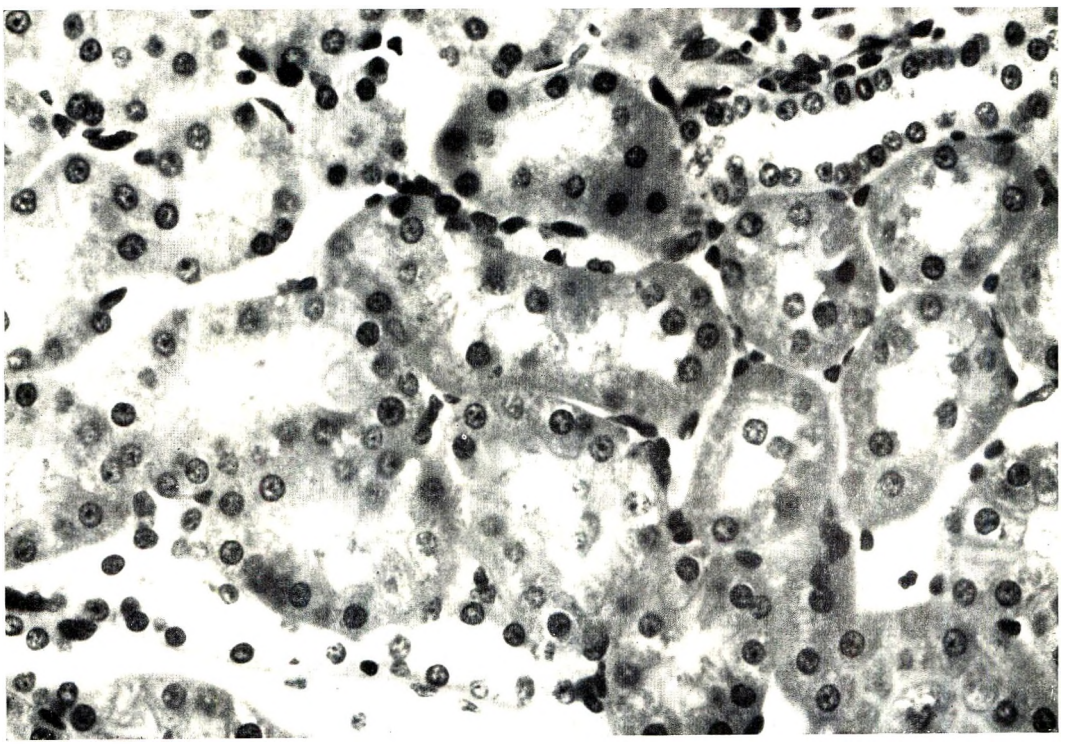

Fig. 1 An area of cortex from a normal rat kidney with proximal tubules predominating. H&E. × 336.

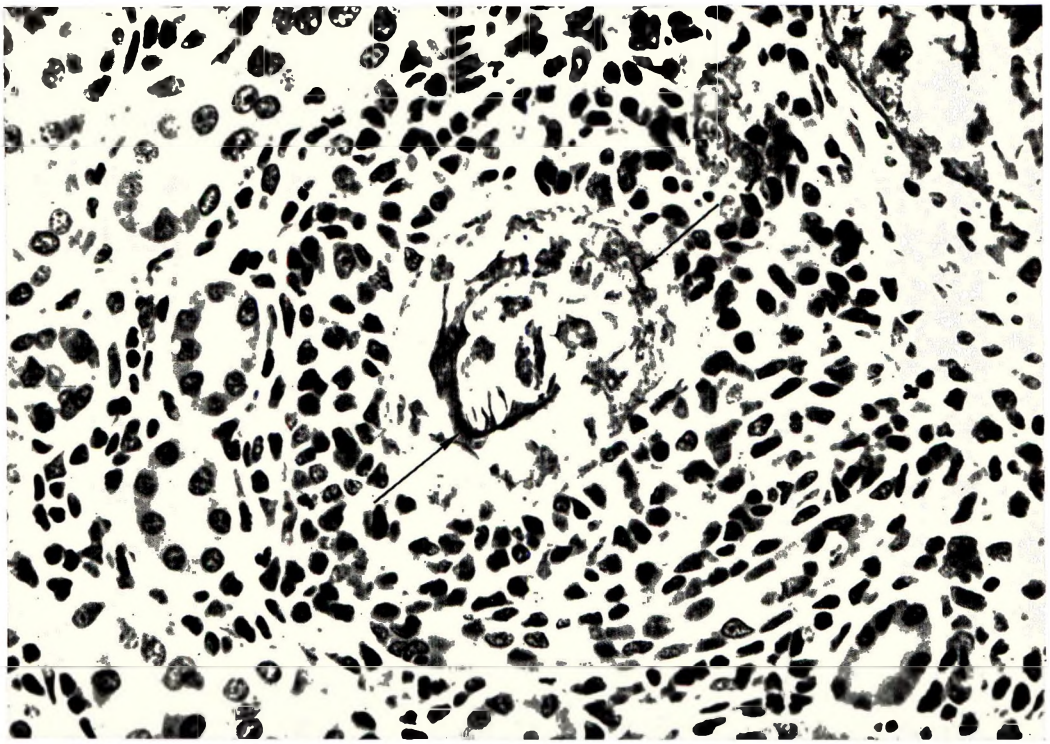

Fig. 2 Rat kidney after 8 days of a magnesium-deficient diet. In the outer medulla an inflammatory reaction with round cells predominating is surrounding a calcified area (arrow). H & E. × 336.

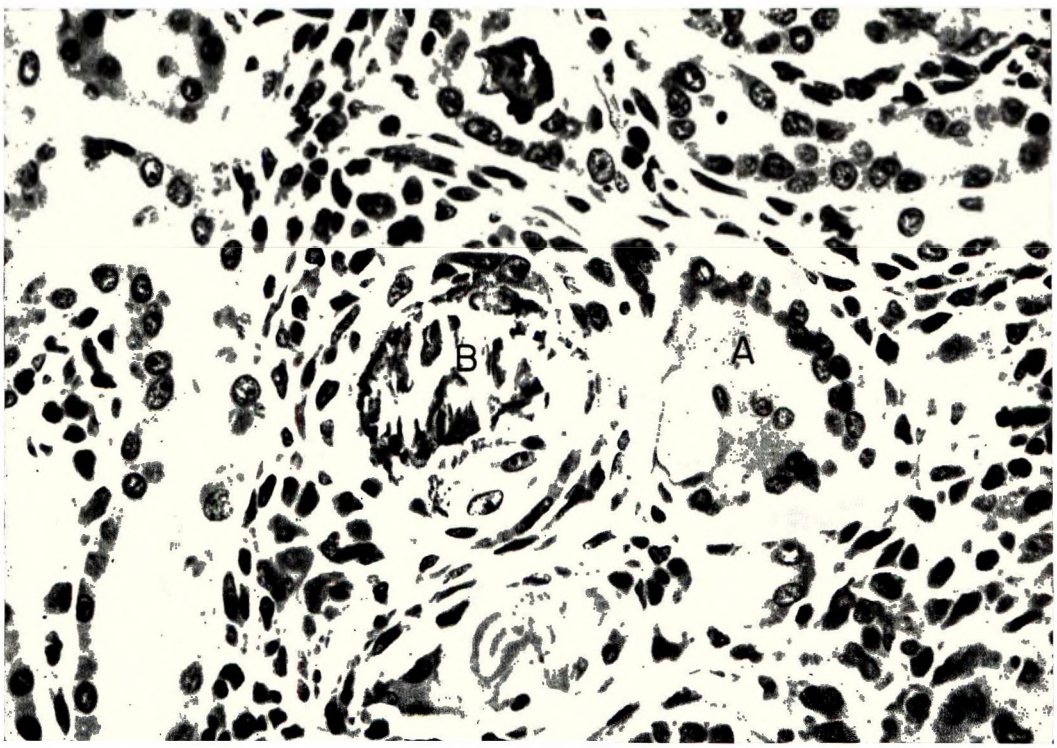

Fig. 3 Kidney after 8 days of a magnesium-deficient diet. A small giant cell (*A*) with many peripherally located nuclei is adjacent to a calcium filled tubule (*B*). H & E. × 336.

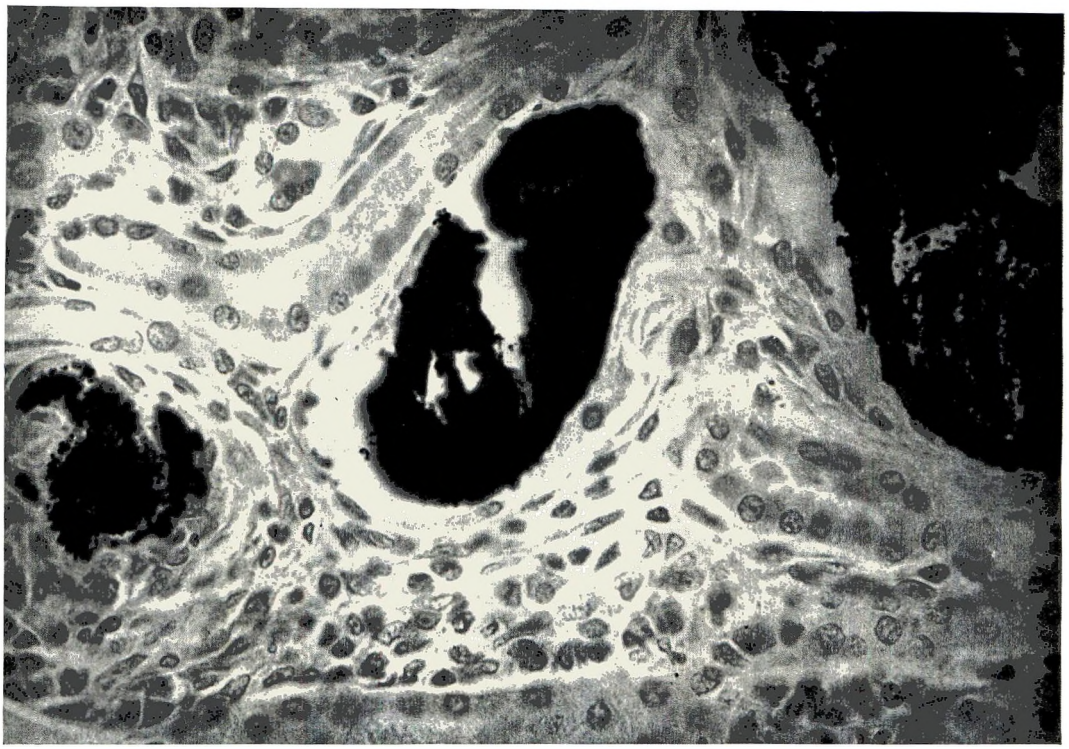

Fig. 4 Kidney after 19 days of a magnesium-deficient diet. Intraluminal calcifications (black areas) may be seen. Von Kossa. × 336.

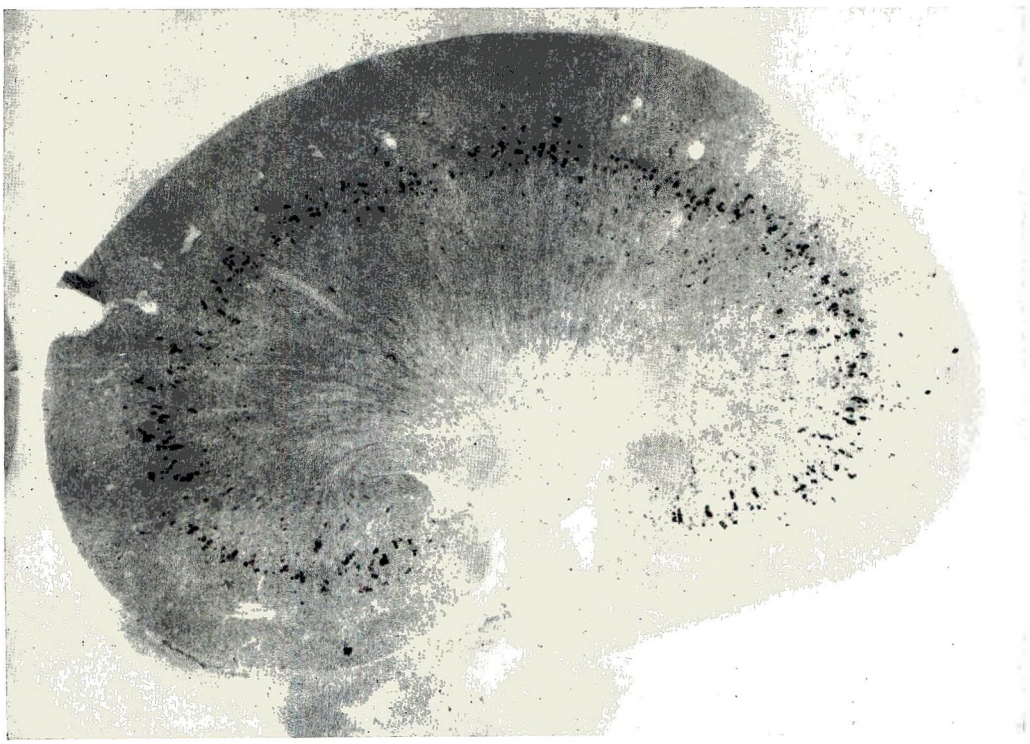

Fig. 5 Kidney after 19 days of a magnesium-deficient diet. Calcification (black areas) is present throughout the outer medulla and is especially dense near the corticomedullary junction. Von Kossa. Approx. × 5.

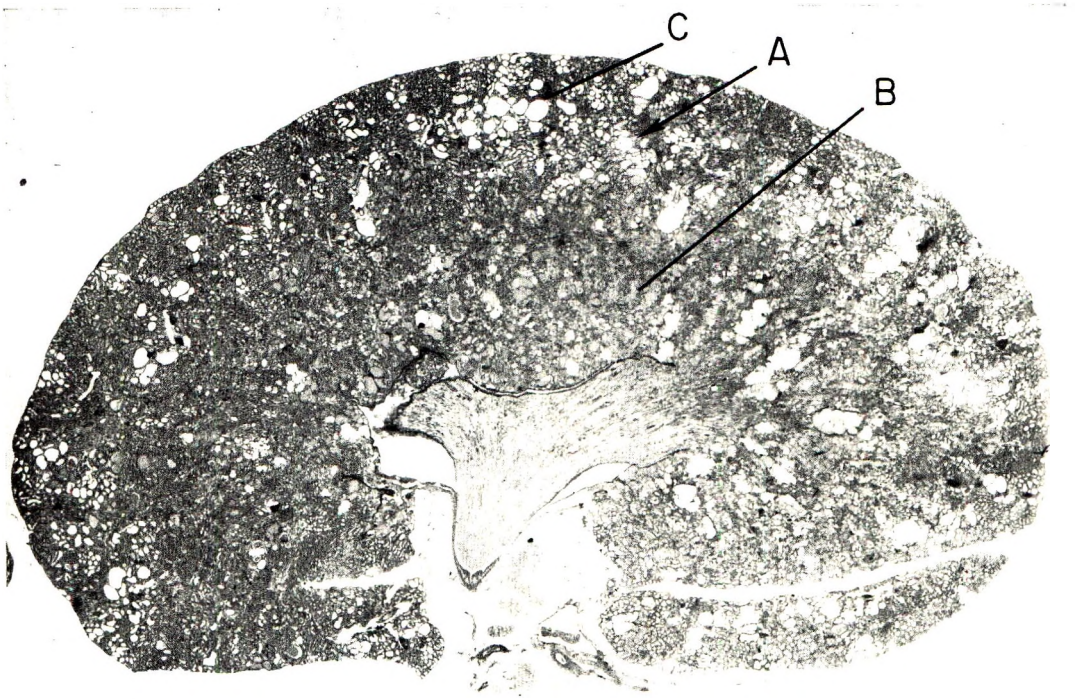

Fig. 6 Kidney after 6 months of a magnesium-deficient diet. There are areas of tubular dilatation (*A*), and some tubules filled with colloid (*B*). The darker areas (*C*) are calcification under higher power. H & E. Approx. × 5.

bling up to 30 closely packed, epithelial cell nuclei (figs. 7–9). The nuclei appeared to be identical to those of the collecting tubular cells, and did not appear to be those of intercalated cells. These collections of nuclei often bulged slightly into the tubular lumen. Not infrequently, four or five different-size groups of such nuclei were seen in one section of the same tubule. Even after colchicine administration, no mitoses were seen in these nuclear collections. These groups of nuclei were often associated with one or two small, round cells.

These cells, probably lymphocytes, had a scant cytoplasm and stained heavily with nuclear stains. They were rarely seen in the normal rat kidney but were seen with regularity either singly or in small groups in the lumen of affected tubules, or associated with the collections of nuclei in the collecting tubules.

Cardiac pathology. Small foci of myocarditis, as previously described in the Fisher 344 rat (16), were seen in 75 to 90% of the animals older than 6 months (table 3, figs. 10 and 11). Since only one section of the heart was examined for each animal, it is not possible to attach much significance to the difference in the frequency of these lesions in groups 1, 2, and 3.

A second type of cardiac lesion was seen in animals fed the low magnesium diet for 3 to 4 weeks, and after 6 months this lesion was found in 75% of the hearts examined. Grossly, these areas appeared as yellow streaks in the myocardium. Microscopically, they consisted of areas of myocardial calcification usually without inflammatory cells being present (figs. 12 and 13). These affected regions ranged in size from one or two fibers to almost one-half of the myocardium, were often subendocardial in location, and were positive for Von Kossa, Alizarin red, and PAS. Sometimes the calcified regions were adjacent to areas of myocarditis, but usu-

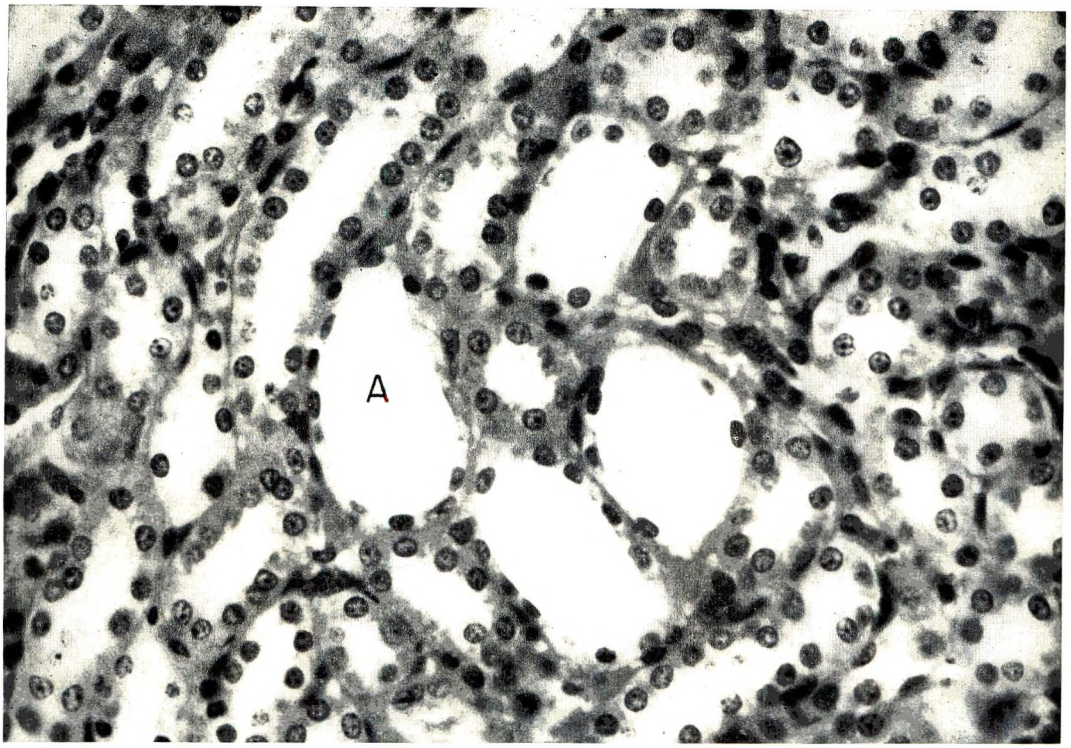

Fig. 7 Section of the outer medulla of the kidney of a rat fed the stock diet for 4 months. Collecting tubules predominate although other tubules probably portions of the loops of Henle (*A*) are present. H & E. × 336.

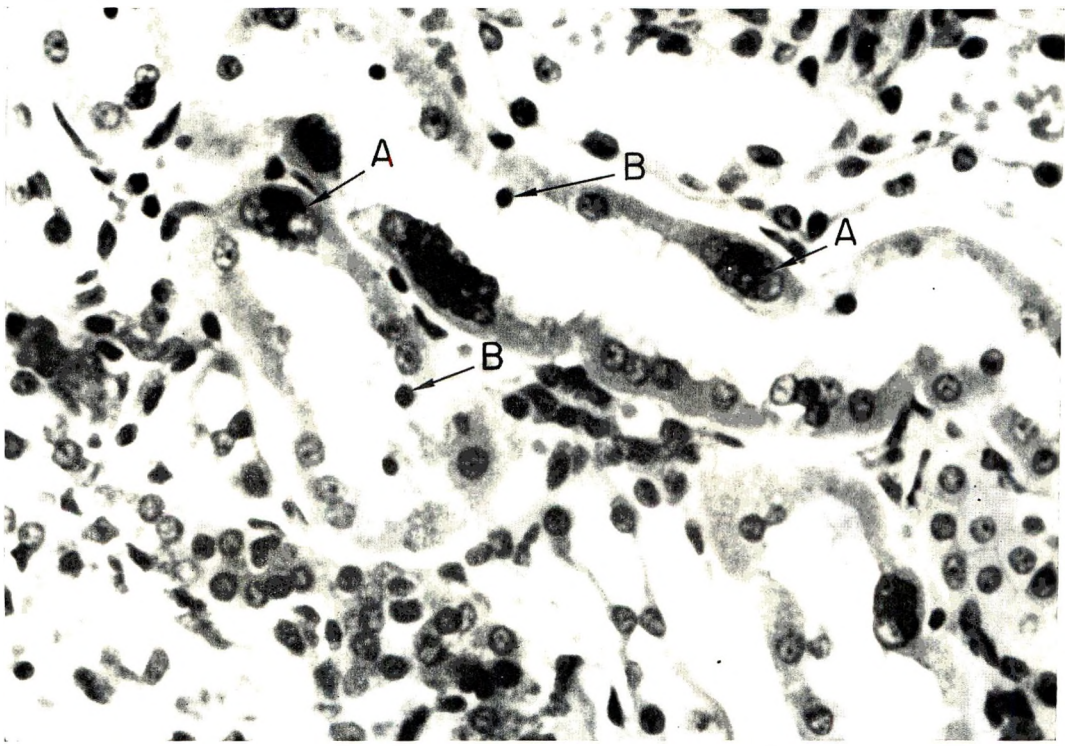

Fig. 8 Kidney after 4 months of a magnesium-deficient diet. A longitudinal section of collecting tubule contains several regions with closely packed grape-like bunches of nuclei (*A*). Darkly staining small lymphocytes (*B*) are near the lumen of the tubule. H & E. × 336.

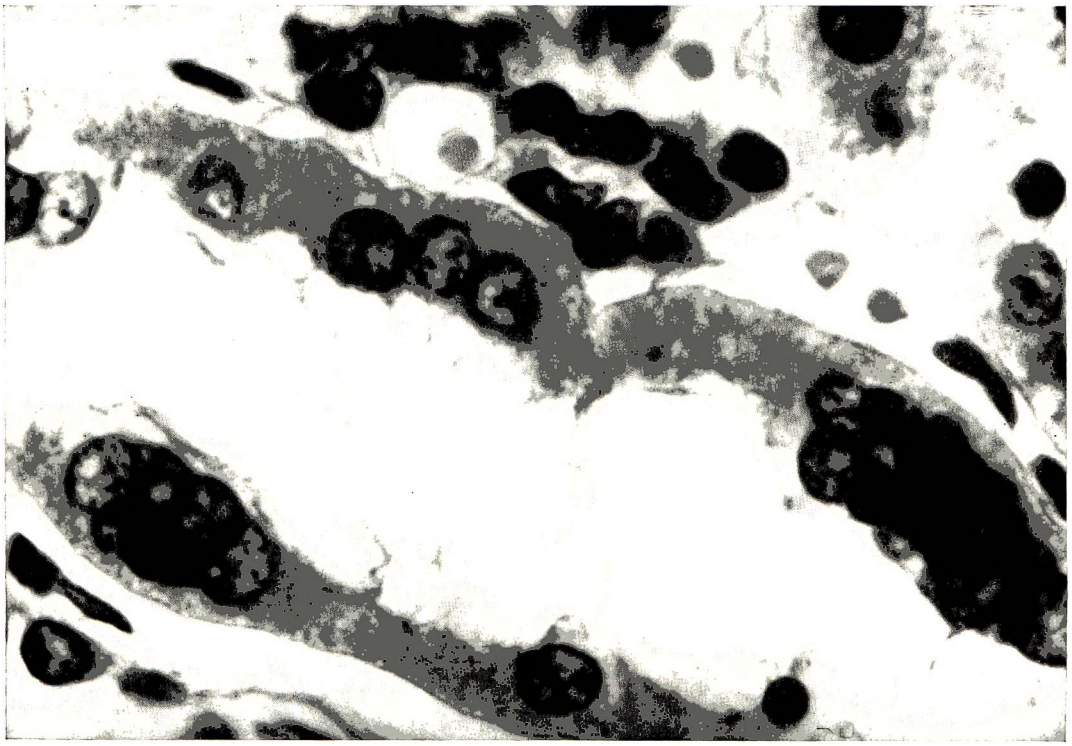

Fig. 9 A portion of the same section as in figure 9, in higher power. H & E. × 865.

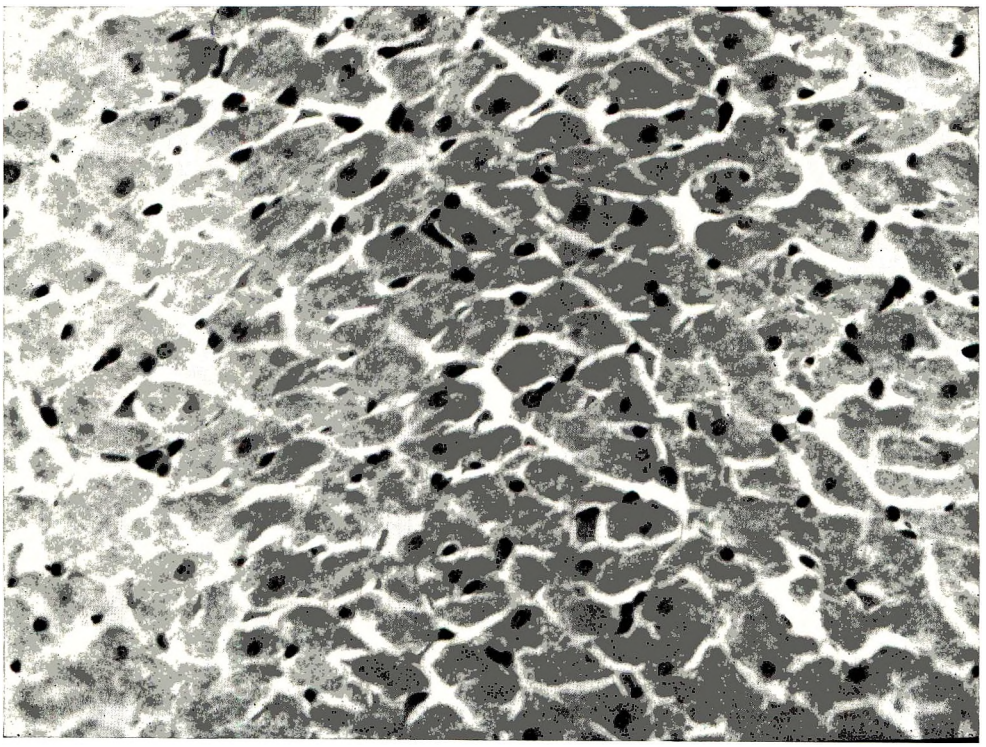

Fig. 10 A section of normal rat heart from an 8-month-old Fisher 344 rat. The pale, large, oval nuclei of the cardiac cells contrast with the interstitial cell nuclei. H & E. × 336.

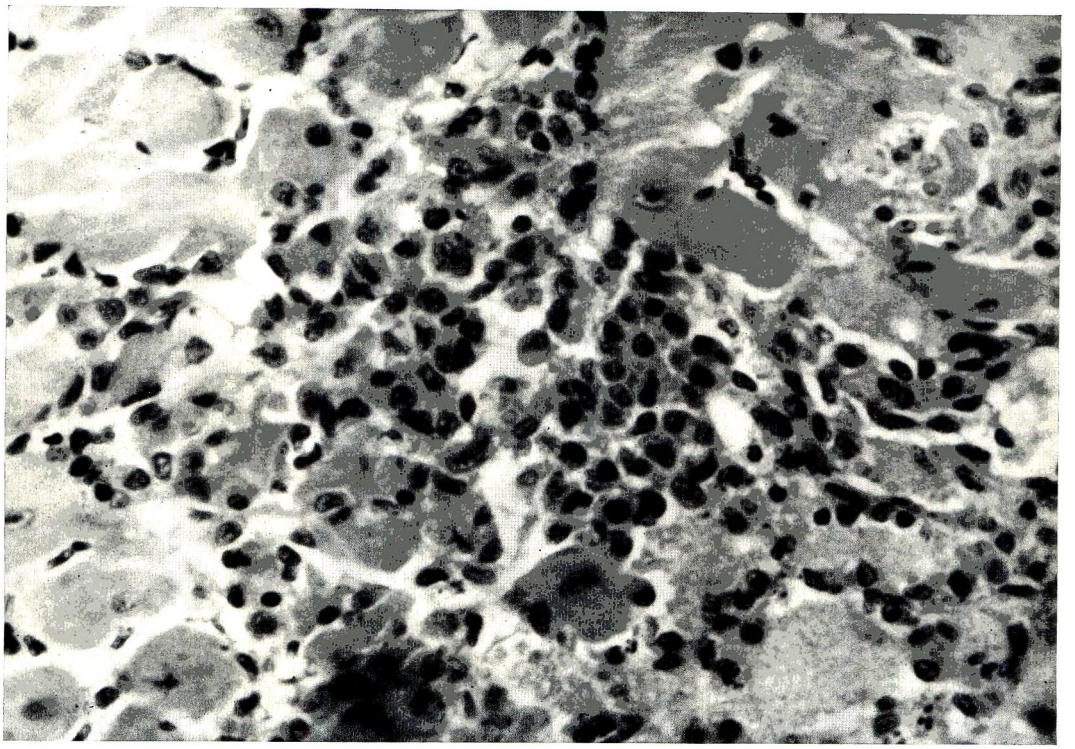

Fig. 11 Heart from an 8-month-old Fisher 344 rat. An area of myocarditis is present. H & E. × 319.

TABLE 3

Types of cardiac lesions found after 7 months of dietary deficiency in Fisher 344 rats

Diet	No. of hearts examined	No. with lesions and type of lesion	
		Myocarditis	Myocardial calcification
Stock	10	9	1
Low magnesium	16	10	11
Low magnesium, vitamin E-deficient	44	34	20

ally there was no relationship between the 2 kinds of lesions, nor was there a difference between the lesions in the vitamin E-deficient or the vitamin E-supplemented animals. Similar calcified areas were present in Sprague-Dawley rats fed the low magnesium diet.

Muscle pathology. Samples of the left psoas muscle were examined after week 24 and until week 29 of the experiment. Stock-fed and the low magnesium-fed animals had no definite muscle lesions. The rats fed the low magnesium, vitamin E-deficient diet had 2 kinds of muscle lesions. One was the muscle degeneration usually associated with vitamin E deficiency ((17) and figs. 14 and 15). The lesions were scored semi-quantitatively and there was no increase in their severity between 24 and 29 weeks of the deficiency diet.

In addition, about 30% of the animals with the typical lesions of vitamin E deficiency had a more intense degenerative lesion associated with short, yellow streaks in the muscles. These areas were often adjacent to those lesions due to vitamin E deficiency, and contained clumps of calci-

Fig. 12 Heart after 5 months of a magnesium deficient diet. The myocardium is diffusely calcified (dark black staining areas). Von Kossa. Approx. × 5.

fication of varying sizes and forms without the regular patterns of the renal calcifications (fig. 16). Regenerating muscle buds, areas of mild to moderate fibrosis, and enlarged sarcolemmal nuclei were associated with these calcifications. After colchicine administration, many mitoses were noted in these areas. These calcified lesions were seen in those animals which had the more highly graded degenerative lesions (table 4). This association was not statistically significant.

Teeth. The teeth of the animals fed the stock diet and the low magensium diet were grossly normal. In the low magnesium, vitamin E-deficient animals there was depigmentation of the lower incisors beginning after 6 weeks, followed by upper incisor depigmentation beginning after 4 months. The consistent and early involvement of the lower incisors is unusual in vitamin E deficiency (18, 19) and is not seen in magnesium deficiency (20, 21). It may be related to the combined deficiencies, the strain of rat used, or the amount of fat in the diet (18). The dialuric acid test was positive in the animals from group 3, and negative in the group 1 and 2 animals tested after 22 weeks of the experiment.

Other findings. The testes of some animals had degeneration of the seminal epithelium, including giant cell formation after being fed the vitamin E-deficient diet for 7 months.

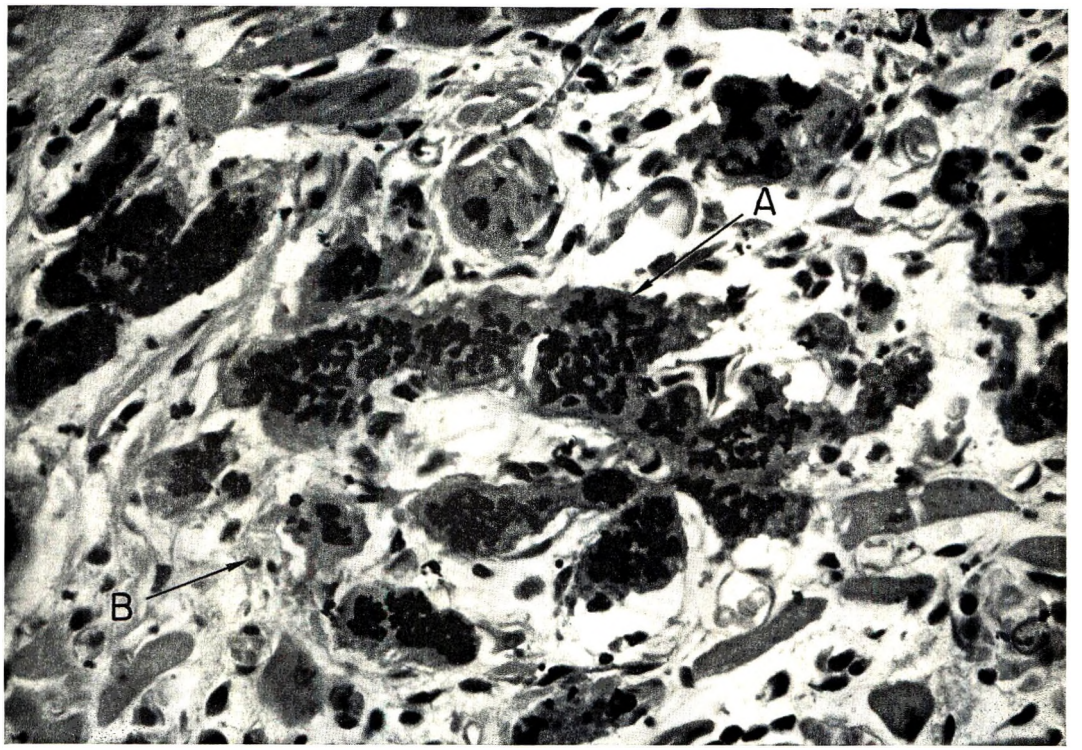

Fig. 13 Heart after 5 months of a magnesium-deficient diet. The myocardial fibers are calcified (*A*) while the interstitial tissue shows no calcification. Only rare inflammatory cells are seen (*B*). Most areas of calcification were without inflammation. H & E. × 319.

TABLE 4

Skeletal muscle lesions after magnesium and vitamin E deficiencies [1]

	Lesions typical of vitamin E deficiency	Lesions typical of vitamin E deficiency plus calcified areas
No. of lesions	19	8
Avg regeneration score	1.3	2.0
Avg degeneration score	2.0 ± 0.2 [2]	3.4 ± 0.9
95% confidence limits of mean degeneration score	1.6 to 2.4	1.3 to 5.5

[1] Twenty-seven animals were studied after 24 to 29 weeks of combined dietary deficiencies. See methods for the scoring technique.
[2] SE of mean.

The parathyroids, liver, spleen, stomach, were unremarkable.

DISCUSSION

The magnesium requirement of the rat is interrelated with the dietary requirements and levels of calcium (9–11), phosphorus (10, 11, 22), protein (23), and lipids (24). In this experiment, the diet contained 1.16% calcium, 0.95% phosphorus, and although it contained 0.019% magnesium, extensive cardiac and renal lesions attributable to magnesium deficiency were produced. These lesions appeared as marked as those which occur when diets containing less than 0.001% magnesium are used.

Tufts and Greenberg (25), using a diet with 0.87% calcium and 0.80% phosphorus — values comparable to those used in this experiment — found 0.005% magnesium to be the borderline value for ade-

Fig. 14 Section of psoas muscle after 26 weeks of a stock diet. Normal muscle fibers are present with faint cross-striations (arrow) visible. H & E. × 336.

quate growth and for the prevention of the signs of magnesium deficiency, but there was an increase in the renal calcium in their animals. Hegsted et al. (9) suggested that with diets containing between 0.006 and 0.012% magnesium there was no apparent effect of increasing the calcium level from 0.2 to 1.8%. McAleese and Forbes (10), and Forbes (11) studied the effects of varying dietary ratios of calcium, magnesium and phosphorus, and showed that magnesium deficiency was produced when calcium and phosphorus were added to diets already low in magnesium. The former authors emphasize that different levels of magnesium are needed when different parameters of deficiency such as growth, blood magnesium level or bone magnesium level are used.

The present study leads me to conclude that with the diet as specified containing 0.95% phosphorus and 1.16% calcium, that 0.19% magnesium is insufficient to prevent the cardiac and renal damage

of magnesium deficiency. A dietary level of 0.064% is sufficient to prevent these lesions.

The appearance of magnesium deficiency despite normal weight gain emphasizes that growth per se is a poor criterion of the nutritional adequacy of a diet. The absence of symptomatology made the detection of the magnesium deficiency in the present study more difficult. Tufts and Greenberg (25) noted a similar variability or absence of symptomatology when their diet contained borderline amounts of magnesium.

The renal lesion in severe magnesium deficiency has recently been restudied by several investigators (1, 7, 26–31) and most authors agree that the primary lesion is in the broad ascending limb of Henle's loop with resulting intralumenal calcifica-

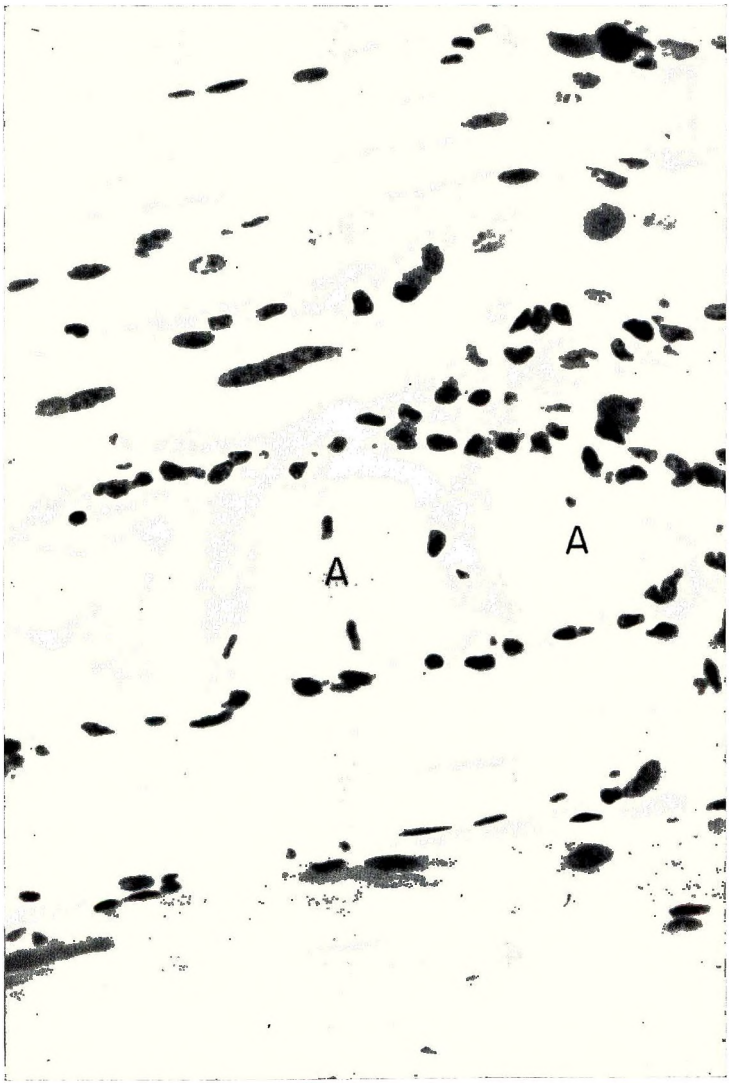

Fig. 15 Psoas muscle after 26 weeks of a magnesium-deficient, vitamin E-deficient diet. An area of hyalin degeneration (*A*), sarcolemmal nuclear proliferation and a few inflammatory cells are present. The normally appearing muscle fibers in the section seem narrower than in the control muscle (fig. 14). H & E. × 319.

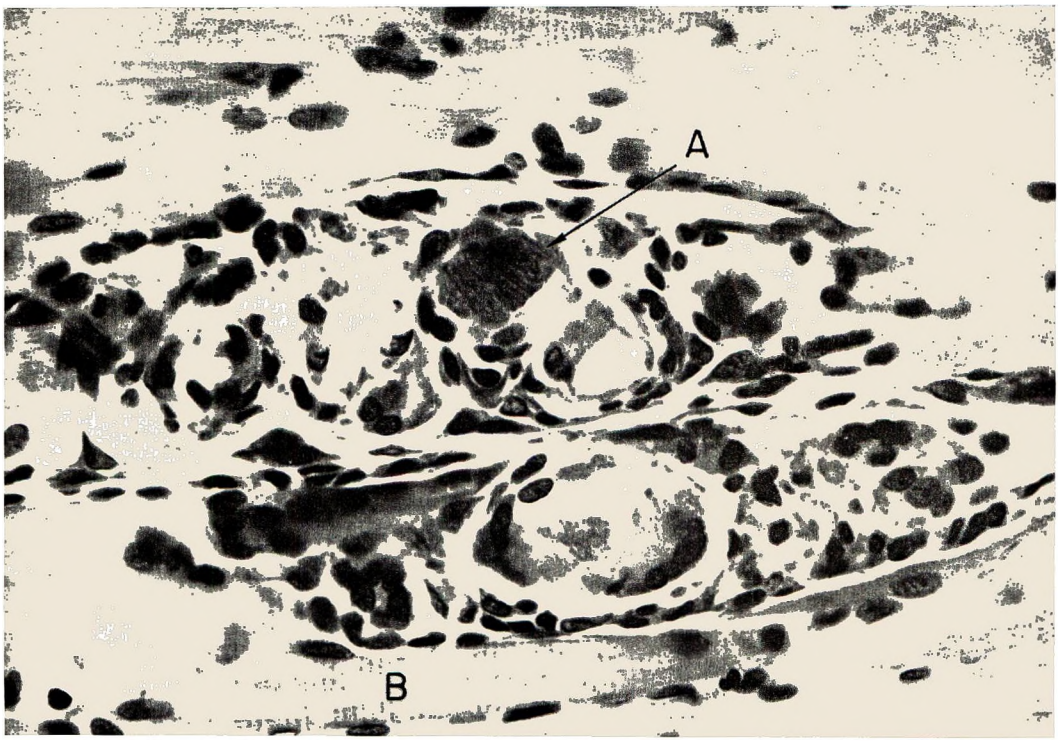

Fig. 16 Psoas muscle after 26 weeks of a magnesium-deficient, vitamin E-deficient diet. An area of marked inflammation with round cells and calcification (arrow A) is adjacent to normal appearing muscle fibers (B) with distinct cross-striations. H & E. × 336.

tion and then tubular damage. Unlike the results of others, calcification occurred in the inner zone of the medulla early in the course of the renal disease. This may have been related to the high calcium and phosphorus content in the diet, with the more rapid calcification of damaged tissues under these conditions. The location and characteristics of the lesion, the dietary analyses, and the experiments with magnesium replacement make other causes of renal calcification, such as hypervitaminosis D, linoleic acid deficiency, and pyridoxine deficiency, unlikely. Although the phosphorus content of the diet was high, the lesions differed in character, location, and course from those described due to phosphate loading (32).

An unusual feature of the renal pathology was the proliferation of the cells of the collecting tubules in the outer medullary zone. These cells appeared to be the usual epithelial type and not the intercalated cell which proliferates in potassium deficiency (33). There was no morphological evidence of potassium deficiency, although some potassium deficiency accompanies severe magnesium deficiency (7). The exact mode of formation of these cells was not clear. They did not appear to be resolving foreign body giant cells. No mitotic figures were seen in them even after colchicine treatment. They may have resulted from the aggregation of epithelial cells which were not normally sloughed off because of the unusual mineral balance in these animals.

Several forms of tubular multinuclear cells besides the one seen in potassium deficiency have been described with increasing age (34), chloride deficiency (35), and as normal variants (36). A lesion similar to the one described has not been noted previously in magnesium deficiency. Further studies, including autoradiography, may be necessary to elucidate the pathogenesis of this lesion.

The cardiac pathology of severe magnesium deficiency has been described differently by several authors (2, 6, 37). Recently, Heggtveit et al. (4) produced profound magnesium deficiency in the rat with subsequent myocardial necrosis and exudative inflammation. Many of the lesions were accompanied by varying degrees of calcification and an occasional animal had only myocardial calcification.

In the present study, the cardiac lesion consisted of degeneration and calcification usually without any inflammatory response. It is possible that the course of the development of magnesium deficiency in this case was different from that usually seen and was responsible for the absence of inflammation. In view of the brisk inflammatory response in the kidney it appears that the inflammatory response per se is intact.

There were no muscle lesions such as Ko et al. (28), and Heggtveit (3, 38) have described with severe symptomatic magnesium deficiency. However, there was an apparent potentiation of the muscular degeneration of vitamin E deficiency. At this time, it is difficult to know if this was due to the combined effects of magnesium and vitamin E deficiencies, due to the effect of renal disease on the muscle lesion, or whether the low magnesium levels increased the propensity to calcification of the necrotic muscle leading to calcification of the lesion, with this being followed by a subsequent increase in the inflammatory response.

ACKNOWLEDGMENTS

The author is indebted to Drs. J. C. Robinson, K. Snell, and L. Miller for criticism, encouragement, and advice.

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The Mechanism of the Lysine-Arginine Antagonism in the Chick: Effect of lysine on digestion, kidney arginase, and liver transamidinase [1,2]

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ABSTRACT Experiments were performed in chicks to determine the mechanism by which excess dietary L-lysine alters arginine metabolism. Excess lysine also decreased growth and plasma arginine level when a mixture of crystalline amino acids was used to simulate the composition of the basal 18% casein-10% gelatin ration. The postprandial increase in plasma arginine level was comparable in lysine-fed and control chicks. Thus, lysine does not alter arginine metabolism by affecting a digestive or absorptive process. In chicks fed crystalline amino acid diets with varied concentrations of lysine and arginine, changes in growth and plasma lysine and arginine concentrations showed a direct relationship between excess dietary lysine and arginine metabolism. A decrease in plasma arginine level caused by adding 4% L-aspartic acid to the basal ration was reversed by increasing dietary pyridoxine·HCl from 1.6 to 3.2 mg/100 g of ration. The depressed plasma arginine level in chicks fed 2% L-lysine was not altered by 1% creatine but the creatine partially eliminated the growth depression. Lysine also decreased the liver transamidinase level; therefore, this enzyme is probably not implicated in the disposal of arginine. Plasma arginine level decreased significantly within 6 hours after chicks ate diets containing excess lysine; the kidney arginase response — an increase to 5 to 10 times control values — occurred 2 to 4 days later. Because of its time relationship the arginase response is probably not physiologically significant. It is postulated that lysine causes an immediate loss of arginine from tissue, of undetermined nature, and that the increase in arginase is caused by removal of product repression.

It is now generally recognized that the addition of excess L-lysine to the diet of the young chick increases the requirement for arginine (1–4). This increased requirement is manifested by decreased growth rate, symptoms of arginine deficiency, and decreased plasma arginine level (1), all of which are reversed by adding arginine to the diet. The relationship between lysine and arginine is of particular interest because the antagonism is readily demonstrated with relatively low levels of lysine, 0.5 to 2.0% of L-lysine added to a ration complete in all respects including protein. Elucidation of the mechanism by which lysine affects the metabolism of arginine may explain the dependency of the arginine requirement of the chick on the composition of the diet (5). Although this relationship has been termed an "antagonism," the mechanism remains undefined.

In the chick, which exhibits a definite arginine requirement (6), ornithine cannot spare arginine and hence it is assumed that the chick cannot synthesize arginine. This is probably because it lacks carbamyl phosphate synthetase (7). Therefore, it may be assumed that lysine does not cause the reduction in tissue arginine concentrations or the increase in dietary requirement for arginine by interfering with the synthesis of arginine.

In the chick the lysine-induced low tissue arginine level may reflect a relationship between these 2 amino acids in: 1) digestive-absorptive processes (does lysine interfere with digestion or absorption?); 2) amino acid transport including renal (does lysine cause an increased renal excretion or redistribution of arginine in the tissues?); 3) arginine metabolism (does lysine directly or indirectly alter the intermediary metabolism of arginine?).

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[1] This investigation was supported in part by Public Health Service Research Grant no. A-6336 from the National Institutes of Health.

[2] A preliminary report of this work has appeared: Jones, J. D., S. Petersburg and P. C. Burnett 1966 Effects of lysine on arginine metabolism in the rat and chick. Federation Proc., 25: 240 (abstract).

This report presents data from experiments designed to evaluate these possible mechanisms of the effect of lysine on arginine metabolism in the chick.

EXPERIMENTAL

Commercially obtained Cobb × Ledbrest cockerels were distributed into uniform groups of 10 to 12 chicks each on the basis of body weight, one or more replications per treatment. Chicks were housed in electrically heated batteries with feed and water supplied ad libitum. Except where noted, all chicks were one day old when started on the test regimens. The basal diet (table 1) was altered during the study to contain the B vitamins at increased concentrations. These alterations are noted in the text for appropriate experiments. Amino acids [3] replaced sucrose when they were added to the diets.

The experimental groups received the basal ration supplemented with amino acid(s). Similar groups of birds receiving the basal ration alone served as controls. Durations of experiments are indicated in the tables. Levels of amino acids in the crystalline amino acid diets were selected to simulate amino acid distribution in the basal casein-gelatin diet with the exception of the variables, lysine and arginine. All amino acids were in the L-form except DL-δ-hydroxylysine. Rations were fed in the dry state; half of the glutamic acid was added as L-glutamine and half of the aspartic acid was added as L-asparagine. The HCl of the hydrochlorides of the basic amino acids in the basal ration was neutralized with $NaHCO_3$. The amino acid composition of the basal casein-gelatin ration was determined by ion exchange chromatography and has been reported previously (1). Alterations in the dietary concentrations of some amino acids were made after the first experiment of this type and are noted in the text.

TABLE 1

Basal ration [1,2]

	%
Casein	18
Gelatin	10
Salts V [3]	6
Corn oil	4
Glycerol	1
L-Methionine	0.3
Choline·Cl	0.2
Inositol	0.1
	mg/100 g ration [4]
Thiamine·HCl	0.6
Riboflavin	0.9
Niacin	5.0
Ca pantothenate	2.0
Pyridoxine·HCl	0.8
Biotin	0.02
Folic acid	0.4
α-Tocopherol	0.3
Vitamin B_{12}	0.003
Menadione	0.05

[1] Sucrose added to make 100%. Diet contains 1.65% lysine, 1.30% arginine, 0.74% K, and 0.40% Na by analysis.
[2] Vitamin D_3 (240 ICU) and vitamin A (2,400 units) given orally each week.
[3] This is the mixture described by Briggs and co-workers (8).
[4] See text for alterations of the B vitamin concentrations. In the later experiments these concentrations were doubled except for pyridoxine·HCl and folic acid which were increased to 3.2 and 4.8 mg/100 g of ration, respectively.

Methods of sampling and analysis were those reported previously (1) with the following additional techniques: transamidinase, by the method of Van Pilsum and associates (9); arginase, by the method of Smith and Lewis (10) except that the urea formed was measured by the Auto-Analyzer method of Marsh and co-workers (11) instead of by using α-isonitrosopropiophenone; creatine and creatinine, by the Jaffé reaction as described by Natelson (12).

RESULTS AND DISCUSSION

Effects on digestion or absorption. Digestive enzymes. It was reasoned that L-lysine, an in vitro inhibitor of trypsin and carboxypeptidase B [4] from the chick, could reduce the availibility of arginine by inhibiting the action of these enzymes on protein within the gastrointestinal tract or by decreasing the production of these enzymes by the pancreas. Four experiments were performed [5] to determine whether dietary lysine affected the concentration of these 2 enzymes in the pancreas of chicks. In only one of these trials did lysine depress both trypsin and carboxypeptidase B, whether expressed per bird, per unit body weight, or per gram of pancreas, although it consistently de-

[3] L-Lysine hydrochloride and crystalline B vitamins were kindly furnished by Merck and Company, Rahway, New Jersey. Other crystalline L-amino acids were purchased from General Biochemicals, Chagrin Falls, Ohio.
[4] Unpublished data.
[5] Unpublished data.

pressed growth rate after 14 to 22 days on the test regimen in all experiments.

Although the results do not allow definite conclusions, it appears unlikely that high dietary lysine level, and the resultant increased tissue lysine content, alters hydrolysis of arginine peptides by reducing the production of these enzymes by the pancreas. (Similar conclusions were drawn from data obtained in rats (13).)

Digestion and absorption. If lysine interfered with arginine utilization by altering the digestion of dietary proteins, the effect of lysine should not be apparent when all dietary nitrogen is furnished as crystalline amino acids. Therefore, crystalline amino acid rations, simulating the basal ration, and identical rations with added lysine and salts were fed to one-day-old chicks. The data shown in table 2 indicate that relatively good growth can be obtained with such crystalline amino acid rations and that lysine causes a significant growth depression regardless of whether the dietary nitrogen is furnished as protein (casein and gelatin) or as crystalline amino acids. The effects of salts are also shown in this experiment. In chicks receiving the casein-gelatin ration, neutralizing the HCl accompanying the lysine, arginine, and histidine with $NaHCO_3$ or $KHCO_3$ did not significantly reduce the growth depression caused by lysine. However, addition of NaCl, equivalent to the NaCl formed in neutralizing a ration containing 2% L-lysine·HCl with $NaHCO_3$, to the basal ration did significantly depress the growth rate of the chicks. When similar manipulations with salts were performed with rations composed of crystalline amino acids, none of the additions were either beneficial or harmful. Because the basal ration in this case had added $NaHCO_3$ to neutralize the hydrochlorides of the basic amino acids, any possible beneficial effect of neutralizing the hydrochloride accompanying the lysine was probably masked by the adverse effect of the already high salt concentration. A response to potassium was not noted with either ration containing added lysine. Potassium acetate has been reported (2) to give a growth response when added to a ration containing excess lysine and 0.41% potassium (our calculation). The potassium content of the ration used in the experiments reported here was 0.74%.

From these experiments we conclude tentatively that lysine does not interfere with the digestion or absorption of arginine and that any effects that salts exert are not primarily responsible for the relationship between lysine and arginine.

TABLE 2

Effect of L-lysine and salts on growth of chicks fed casein-gelatin ration or crystalline amino acid mixture

	Treatment	Gain in body wt
		g [1]
	18% casein-10% gelatin	
1	Control	130.5 ± 5.5
2	Diet 1 + 2% L-lysine	61.7 ± 5.6 [2]
3	Diet 2 neutralized with $NaHCO_3$	76.5 ± 6.5 [2]
4	Diet 1 + NaCl equivalent to diet 3	114.0 ± 4.9 [3]
5	Diet 2 neutralized with $KHCO_3$	76.2 ± 8.8 [2]
	Crystalline amino acid mixture [4]	
6	Control	111.3 ± 5.3
7	Diet 6 + 2% L-lysine	67.6 ± 5.8 [2]
8	Diet 7 neutralized with $NaHCO_3$	47.1 ± 4.5 [2]
9	Diet 6 + NaCl equivalent to diet 8	104.4 ± 8.7
10	Diet 7 neutralized with $KHCO_3$	52.4 ± 6.4 [2]

[1] Mean ± SE at 13 days.
[2] Different from control, $P \leq 0.01$.
[3] Different from control, $P \leq 0.05$.
[4] Simulating diet 1, neutralized with $NaHCO_3$. The percentages of amino acids were: alanine, 1.56; arginine, 1.30; asparagine, 0.88; aspartic acid, 0.89; cystine, 0.08; glutamine, 2.29; glutamic acid, 2.30; glycine, 2.65; histidine, 0.51; DL-δ-hydroxylysine, 0.10; hydroxyproline, 0.96; isoleucine, 1.03; leucine, 1.79; lysine, 1.65; methionine, 0.87; phenylalanine, 1.03; proline, 3.12; serine, 1.28; threonine, 0.90; tryptophan, 0.25; tyrosine, 1.05; and valine, 1.28.

To test this hypothesis further it was reasoned that if lysine acted by decreasing the digestion or absorption of arginine, there should be a gross alteration in plasma arginine level in the early postprandial state. One-day-old chicks were fed either the basal ration or basal ration plus 2% L-lysine for 12 to 26 days. They were fasted for 13 hours and then a test meal of either basal or basal plus lysine ration (1 ml of a ration-water slurry [2:3] per 20 g body weight in experiments A and C and per 40 g body weight in experiment B) was placed in the crop by tube at zero time. At 0.5, 1, 2, 3, and 4 hours after this test meal, blood was removed from 5 or 6 chicks per treatment for determination of "free" arginine and lysine by microbiologic assay; each chick supplied blood at only one interval (although this single sampling introduces considerable variability into the data, it was necessary to obtain enough plasma for the analysis).

Plasma lysine concentrations peaked at one-half hour in control chicks fed the basal ration and then appeared to decrease until 4 hours later (table 3). However, administration of the lysine-containing ration to control chicks in experiment A resulted in increased postprandial plasma lysine concentrations which did not return to normal within 4 hours. Observations on emptying of crops of chicks given 1 ml of slurry per 20 g of body weight showed that a considerable quantity of the ration remained in the crop 2 hours after feeding. Therefore, the experiment was repeated with half the quantity of ration (exp. B, table 3). This reduction resulted in lysine values that more nearly approximated what would be expected with a test meal, lysine increasing to a maximum and then decreasing to the 4-hour interval.

Plasma arginine concentration reached a maximum between one-half hour and 2 hours after the test meal in all instances, regardless of pretreatment or test ration; it then tended to decrease toward the fasting concentration or lower in chicks receiving a test meal similar to their pretreatment ration. In those chicks receiving the control pretreatment ration (basal) and then the lysine-supplemented test meal, the concentration of plasma arginine at 4 hours approached that observed in chicks fed lysine for longer intervals (table 3: exp. A, line 2, compared with exp. C, line 2). The similarity in maximal increases in plasma arginine level suggests that the increase reflects the concentration of arginine in the test meal rather than the metabolism of arginine after absorption. If lysine affected arginine utilization by altering digestion or absorption, such

TABLE 3

Plasma arginine and lysine concentration in chicks given basal ration or basal ration plus lysine

Exp.	Pretreatment diet [2]	Test meal [3]	Time after test meal,[1] hours					
			0	0.5	1	2	3	4
		Plasma arginine, μg/ml						
A	Basal	basal	56	96	75	102	67	83
	Basal	L-lysine, 2%	56	90	82	65	64	50
B	Basal	basal	40	60	41	50	47	47
	Basal	L-lysine, 2%	40	45	63	29	22	30
C	Basal	basal	101	139	94	104	112	72
	L-lysine, 2%	L-lysine, 2%	25	42	48	41	42	33
		Plasma lysine, μg/ml						
A	Basal	basal	197	207	206	147	185	133
	Basal	L-lysine, 2%	197	500	577	387	465	434
B	Basal	basal	257	298	179	178	156	156
	Basal	L-lysine, 2%	257	392	365	345	186	183
C	Basal	basal	243	291	193	193	195	217
	L-lysine, 2%	L-lysine, 2%	418	642	558	619	651	512

[1] All chicks were fasted for 13 hours before receiving the test meal.
[2] Duration of pretreatment regimen: experiment A, 26 days; experiment B, 12 days; experiment C, 15 days.
[3] Test meal consisted of a slurry of ration-water (2:3): experiments A and C, 1 ml/20 g body weight; experiment B, 1 ml/40 g body weight.

comparable increases in plasma arginine concentration should not occur. These data, interpreted according to Longenecker and Hause (14), indicate that arginine becomes limiting in the lysine-supplemented ration and that the effect of lysine observed at later intervals can be shown as early as 4 hours after the chick has received the supplemented ration.

Lysine-arginine relationship. To determine whether a direct relationship exists between lysine and arginine, when other factors remain constant, crystalline amino acid diets were used, the only variations being in the concentrations of these 2 amino acids. The diets were neutralized with $NaHCO_3$. The criteria used were growth and bone composition after 12 days on the regimens (table 4). The diet that contained 1.65% L-lysine and 1.30% L-arginine was similar in amino acid composition to the basal (casein-gelatin) ration, and, when 2% L-lysine was added to it, a marked depression in growth resulted. The effect of lysine on growth of chicks fed diets similar to those shown in table 2 was comparable. With increased level of lysine intake, increased arginine intake was required to obtain the best growth.

The gross bone composition appeared to be a sensitive index of lysine excess or arginine deficiency in chicks in our earlier studies (1), but it did not show the expected effect in the present experiments. (In earlier experiments addition of 2% L-lysine to casein-gelatin diets decreased tibia ash from 35% to 28% and increased tibia fat from 18% to 30%.) There was no change in bone ash, and bone fat increased only in the lysine-deficient groups. This was the major difference noted between the protein diets and the amino acid diets.

Effects on plasma lysine and arginine concentrations were studied, with additional levels of lysine and arginine added to the diet and omission of those combinations which were obviously deficient in either lysine or arginine (table 5). In this experiment, hydroxyproline and hydroxylysine were omitted from the amino acid mixture. Plasma arginine and lysine were determined by microbiologic analysis. At any dietary level of arginine, an increase in dietary lysine caused a corresponding decrease in growth rate and plasma arginine concentration but a corresponding increase in plasma lysine concentration. (An inverse response of plasma arginine concentration to dietary lysine has also been observed recently by Zimmerman and Scott (15).) At a constant dietary level of lysine (with one exception — growth at 1.25% lysine and 2.0% arginine), chicks responded to increased dietary arginine by increased growth and plasma arginine concentration. The plasma lysine concentration appears to reflect the growth rate of

TABLE 4

Effect of variations in dietary lysine and arginine (crystalline amino acid diets) on weight gain and tibia composition in chicks [1]

L-Arginine	Wt gain and bone values	L-Lysine, %			
		0.70	1.00	1.65	3.65
%					
0.65	Wt gain, g	30	41	—	—
	Bone ash, %	28.3	30.9	—	—
	Bone fat, %	34.4	27.0	—	—
1.00	Wt gain, g	26	79	75	—
	Bone ash, %	28.9	32.5	31.2	—
	Bone fat, %	33.9	21.3	22.2	—
1.30	Wt gain, g	—	59	77	37
	Bone ash, %	—	32.0	30.9	33.6
	Bone fat, %	—	20.3	18.8	16.2
1.95	Wt gain, g	—	—	84	67
	Bone ash, %	—	—	29.5	31.8
	Bone fat, %	—	—	13.0	16.6

[1] Values obtained after 12 days on regimen.

TABLE 5

Effect of variations in dietary lysine and arginine (crystalline amino acid diets) on weight gain and plasma lysine and arginine concentrations in chicks [1]

L-Arginine	Wt gain and plasma values [2]	L-Lysine, %			
		1.00	1.25	2.25	3.25
%					
1.00	Wt gain, g	107	97	32	17
	Lysine, μg/ml	18.2	71.0	853.2	912.1
	Arginine, μg/ml	32.0	10.9	7.4	5.0
1.30	Wt gain, g	—	129	65	40
	Lysine, μg/ml	—	30.8	268.1	1,417.5
	Arginine, μg/ml	—	42.5	17.5	7.7
2.00	Wt gain, g	—	102	87	70
	Lysine, μg/ml	—	68.6	399.3	953.3
	Arginine, μg/ml	—	127.7	30.8	25.3

[1] Values obtained after 14 days on regimen.
[2] Plasma amino acid values were determined microbiologically.

the chicks: those growing more had lower plasma lysine concentrations at the same dietary levels in some of the comparisons. Although the responses of the chick in growth and plasma amino acid concentrations are interrelated, are functions of food intake, and are probably influenced by other factors, it is apparent that a direct relationship exists between lysine and arginine.

Possible mechanisms. The lysine-arginine relationship may reflect an effect of lysine on metabolic processes involving arginine. The need for arginine could be increased by:

1. An increase in protein synthesis. Because the chicks do not grow well when fed excess lysine this mechanism is not very likely.
2. An increase in synthesis of creatine. This might be evidenced by an increase in arginine-glycine transamidinase activity.
3. An increase in degradation of arginine, possibly by the action of kidney arginase. Although arginase activity is low in chick kidney, it has been estimated in chicks slightly older than those used here that this enzyme degraded, to urea, about 30% of the dietary arginine (16).

Arginase. In the chick, arginase activity has been related to the arginine content of the diet or to the arginine requirement of the chicks studied. Smith and Lewis (10) reported that chick kidney arginase activity was similar in 2 groups of chicks receiving crude diets containing 0.8 and 1.2% arginine. O'Dell and colleagues (17), using chicks fed a "semipurified diet that contained a level of arginine that supported a submaximal growth rate," found that kidney arginase activity was inversely correlated with growth. Nesheim [6] determined kidney arginase activity in strains of chicks with high and with low arginine requirements. He found that addition of arginine to a ration adequate in arginine increased arginase activity in both strains but that an arginine-deficient casein diet markedly increased arginase activity only in the strain with a high arginine requirement. He also noted increased concentrations of lysine in the tissues of chicks of the high-arginine requirement strain receiving arginine-deficient or arginine-supplemented diets. He related the difference between strains to a decreased ability of the high-arginine requirement strain to metabolize lysine.

The stimulus for the changes in arginase activity noted by others may be lysine, a competitive, in vitro inhibitor [7] of arginase in kidney tissue from control chicks.

Interpretation of results is difficult because the in vitro assay of arginase activity in the presence of excess substrate is artificial in that it tends to remove the effect of lysine on arginase activity. (At this time the concentrations of the B vitamins in the diet were doubled with the exception of folic acid which was increased from 0.4 to 4.8 mg/100 g of diet.)

[6] Personal communication, M. C. Nesheim.
[7] Unpublished data.

Preliminary experiments were performed to determine whether kidney arginase activity could be altered by adding lysine to the diet. Another group of chicks received 4% L-aspartic acid added to the basal ration because Sauberlich (18) reported that aspartic acid and tryptophan reduced the plasma level of arginine in rats. Thus, if both lysine and aspartic acid would reduce the plasma arginine level but have different effects on kidney arginase activity, it might be possible to evaluate the significance of an arginase response. In chicks on the 2% L-lysine regimen for 14 days, plasma arginine level decreased and kidney arginase activity increased 3.5-fold; with the 4% L-aspartic acid regimen, plasma arginine decreased from 47 to 26 μg/ml (measured microbiologically) but there was no effect on growth rate or arginase activity.

Experiment A in table 6 was designed to confirm these results and to evaluate the significance of arginase and transamidinase as mediators of the lysine effect on arginine. The treatments, 2% L-lysine, 2% L-arginine, 4% L-aspartic acid, and 2% L-tryptophan, were anticipated to alter the plasma arginine, possibly by altering the activity of arginase or transamidinase. Arginine, the substrate for these enzymes, and other amino acids were determined by ion exchange chromatography. Creatine also was determined because it causes a drastic reduction in transamidinase activity in the chick liver (19). Only those diets containing increased lysine or tryptophan significantly depressed the growth of the chicks. Plasma arginine level appeared to be reduced by lysine, aspartic acid, and tryptophan; arginine increased it. Lysine caused a marked increase in plasma lysine level while aspartic acid depressed it. Although the effect was highly variable, lysine, aspartic acid, and tryptophan significantly reduced the liver creatine concentration. The liver transamidinase activity was lower in the control and lysine-fed groups and was not related to the creatine concentration. Kidney arginase activity was increased significantly by lysine and arginine, increased slightly by tryptophan, and was not altered by aspartic acid.

We concluded from this experiment that 1) plasma arginine concentration can be altered by means other than dietary lysine or increased kidney arginase activity because dietary aspartic acid decreased the plasma arginine level without affecting kidney arginase activity; 2) the chick can grow well with a decreased plasma arginine level; 3) loss of arginine via trans-

TABLE 6

Plasma lysine and arginine, kidney arginase, liver transamidinase, and growth in chicks fed diets supplemented with high levels of a single amino acid at 2 levels of pyridoxine

Amino acid added to basal ration	Wt gain	Plasma [1]		Liver		Kidney
		Arginine	Lysine	Creatine	Transamidinase	Arginase
	g	*μg/ml*	*μg/ml*	*μg/g*	*μmoles GAA/hr/g*	*μmoles urea/hr/g*
Experiment A,[2] 1.6 mg pyridoxine·HCl/100 g ration						
None	122 ± 8 [3]	35 ± 12	184 ± 9	180 ± 40	11 ± 1	1,400 ± 200
L-Lysine, 2%	70 ± 4 [4]	16 ± 1	470 ± 40 [4]	61 ± 14	13 ± 2	2,300 ± 100 [4]
L-Arginine, 2%	123 ± 5	91 ± 12	140 ± 17	110 ± 30	20 ± 2 [4]	4,400 ± 900 [5]
L-Aspartic, 4%	118 ± 6	19 ± 3	70 ± 20 [4]	52 ± 14 [5]	20 ± 2 [4]	1,100 ± 300
L-Tryptophan, 2%	101 ± 6 [5]	21 ± 2	160 ± 70	75 ± 20 [5]	19 ± 3 [5]	2,100 ± 500
Experiment B,[6] 3.2 mg pyridoxine·HCl/100 g ration						
None	182 ± 5	38 ± 8	150 ± 20	190 ± 20	21 ± 1	2,700 ± 800
L-Lysine, 2%	73 ± 6 [4]	20 ± 3 [4]	800 ± 100 [4]	200 ± 20	10 ± 2 [4]	14,000 ± 4,000 [4]
L-Arginine, 2%	188 ± 6	201 ± 26 [4]	115 ± 7	210 ± 20	13 ± 2 [4]	12,800 ± 800 [4]
L-Aspartic, 4%	157 ± 5 [4]	42 ± 6	138 ± 11	190 ± 10	22 ± 1	2,000 ± 200
L-Tryptophan, 2%	157 ± 6 [4]	33 ± 4	180 ± 20	160 ± 20	17 ± 1 [5]	7,000 ± 1,000 [5]

[1] Amino acids were determined by ion exchange chromatography.
[2] Samples were obtained after 12 days on regimens.
[3] Mean ± SE.
[4] Difference from control, $P \leq 0.01$.
[5] Difference from control, $P \leq 0.05$.
[6] Samples were obtained after 15 days on regimens.

amidinase is probably not the cause of the increased arginine requirement; and 4) the increased kidney arginase activity may indicate the metabolic pathway causing the loss of arginine.

Normal amino acid metabolism is highly dependent on the level of dietary pyridoxine. Although the concentration we used was in excess of the estimated requirement in the chick (20), and the growth depression caused by excess lysine has been reported (21) to occur with diets limiting in pyridoxine, the experiment was repeated with the pyridoxine level of the diet doubled (table 6, exp. B). (Except where specifically indicated, this new level was used for the remaining experiments.) At the increased level of pyridoxine, lysine, aspartic acid, and tryptophan all depressed growth rates significantly. Only lysine significantly reduced plasma arginine level. In contrast with the previous experiment, liver creatine concentration was not altered. Lysine, arginine, and tryptophan decreased the liver transamidinase activity. Aspartic acid again did not alter arginase activity significantly, whereas the arginase response to lysine and arginine was more pronounced ($P \leq 0.01$); tryptophan also increased arginase activity ($P \leq 0.05$). Thus, pyridoxine did not eliminate the effect of excess lysine and appeared to increase the response of kidney arginase to lysine.

Since, in chicks fed 4% L-aspartic acid, plasma arginine concentration appeared to be dependent on pyridoxine intake, that portion of the experiment was repeated. The data (table 7) confirm the previous results in that aspartic acid depressed plasma arginine level at the lower (1.6 mg/100 g) but not at the higher (3.2 mg/100 g) pyridoxine·HCl level. Plasma lysine again tended to be decreased with added dietary aspartic acid. Transamidinase and arginase were not altered by the experimental variables. Thus, the relationship between dietary aspartic acid and plasma arginine level is dependent on the pyridoxine consumption but is not reflected in an altered activity of either arginase or transamidinase.

Creatine and transamidinase. Creatine and transamidinase are of interest not only because they may be involved in the catabolism of arginine but also because a decrease in tissue arginine level may limit creatine synthesis. That creatine spares arginine in chicks fed an arginine-deficient ration was first reported by Almquist and associates (22) in 1941. To determine the relationship between lysine or arginine and creatine, chicks were fed rations containing either 1% creatine or 2% L-lysine or both and their growth and plasma arginine and lysine levels were compared with those of control chicks (table 8, exp. A). Lysine caused a growth depression which was partially reversed by dietary creatine. The plasma arginine concentration was increased by dietary creatine alone but creatine did not alter the decrease caused by lysine. When the experiment was repeated with higher levels of all B vitamins (table 8, exp. B), similar results were obtained. In addition, arginase and transamidinase activities were determined; lysine depressed

TABLE 7

Plasma lysine and arginine, kidney arginase, liver transamidinase, and growth of chicks fed 4% aspartic acid at 2 levels of dietary pyridoxine [1]

Amino acid added to basal ration	Wt gain	Plasma		Liver	Kidney
		Arginine	Lysine	Transamidinase	Arginase
	g	*μg/ml*	*μg/ml*	*μmoles GAA/hr/g*	*μmoles urea/hr/g*
	1.6 mg pyridoxine·HCl/100 g ration				
None	173 ± 9 [2]	92 ± 14	120 ± 20	21 ± 3	2,800 ± 900
L-Aspartic, 4%	156 ± 10	31 ± 3 [3]	85 ± 10	24 ± 3	1,500 ± 200
	3.2 mg pyridoxine·HCl/100 g ration				
None	185 ± 9	76 ± 5	135 ± 12	22 ± 3	1,700 ± 200
L-Aspartic, 4%	186 ± 6	69 ± 4	119 ± 10	19 ± 4	1,900 ± 200

[1] Samples were obtained after 14 days on regimens.
[2] Mean ± SE.
[3] Difference from control, $P \leq 0.01$.

TABLE 8

Effect of dietary lysine and creatine on plasma arginine and lysine, growth, kidney arginase, and liver transamidinase

Amino acid added to basal ration	Wt gain	Plasma		Liver	Kidney
		Arginine	Lysine	Transamidinase	Arginase
	g	*μg/ml*	*μg/ml*	*μmoles GAA/hr/g*	*μmoles urea/hr/g*
Experiment A,[1] 1.6 mg pyridoxine·HCl/100 g ration					
None	209 ± 10 [2]	49 ± 4	270 ± 40	—	—
L-Lysine, 2%	84 ± 11 [3]	30 ± 1 [3]	480 ± 50 [3]	—	—
Creatine, 1%	196 ± 8	82 ± 8 [3]	116 ± 9 [3]	—	—
L-Lysine, 2% + creatine, 1%	131 ± 5 [3]	33 ± 3 [3]	440 ± 50 [3]	—	—
Experiment B,[4] 3.2 mg pyridoxine·HCl/100 g ration					
None	147 ± 9	60 ± 8	143 ± 13	25 ± 1	1,600 ± 300
L-Lysine, 2%	71 ± 8 [3]	23 ± 2 [3]	380 ± 50 [3]	13 ± 2 [3]	15,000 ± 2,000 [3]
Creatine, 1%	155 ± 5	80 ± 7	102 ± 10 [5]	2 ± 1 [3]	1,400 ± 200
L-Lysine, 2% + creatine, 1%	102 ± 10 [3]	—	—	—	—

[1] B vitamins in experiment A were as shown in table 1. Samples were obtained after the chicks had received the diets for 15 days.
[2] Mean ± SE.
[3] Difference from control, $P \leq 0.01$.
[4] B vitamins in experiment B were two times those shown in table 1 except for pyridoxine·HCl and folic acid which were 3.2 and 4.8 mg/100 g diet. Samples were obtained after the chicks had received the diets for 14 days.
[5] Difference from control, $P \leq 0.05$.

transamidinase (as in experiment B, table 6) and increased arginase about tenfold. Creatine did not affect arginase but severely depressed transamidinase. (This effect of creatine on transamidinase is well documented (19) and should give considerable confidence to the observation of depression of the enzyme by lysine.) The depression in kidney transamidinase activity noted by Van Pilsum (23) in rats fed a protein-free diet can be assumed to be due to a different mechanism.

Because addition of creatine to the basal ration increases the plasma arginine level, it can be said that it spares arginine. However, because chicks fed additional lysine respond to dietary creatine by showing increased growth without increase of plasma arginine concentration, creatine per se must become limiting when lysine is fed. There may be less creatine formed, compared with that with the basal ration, because of the decreased transamidinase activity and tissue arginine concentration. The persistence of the decreased plasma arginine concentration in the chick fed creatine indicates that the mechanism by which lysine controls the concentration of arginine is unaffected by growth or transamidinase activity. This makes it less likely that arginine is disposed of via transamidinase action. It is probable that synthesis of creatine is reduced by the decrease in precursor or by the presence of lysine. This is supported by the fact that creatine substitutes for arginine in the presence of lysine, as shown by its tendency (table 8) to reverse the growth-depressing effect of lysine. (Under similar conditions, it had little effect on growth in the rat, in which lysine does not depress plasma arginine concentration.) [8]

Time relationships. To relate the effect of lysine in decreasing plasma arginine level to the increase in kidney arginase activity, plasma arginine and lysine levels and kidney arginase activity were determined in chicks that had been allowed access to diets for different time intervals. One-day-old chicks were fed the basal ration for 3 days, fasted for 5 hours, and then allowed access to one of two diets, basal ration or basal ration plus 2% L-lysine. At each of the intervals noted (table 9), 6 chicks per group were taken for the determination of plasma lysine and arginine levels, kidney arginase activity, and gain in body weight. The intervals

[8] Unpublished data.

TABLE 9

Relationship between responses in plasma arginine and kidney arginase to dietary lysine and time on regimen

Parameter	Diet	Interval, days						
		0.25	1	2	4	7	10	14
				Experiment A				
Plasma arginine, μg/ml	control	41 ± 6 [1]	45 ± 1	48 ± 2	48 ± 4	34 ± 4	46 ± 2	60 ± 8
	2% L-lysine	27 ± 4	13 ± 5 [2]	15 ± 4 [2]	14 ± 1 [2]	13 ± 1 [2]	15 ± 1 [2]	23 ± 2 [2]
Plasma lysine, μg/ml	control	155 ± 6	176 ± 9	173 ± 9	170 ± 20	150 ± 30	110 ± 20	143 ± 13
	2% L-lysine	400 ± 40 [2]	350 ± 50 [3]	480 ± 20 [2]	505 ± 7 [2]	427 ± 4 [2]	440 ± 30 [2]	380 ± 50 [2]
Kidney arginase, μmoles urea/g/hr	control	480 ± 80	1,200 ± 300	2,100 ± 600	2,800 ± 800	3,100 ± 500	2,700 ± 600	1,600 ± 300
	2% L-lysine	600 ± 100	2,200 ± 600	5,000 ± 1,000	27,000 ± 4,000 [2]	15,000 ± 3,000 [2]	16,000 ± 4,000 [2]	15,000 ± 2,000 [2]
Gain in body wt, g	control	—	7.0	12 ± 1	21 ± 3	43 ± 6	94 ± 7	147 ± 9
	2% L-lysine	—	7.0	13 ± 1	21 ± 3	49 ± 5	61 ± 8 [3]	71 ± 8 [2]
				Experiment B				
Plasma arginine, μg/ml	control	62 ± 3	43 ± 3	36 ± 6	51 ± 1	54 ± 5	—	82 ± 8
	2% L-lysine	31 ± 2 [2]	28 ± 5	33 ± 3	31 ± 1	23 ± 3 [2]	—	27 ± 1 [2]
Plasma lysine, μg/ml	control	140 ± 20	142 ± 6	140 ± 20	230 ± 20	290 ± 20	—	100 ± 10
	2% L-lysine	382 ± 6 [2]	400 ± 40 [2]	480 ± 30 [2]	700 ± 40 [2]	570 ± 40 [2]	—	260 ± 30 [2]
Kidney arginase, μmoles urea/g/hr	control	1,000 ± 200	600 ± 100	1,600 ± 500	5,000 ± 2,000	4,500 ± 400	—	3,000 ± 2,000 [3]
	2% L-lysine	1,100 ± 200	650 ± 80	3,000 ± 1,000	17,000 ± 3,000 [2]	13,000 ± 2,000 [2]	—	19,000 ± 5,000 [3]
Gain in body wt, g	control	—	7.1	13 ± 3	41 ± 4	73 ± 7	—	250 ± 10
	2% L-lysine	—	4.6	8 ± 3	21 ± 2 [2]	42 ± 4 [2]	—	42 ± 9 [2]

[1] Mean ± SE.
[2] Difference from control, $P \leq 0.01$.
[3] Difference from control, $P \leq 0.05$.

were chosen because our data on plasma amino acid levels after a test meal (table 3), as well as those of Dean and Scott (24), indicated that the plasma arginine level is depressed as early as 4 to 6 hours after consumption of a diet high in lysine. In experiment A, table 9, lysine depressed the plasma arginine by 6 hours and that depression was maintained through 14 days. Although kidney arginase activity started to increase as early as one day, the first significant difference occurred at 4 days.

Similar results were obtained when the experiment was repeated (table 9, exp. B). Lysine significantly depressed the plasma arginine level by 6 hours but the arginase values were not affected significantly until the fourth day. Although the effect of lysine on arginase may have become apparent earlier in experiment A than in experiment B, the increase still occurred considerably later than the change in plasma arginine level. (This response in arginase activity is also slower than that observed in rats with a change from 30% to 60% dietary protein, which is thought to be a substrate induction (25).) Because lysine is a competitive inhibitor of arginase from kidney of control chicks [9] and the response in arginase is delayed, it is very unlikely that this change in activity is a significant factor in the initial decrease in plasma arginine concentration.[10]

Tissue distribution. An experiment (table 10) was performed to determine whether the initial decrease in plasma arginine concentration was caused by a redistribution of arginine in the tissues. The concentrations of "free" dibasic amino acids in plasma, liver, and muscle were determined in 5-day-old chicks that had received basal rations for 4 days, been fasted 4 hours, and then been given access to the test rations (basal ration or basal ration plus 2% L-lysine) for 6 hours before the samples were obtained. The muscle and liver lysine concentrations were a reflection of the high plasma lysine concentration. Dietary lysine decreased muscle and plasma arginine levels but did not alter liver arginine measurably. Tissue ornithine concentration followed that of arginine, being proportionately lower in muscle and plasma but unchanged in liver. Because we chose to analyze the kidneys of these chicks for arginase activity, which was unaltered by dietary treatment, instead of for amino acids, kidney concentration data are not available. The data from this experiment indicate 2 things: 1) Lysine does not cause any great alteration in distribution of arginine to account for the decrease in plasma arginine concentration (that is, the tissues do not accumulate arginine). 2) The initial decrease in plasma arginine concentration is probably not due to hydrolysis of arginine by arginase because ornithine does not accumulate in plasma, muscle, or liver.

Other metabolic pathways. We have not investigated other pathways for arginine catabolism in the chick. In one of

[9] Unpublished data.

[10] In experiments in which chicks were placed on the test regimens at different ages, the arginase response was variable while the plasma arginine level was invariably decreased.

TABLE 10

Effect of dietary lysine on tissue concentrations of free arginine, lysine, and ornithine 6 hours after access to diets [1]

Amino acid	Diet	Amino acid concentration		
		Plasma	Liver	Muscle
		μmoles/100 ml	*μmoles/100 g*	*μmoles/100 g*
Lysine	control	87.6	59.1	593
	L-lysine, 2%	287.0	223.8	1,145
Arginine	control	19.1	7.3	84.7
	L-lysine, 2%	13.0	6.5	48.4
Ornithine	control	6.5	5.1	20.7
	L-lysine, 2%	3.9	4.8	13.4

[1] One-day-old chicks were maintained with the control ration for 4 days, fasted for 4 hours, and then given one of the two diets ad libitum. Six hours later they were killed and equal portions of tissues from three or four chicks were pooled per sample. Each value represents a mean of three or more samples.

these, suggested by Klain and Johnson (26), arginine may go to uric acid more directly than through glycine as an intermediate. Lewis (4) recently presented data showing that chick liver preparations can oxidize lysine and other amino acids by a process not involving transdeamination and L-glutamic dehydrogenase and that the activity of one of the liver fractions in respect to lysine was somewhat increased in chicks fed additional lysine. He suggested that there is an increase in oxidative catabolism in response to excess amino acid(s), which would then emphasize the inadequacy of the limiting amino acid and might account for some of the unexplained relationships between amino acids.

Data presented here showing an interrelationship among arginine, aspartic acid, and pyridoxine may be an indication of another route of arginine catabolism.

An arginase response to lysine similar to that observed in chicks has recently been reported in Chang's liver cells, which also respond to ornithine, valine, and leucine (other inhibitors of arginase) (27). Rat liver arginase activity is not altered by dietary lysine (28),[11] probably because the rat can synthesize arginine while the chick and Chang's liver cells cannot because they lack carbamyl phosphate synthetase and ornithine transcarbamylase, respectively. The similarities of the arginase response of the chick and Chang's liver cells suggest that a similar control mechanism is operating. Eliasson and Strecker (27) postulate that, for Chang's liver cells, "lysine decreases the rate of formation of a product of arginine metabolism that otherwise would accumulate in the cells and function to repress arginase synthesis." These investigators found that proline, a product of arginine metabolism, decreased arginase activity in their cultured cells. We have not measured arginase activity in chicks fed additional proline, but we have found that tissue concentrations of proline are increased after 17 days on an increased dietary lysine regimen (1). Also, Fisher and associates (29) could not demonstrate that proline could spare arginine in adult roosters receiving a 35% casein diet; if proline acted as an arginase repressor in the chick in vivo, this would be evidence that arginase is of no significance in the lysine-arginine relationship.

We concluded earlier (1, 13) that dietary lysine did not markedly interfere with transport of arginine by the tissues on the basis of data obtained from chicks and rats in which plasma-to-muscle ratios of arginine were not altered appreciably by high dietary and tissue concentrations of lysine. Similarly, by extrapolation from data obtained with rats in which high plasma lysine levels caused by high dietary lysine intakes did not cause an appreciable loss of arginine via the urine (13) and from data which showed a urinary loss of only 1.3% of the arginine consumed by 4- to 5-week-old chicks fed either a 25% casein-10% gelatin or 35% casein ration (16), it appears that kidney tubular transport is not involved in the increased arginine requirement of chicks fed casein rations. If the increased requirement for arginine noted in chicks fed a 35% casein ration was caused by the high lysine content, the mechanism would be similar to that occurring when lysine is added to an 18% casein-10% gelatin ration. This relationship should be reflected in increased plasma lysine and decreased arginine concentrations. Because competition for renal tubular reabsorption between lysine and arginine does occur in adult roosters infused with L-lysine,[12] it is conceivable that if plasma lysine concentration were increased sufficiently a urinary loss of arginine could occur. (Our unpublished data indicate that the rooster kidney may lose arginine with an increase of plasma lysine concentration of less than threefold.) Because the plasma amino acid concentrations are not known in the chicks fed a 35% casein ration, it may be prudent to determine experimentally whether 2% L-lysine added to an 18% casein-10% gelatin ration does cause an initial loss of arginine via the urine, which thus could be the initial cause of the immediate decrease in plasma arginine concentration.

COMMENT

The data presented here indicate that the effects of lysine on arginine metabolism may be separated into 2 phases: 1)

[11] Unpublished data.
[12] Unpublished data.

an early decrease in tissue arginine concentration, and 2) a delayed response in kidney arginase activity. Although an increase in dietary lysine eventually causes an increase in kidney arginase activity, this is probably not the primary cause of the lysine-arginine antagonism. Nor it is likely that increased metabolism of arginine by transamidinase to creatine is implicated in the antagonism, although growth may be limited by the decreased formation of creatine. The primary effect of lysine could be increased catabolism of arginine within the chick or competition between lysine and arginine at the renal tubular absorptive surface, either of which would result in an early decrease in plasma arginine concentration. If the competition between lysine and arginine in the renal tubules of the chick resembles that in older birds, it would result in an early renal loss of arginine. This process would also serve to maintain a decreased plasma arginine concentration. The decrease in plasma arginine level coupled with an in vivo inhibition of arginase by lysine may decrease the rate of formation of a product(s) of arginine metabolism that otherwise would accumulate and repress arginase synthesis; this may explain the delayed increase in kidney arginase activity. There is no clear-cut evidence to support a conclusion on the role of the kidney in the antagonism.

Experiments are in progress to determine the immediate metabolic fate of arginine in chicks fed a high lysine diet and the cause of the increase in kidney arginase activity.

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Effect of Dietary Carbohydrate on the Serum Protein Components of Two Strains of Rats

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ABSTRACT To determine the influence of dietary carbohydrate on serum protein components as resolved by moving boundary electrophoresis, BHE and Wistar-strain rats were fed high cholesterol diets containing sucrose, cornstarch or glucose. Carbohydrate-induced differences observed depended on age and strain and on state of fast. At 150 days fasted BHE rats fed glucose had pre-albumin (PA) more often in their sera, and lower albumin and α_1-globulin and higher γ-globulin concentrations than rats fed sucrose or cornstarch. At 350 days a high level of PA was observed when the diet contained sucrose. In nonfasted BHE rats fed sucrose, PA incidence was high at 150 and 350 days; it was significantly higher than with cornstarch at 150 days. Fasted Wistar rats, in contrast, had a higher PA incidence at 150 days with sucrose than with glucose but a low incidence regardless of carbohydrate at 350 days. In all except 350-day-old fasted rats, albumin and α_1-globulin concentration tended to be lower with cornstarch than with glucose. At 350 days, α_2- and β-globulin levels were lower with starch than with glucose or sucrose when animals were fasted and higher when nonfasted. Regardless of diet or state of fast, PA level was directly related to level of serum noncholesterol lipids.

Previous studies in this laboratory have shown that both type and level of dietary fat produce significant changes in the concentrations of most of the serum protein components of the BHE-strain rat (1). Comparison of the nutritional response of this strain [1] with two other strains of rats, Wistar and Holtzman, with three different diets have led to the conclusion that certain measurements are influenced by strain of rat as well as by diet and that differences in response among strains must be related to differences in inherent metabolic characteristics of the rat (2). Examination of the sera of some of the rats from each of these strains revealed several significant differences in the blood serum proteins among the strains and these differences varied with diet (3). A significant interaction of strain and diet was found for pre-albumin (PA) and for β-globulin. Evaluation, therefore, of the nutritional response of a group of rats to different diets based on the amounts of serum pre-albumin and β-globulin must be considered with respect to the strain used. The pre-albumin fraction has been shown to be associated with lipid material,[2] whereas the β-globulin fraction is known to contain a component, β-lipoprotein, which plays a significant role in the transport of lipid.

The conversion of dietary carbohydrate to fat is well-established. All carbohydrates, however, do not exert the same effect on fat metabolism (4, 5). The present paper reports results of the effect of type of dietary carbohydrate on the serum proteins of BHE and Wistar rats fed for two different periods of time.

EXPERIMENTAL

Male BHE or Wistar rats were weaned at 21 days of age and housed individually in a room maintained at approximately 27° and 45% relative humidity. Each animal was fed ad libitum one of three nutritionally adequate diets containing 25 g whole dried cooked egg and 39 g carbohydrate/100 g diet; only the type of carbohydrate was varied. The composition of the diet in which sucrose was the main carbohydrate has been reported (6). Glucose or

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[1] Mixed strain started in 1942 by breeding an albino Yale strain obtained from Columbia University with a black and white hooded strain from Pennsylvania State College.

[2] Lakshmanan, F. L. 1963 Factors influencing the presence of rapidly migrating serum protein component(s), PA. Federation Proc., 22: 608 (abstract).

cornstarch replaced sucrose in the other 2 diets. Water was accessible at all times. Animals were killed at 150 or 350 days of age. Some rats were fasted for approximately 17 hours before killing; others were allowed access to food. The procedures of anesthesia, blood removal, moving boundary electrophoresis, total protein and nonprotein nitrogen content of the sera (1) and total urine protein (6) have been described. Paper electrophoresis was performed on the serum according to a method described in an industrial manual.[3] Amidoschwarz 10 B (7) and Sudan black B (8) were used to stain protein and lipid, respectively. Total serum lipid was extracted, saponified and acidified; total fatty acids were extracted and measured colorimetrically (9, 10). For the purpose of discussion the total fatty acid content of serum will be referred to as total noncholesterol lipid to distinguish it from the free fatty acid or nonesterified fatty acid content of serum. Significant differences within a group for moving boundary electrophoretic analyses were determined by means of Student's *t* test. Chi-square analysis was used to test the significance of the percentage of rats with pre-albumin in their sera (11). Correlation coefficients were calculated (12) to evaluate the relationship between the various serum protein fractions and total urine protein as well as between the serum protein and total noncholesterol lipid content of serum.

RESULTS

Table 1 shows for both strains of rats fasted overnight the effect of dietary carbohydrate and age on the serum protein components determined by moving boundary electrophoresis. A combined value for albumin and α_1-globulin is given because of the poor separation of these components in certain sera. Mobilities of the individual protein fractions were not affected by diet, strain, age or state of fast. Regardless of dietary carbohydrate the average total protein content of the serum remained fairly constant in both strains. A few significant differences in amounts occurred with age, between strains, and between fasted and nonfasted animals but were small and did not affect interpretation of the results pre-

[3] Beckman/Spinco Division of Beckman Instrument Company.

TABLE 1

Effect of diet, age and strain on the serum protein components of fasted rats

Diet and age	Total no. rats	Total protein	Rats with PA [1]	Concentration of protein components, % of total				
				PA	Albumin and α_1-globulin	α_2-Globulin	β-Globulin	γ-Globulin
		g/100 ml	%					
				BHE strain				
150 days of age								
Sucrose	11	6.2±0.1 [2]	27	1.5±0.8	71.9±2.8	8.2±0.9	14.7±0.6	3.7±1.1
Starch	7	6.3±0.1	14	0.7±0.7	70.3±2.0	8.4±0.7	15.5±1.0	5.1±0.8
Glucose	9	6.2±0.3	78	2.4±0.6	63.3±1.5	9.8±0.6	16.8±0.7	7.7±0.9
350 days of age								
Sucrose	13	6.5±0.1	92	9.3±1.6	70.5±1.0	7.3±0.4	10.6±0.9	2.2±0.5
Starch	6	6.3±0.1	50	4.5±2.5	68.3±1.7	8.2±0.8	14.9±1.8	4.1±1.1
Glucose	4	6.2±0.1	75	3.5±1.4	68.4±2.2	8.7±1.2	14.7±1.7	4.7±1.8
				Wistar strain				
150 days of age								
Sucrose	5	6.8±0.2	100	5.8±0.6	60.3±0.9	8.1±0.5	16.3±0.6	9.4±0.7
Starch	6	6.3±0.3	67	1.8±0.6	61.8±1.7	10.4±0.7	18.8±0.7	7.1±0.8
Glucose	3	7.0±0.4	33	1.3±1.3	66.0±2.1	8.4±0.2	17.8±0.9	6.5±1.9
350 days of age								
Sucrose	6	6.8±0.1	17	0.4±0.4	65.6±1.7	8.3±0.6	18.4±0.8	7.3±0.7
Starch	7	6.5±0.2	14	0.5±0.5	70.7±2.8	7.3±0.6	15.4±1.5	6.1±1.2
Glucose	8	6.5±0.1	25	0.6±0.4	60.6±0.7	9.7±0.7	19.6±1.0	9.5±1.3

[1] Pre-albumin.
[2] Mean ± SE.

sented as percentage of total protein. The differences in growth of both strains of rats due to dietary carbohydrate [4] were relatively small and do not explain the following differences observed in serum protein concentrations due to diet, age and strain.

The percentage of rats having PA in their sera is shown because pre-albumin occurs only in some sera. The significance of the differences of the concentrations of pre-albumin in serum varies with the kind of groups being compared, namely, whether comparisons were made between the average PA calculated for the total number of rats examined or between the average PA calculated for only those rats having PA in their sera. The former value reflects the incidence of PA, whereas the latter indicates the level of PA present when detected. Evaluation of the data has been made both ways when necessary. The average PA concentration for the total number of rats in each group is shown in table 1; the amount present in sera of rats having PA may be calculated from the percentage of rats with PA.

Fasted BHE rats. At 150 days of age rats fed sucrose or starch had similar amounts of the individual serum proteins. Pre-albumin was often observed when the dietary carbohydrate was glucose but incidence was low when the carbohydrate was starch or sucrose. When only rats having PA were compared, the concentration of pre-albumin was higher ($P < 0.05$) in the sera of rats fed sucrose than those fed glucose. Glucose-fed rats had significantly lower amounts of albumin and α_1-globulin ($P < 0.05$), and higher amounts of β-globulin ($P < 0.05$) and γ-globulin ($P < 0.05$) than sucrose-fed rats.

At 350 days of age, PA incidence remained high for BHE rats fed glucose. The level of pre-albumin was higher in the sera of sucrose-fed rats than in the sera of glucose-fed rats ($P < 0.05$). β-Globulin was the only other protein affected by diet at this age. Sucrose-fed rats had the lowest amount of this component. The difference was significant at the 5% level of probability between the sucrose- and starch-fed rats. Both incidence and level of pre-albumin increased and β-globulin decreased with age when the diet contained sucrose ($P < 0.01$).

Fasted Wistar rats. At 150 days of age dietary sucrose resulted in the highest incidence of pre-albumin, and glucose the lowest. The amount present in the sera of sucrose-fed rats was significantly higher than the amount in starch-fed rats ($P < 0.01$). The PA concentrations of glucose-fed and sucrose-fed rats were also significantly different ($P < 0.05$) but the incidence was a contributing factor to the significance. Albumin plus α_1-globulin concentration was highest in sera of glucose-fed young Wistar rats and was significantly higher ($P < 0.05$) than the amount present in the sucrose-fed rats. α_2-Globulin concentration of rats fed starch was significantly higher than that of rats fed sucrose ($P < 0.01$) or glucose ($P < 0.05$). Starch-fed animals also had a higher level of β-globulin than sucrose-fed animals ($P < 0.05$).

At 350 days of age pre-albumin incidence was low in the sera of Wistar rats. Both incidence and level were similar for the three dietary carbohydrates. A significant difference, however, was observed between glucose- and starch-fed Wistar rats for all the other serum protein components. Albumin and α_1-globulin levels were lower ($P < 0.01$), α_2- ($P < 0.05$), β- ($P < 0.05$) and γ- ($P < 0.05$) globulin levels higher when glucose was fed than when starch was fed. Sucrose-fed Wistar rats had intermediate values for most components; the albumin and α_1-globulin concentration was significantly different from those of glucose-fed rats ($P < 0.05$).

An age effect was observed with all three carbohydrates in this strain of rats. PA occurred less often in the sera of the 350-day-old rats than in the sera of the 150-day-old rats when the dietary carbohydrate was sucrose ($P < 0.05$) or starch. Higher concentrations of serum albumin and α_1-globulin were observed in the older animals fed sucrose ($P < 0.05$) or starch ($P < 0.05$) than in the younger animals; the opposite occurred when glucose was fed ($P < 0.05$). α_2-Globulin level was significantly less ($P < 0.01$) in the older Wistar rat than in the younger animals fed starch.

[4] Unpublished data, manuscript in preparation, M. Fisher, E. Conway, D. D. Taylor and M. Adams, 1967.

Strain differences in fasted rats. Strain differences were apparent when comparisons were based on the response of BHE and Wistar rats to the same diets. At 150 days of age pre-albumin was observed more often in the sera of Wistar rats than BHE rats fed sucrose ($P < 0.05$) or starch; the opposite was observed with glucose. Albumin and α_1-globulin concentrations were higher ($P < 0.01$) in BHE rats than in Wistar rats when the dietary carbohydrate was sucrose or starch. β-Globulin levels were generally lower in the BHE strain than in the Wistar strain. The difference between the 2 strains was significant ($P < 0.05$) in the starch-fed rats. Less γ-globulin was observed in BHE rats than in Wistar rats ($P < 0.01$) when sucrose was fed.

At 350 days of age significant strain differences were observed with dietary sucrose or glucose but not with starch. Pre-albumin incidence tended to be high in BHE rats but low in Wistar rats and the difference in PA incidence between the 2 strains was significant when sucrose was fed ($P < 0.01$). Albumin and α_1-globulin concentrations were higher in BHE rats than in Wistar rats fed sucrose ($P < 0.05$) or glucose ($P < 0.01$). The β- and γ-globulin levels were lower in the BHE strain than in the Wistar strain when the dietary carbohydrate was sucrose ($P < 0.01$) or glucose ($P < 0.05$).

Table 2 summarizes similar data for non-fasted BHE and Wistar rats.

Nonfasted BHE rats. In contrast with the fasted group of rats the only difference observed with dietary carbohydrate was in pre-albumin. At 150 days of age pre-albumin occurrence differed significantly ($P < 0.05$) between rats fed starch and those fed sucrose. At 350 days of age dietary carbohydrate had no significant effect on any of the serum proteins.

A significant age effect was observed in the PA concentration of sucrose-fed rats. When rats having pre-albumin in their sera were compared, the level of PA was lower in 350- than in 150-day-old rats ($P < 0.01$).

Nonfasted Wistar rats. At 150 days of age no significant dietary differences were established from the limited number of sera examined. At 350 days of age, however, albumin and α_1-globulin concentra-

TABLE 2

Effect of diet, age and strain on the serum protein components of nonfasted rats

Diet and age	Total no. rats	Total protein	Rats with PA [1]	Concentration of protein components, % of total				
				PA	Albumin and α_1-globulin	α_2-Globulin	β-Globulin	γ-Globulin
		g/100 ml	%					
				BHE strain				
150 days of age								
Sucrose	11	6.2±0.3 [2]	73	4.2±0.9	68.1±2.3	8.2±0.8	15.8±0.9	3.8±1.0
Starch	7	5.9±0.4	0	0.0	69.8±2.7	8.1±1.1	18.4±1.6	3.8±1.2
Glucose	9	6.1±0.3	22	1.2±0.8	70.9±2.2	7.8±0.6	16.0±1.0	4.2±1.0
350 days of age								
Sucrose	5	6.7±0.2	60	1.9±0.8	69.7±4.5	7.4±0.6	16.1±2.3	4.9±2.0
Starch	4	6.7±0.1	25	2.3±2.3	69.4±2.1	9.0±0.7	14.9±1.7	4.4±1.8
Glucose	4	6.5±0.2	50	2.8±1.8	67.6±2.0	8.3±0.4	17.1±1.3	4.2±2.7
				Wistar strain				
150 days of age								
Sucrose	3	6.7±0.6	0	0.0	72.3±3.9	6.9±1.7	16.5±1.4	4.3±1.2
Starch	4	6.0±0.1	50	2.2±1.3	60.4±5.3	8.6±0.8	17.9±2.1	10.9±4.1
Glucose	4	6.4±0.1	50	1.2±0.8	65.4±4.4	10.0±1.5	18.0±1.3	5.3±2.2
350 days of age								
Sucrose	6	6.8±0.3	50	1.7±1.0	66.3±1.7	9.2±0.5	17.7±1.5	5.1±0.7
Starch	6	6.6±0.2	0	0.0	60.0±1.6	11.7±0.7	21.5±1.1	6.8±0.8
Glucose	6	6.7±0.3	33	1.4±0.9	67.1±2.3	10.0±0.9	17.2±0.8	4.3±1.5

[1] Pre-albumin.
[2] Mean ± SE.

tion of rats fed starch was lower ($P < 0.05$) than that of rats fed either sucrose or glucose. Amounts of α_2-globulin were higher in starch-fed than in sucrose-fed rats ($P < 0.05$). The β-globulin level was higher when the dietary carbohydrate was starch than when it was glucose ($P < 0.05$).

An age effect was observed in α_2-globulin level of starch-fed rats; 350-day-old rats had more of this component than 150-day-old rats ($P < 0.05$).

Strain differences in nonfasted rats. At 150 days of age no significant differences in the serum protein concentrations were observed between BHE and Wistar rats fed the same diet except that PA incidence was higher in the BHE rat fed sucrose than in the Wistar rat. At 350 days of age, however, strain differences were apparent, particularly in starch-fed rats. Albumin and α_1-globulin level was higher ($P < 0.01$), α_2- ($P < 0.05$) and β- ($P < 0.05$) globulin levels were lower in BHE than in Wistar rats. The α_2-globulin concentration was also lower in the BHE strain than in the Wistar strain when sucrose was fed ($P < 0.05$).

Noncholesterol lipid and PA. In a previous study of the nutritional response of the BHE strain to various kinds of dietary fats [5] it was observed that high total serum cholesterol values paralleled high PA concentrations for many of the diets but no direct relationship between them could be established. In this study some sera were analyzed for noncholesterol lipid content (13). At 150 days of age no relationship could be shown between amount of PA and concentration of noncholesterol lipid in either BHE or Wistar rats. However, a positive relationship ($P < 0.01$) between the 2 measurements was found for 350-day-old BHE rats fed any of the 3 carbohydrates regardless of state of fast and is shown in figure 1. A significant correlation ($P < 0.05$) was also observed for 350-day-old Wistar rats fed sucrose.

Urine proteins. A large percentage of BHE rats develop nephrosis [6] by 350 days of age. To determine any possible relationship between the proteins excreted in the urine and the serum proteins, urine samples containing sufficient protein to analyze without further concentration were separated by moving boundary electrophoresis. The mean amounts of the protein

[5] Lakshmanan, F. L. 1961 Effect of age and dietary fat on rapidly migrating serum protein components. Federation Proc., 20: 367 (abstract).

[6] Unpublished data, manuscript in preparation, A. M. A. Durand, M. Fisher and M. Adams, 1967.

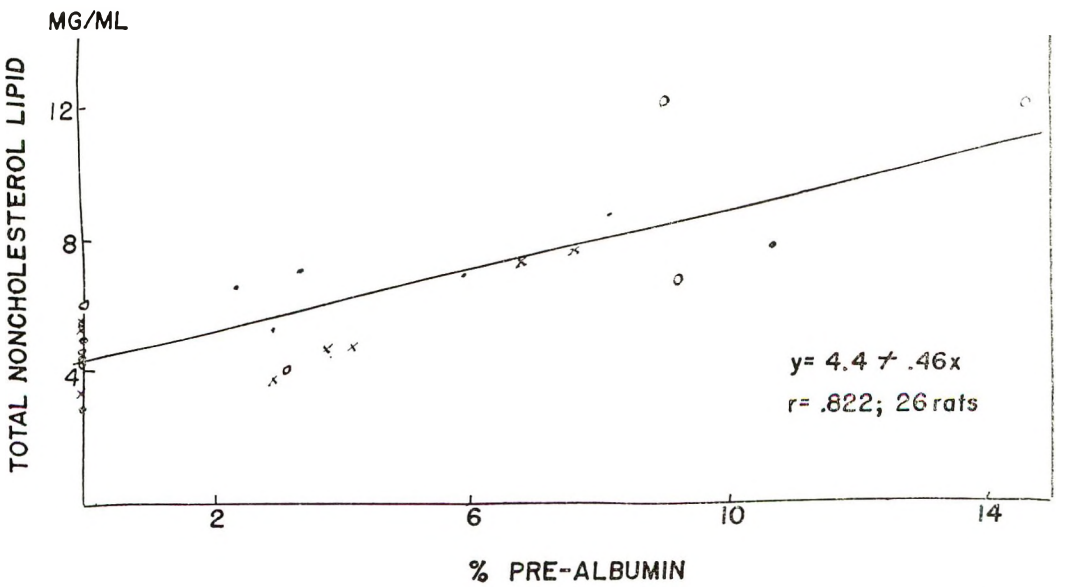

Fig. 1 Relationship of pre-albumin concentration to total noncholesterol lipid content of serum for all 350-day-old BHE rats fed sucrose (●), glucose (×) or starch (○).

components in urine samples collected from 7 BHE rats 350 days of age were 63.6, 12.6, 6.6, 14.5 and 2.8% of the total protein for fractions migrating respectively at the mean rates of 6.6, 5.4, 4.5, 2.8 and 1.7 $cm^2/v/sec$. Mean mobilities for serum fractions — pre-albumin, albumin and α_1-, β-, and γ-globulin — were 7.2, 6.0, 4.5, 2.6 and 1.6 $cm^2/v/sec$, respectively. Mobilities of the proteins in urine and serum did not differ markedly except for the two fastest migrating components. More data will be necessary to establish the significance of the observation and the nature of these 2 proteins. Similar mobilities have been reported by Sellers et al. (14) for individual serum and urine protein components of normal rats and of rats administered renin.

Serum proteins and total urine protein. The relationships between individual serum protein components and total urine protein significant at the 1% level of probability are shown in figure 2. At 350 days of age regardless of diet or state of fast a positive correlation existed between total urine protein and percentage of serum prealbumin but a negative correlation was found between total urine protein and percentage of serum β-globulin. A significant negative correlation was also found between total urine protein and percentage of serum γ-globulin but the scatter was wide. No relationship was observed between total urine protein excreted and total serum protein, albumin and α_1-globulin, or α_2-globulin. Wistar rats excreted very little protein at 350 days of age and no relationship between total urine protein and individual serum protein components was found.

Noncholesterol serum lipid and total urine protein. No relationship between noncholesterol lipid content of sera and total urine protein was observed in rats of either strain at 150 days of age. A positive correlation ($P < 0.01$) between the 2 measurements, however, was found for 350-day-old BHE rats regardless of diet or state of fast (fig. 3).

Serum nonprotein nitrogen (NPN). NPN content was affected by age but not by diet, strain or state of fast. The average nonprotein nitrogen concentration (mg/100 ml) for 150-day-old BHE rats ranged from 27 ± 3 to 38 ± 1 and for 350-day-old BHE rats from 37 ± 3 to 62 ± 23; for 150-day-old Wistar rats from 21 ± 3 to 34 ± 5 and for 350-day-old Wistar rats from 41 ± 3 to 48 ± 3. Variation was less in the Wistar strain than in the BHE strain. The age

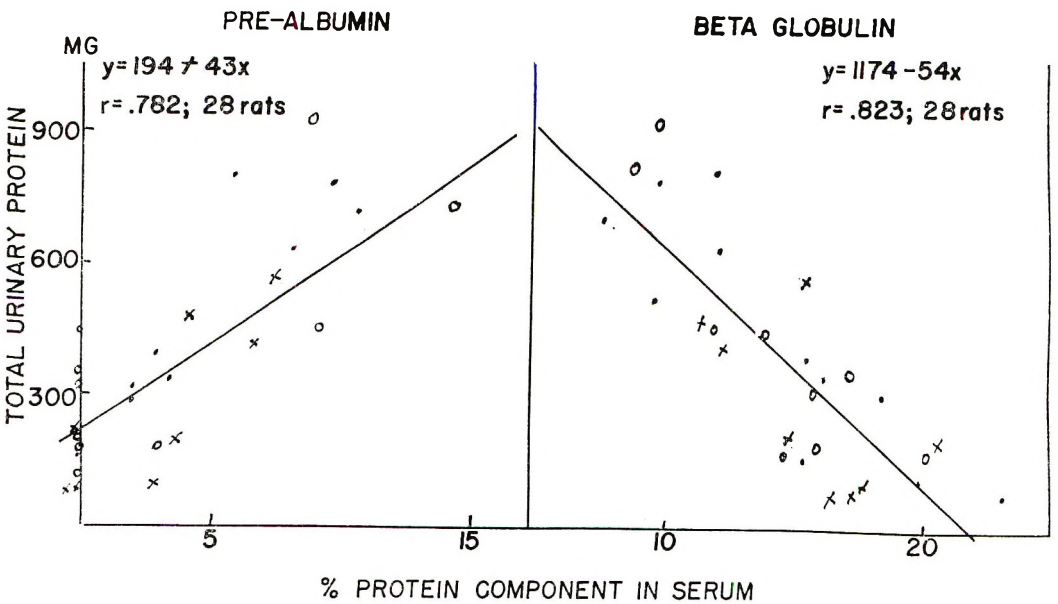

Fig. 2 Relationship of concentration of two serum protein components to urinary protein excreted by all 350-day-old BHE rats fed sucrose (●), glucose (×) or starch (○).

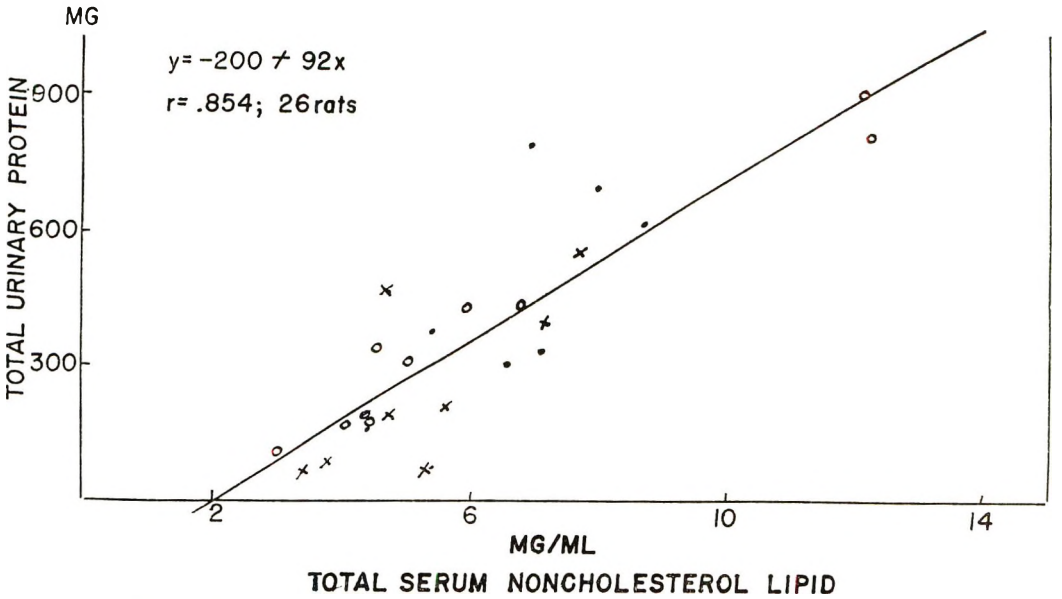

Fig. 3 Relationship of total urinary protein excreted to total serum noncholesterol lipid of all 350-day-old BHE rats fed sucrose (●), glucose (×) or starch (○).

effect was significant for each group of Wistar rats studied except for nonfasted rats fed sucrose and for fasted rats fed starch. This same trend in NPN concentration with age was also observed in the BHE strain but was significant only ($P < 0.05$) for fasted sucrose fed rats.

According to Feher et al. (15) rats would be considered in a uremic condition when blood NPN concentrations rose above 54 mg/100 ml. Regardless of dietary carbohydrate average NPN values in 350-day-old fasted BHE rats were equal to or greater than this value. In contrast, this was not observed in nonfasted older BHE rats nor Wistar rats killed with or without fasting.

Paper electrophoresis. The sera of the animals of this investigation were also examined by paper electrophoresis to determine the nature of the pre-albumin fraction. No protein pre-albumin, however, was detectable using the method recommended in the Beckman manual. By concentrating the serum in a small spot at the point of application as suggested by Ghata (16) instead of a straight line, a protrusion of protein as well as lipid material migrating ahead of albumin could be detected, particularly in sera demonstrated by moving boundary to have pre-albumin. Results of such electropherograms were not quantified. Lipoprotein separation by paper electrophoresis was not distinct for sera of either strain regardless of diet, age or state of fast. One continuum of lipid material was observed from the albumin area or sometimes from ahead of albumin through the β-globulin area. Other investigators (17, 18) also have reported poor fractionation of rat serum lipoproteins by paper electrophoresis.

DISCUSSION

The results have shown that each protein fraction can be affected by changes in dietary carbohydrate but that age, heredity and state of fast were factors contributing to the differences observed in the amounts of the proteins. Coles and Macdonald (19) and Chang and Varnell (20) observed that the chief variation in serum proteins due to type of dietary carbohydrate was in albumin concentration. The former investigators studied the protein-sparing effect that amount as well as type of carbohydrate had on the sedum proteins when a low protein diet was fed to rabbits. The latter investigators were determining whether the influence that the various carbohydrates had on the utilization of wheat

gluten was accompanied by alteration in the serum protein pattern of the Sprague-Dawley rat. Both groups of investigators had used paper electrophoresis which yields a more distinct separation of the albumin and α_1-globulin fraction than moving boundary electrophoresis.

Previous work in this laboratory has shown that state of fast has been found to be a factor contributing to liver size, protein and lipid content, and serum lipid content (13) as well as amounts of serum proteins. At 150 days of age differences in the concentrations of the serum proteins due to diet were almost exclusively confined to fasted rats of either strain; significant differences in liver lipid content due to diet were also confined chiefly to fasted animals. Regardless of state of fast, serum proteins of 350-day-old BHE rats showed no response to kind of carbohydrate except for a high PA level in sucrose-fed fasted animals in which kidney damage was extensive.[6] In contrast, 350-day-old Wistar rats responded to the kind of carbohydrate, and the response differed with the state of fast. At 350 days of age liver lipid content of both strains of rats, however, was affected by diet; fasted and nonfasted animals fed sucrose had more lipid in their livers than those fed glucose. Serum cholesterol levels did not differ with dietary carbohydrate for any of the experimental groups investigated but serum noncholesterol lipid did respond to the kind of carbohydrate in the diet. Noncholesterol serum lipids were generally high in sucrose-fed rats (13).

The development of nephrosis in the BHE strain affects the interpretation of the serum protein patterns. Neither low amounts of total protein nor low levels of albumin in serum, however, were observed in BHE rats maintaining weight and outwardly appearing healthy in spite of nephrotic kidneys. A considerable amount of protein was being excreted in urine of 350-day-old BHE rats and the major fraction in electrophoretic patterns of urine protein migrated at a rate intermediate to prealbumin and albumin. Berg (21) has also reported that serum albumin levels do not change essentially in Sprague-Dawley rats developing spontaneous nephrosis although the predominant protein fraction in nephrotic urine as separated by paper electrophoresis was albumin. Deranged lipid metabolism has been related to the nephrotic syndrome (22). Hyperlipemia in nephrosis may be due to transportation of nonesterified fatty acids by serum albumin since the body depends on these fatty acids for energy. Sera of BHE nephrotic rats were opalescent and some were lactescent indicating increased lipid content but neither hypoalbuminemia nor hyper-β-globulinemia occurred. Hyperpre-albuminemia, however, has been reported (1) to occur with increased kidney damage. With the increased excretion of protein by the kidney more pre-albumin and noncholesterol lipid but less β-globulin were observed in the serum of the nephrotic rat. The rat developing nephrosis spontaneously may be depending on pre-albumin to transport lipid as an alternate supply of energy.

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centimeter(s)	cm
counts per minute	count/min
cubic centimeter(s)	cm^3
cubic millimeter	mm^3
degree(s)	°
degrees of freedom	df (*in tables*)
gram(s)	g
international unit(s)	IU (*to be used only when weight can not be given*)
kilogram(s)	kg
liter(s)	(spell out)
meter(s)	m
microgram(s)	μg (not γ)
micromicrogram(s)	μμg
microcurie(s)	μCi
micron(s)	μ
micromicron(s)	μμ
micromolar (unit of concn)	μM
micromole (unit of mass)	μmole
milligram(s)	mg
milligrams %	(*never use*)
milliliter(s)	ml
millimeter(s)	mm
millimicrogram(s)	mμg
millimicron(s)	mμ
millimole(s)	mmole
molar (mole per liter)	M
parts per million	ppm
per cent	%
probability (in statistics)	*P*
square centimeter	cm^2

[1] Style Manual for Biological Journals 1960 American Institute of Biological Sciences, 2000 P street, N. W., Washington 6, D. C.

square meter	m^2
square millimeter	mm^2
standard deviation	SD
standard error	SE
t (Fisher's test)	*t*
weight (in tables)	wt

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[2] See footnote 1.

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