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RICHARD H. BARNES, *Editor*
Graduate School of Nutrition
Cornell University, Savage Hall
Ithaca, New York

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Biographical Editor

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(1886–1966)

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Harold Hanson Mitchell

— A Biographical Sketch

(1886–1966)

One morning in the spring of 1940 while I (BCJ) was a graduate student in biochemistry at the University of Wisconsin, Professor Hart brought two visitors through the animal laboratory. On asking, I was told that the visitors were Mitchell and Hamilton. The awe with which this information was conveyed is perhaps hard to conceive in our present age of sophistication. However, at that time their book "The Biochemistry of the Amino Acids" was a standard of reference and we had all been exposed to the intricacies of Biological Value. This was my first meeting with Dr. H. H. Mitchell, then at the height of his world famous career. I was fortunate to be interviewed by these distinguished visitors and to be associated with them for the next 25 years at the University of Illinois.

Harold Hanson Mitchell, one of seven children of Charles Page Mitchell and Clara Hanson Mitchell, was born in Evanston, Illinois, on January 22, 1886, and was a life-long resident of Illinois.

He received the Bachelor of Science degree in General Science from the University of Illinois with the class of 1909 and continued his studies at Illinois receiving degrees of Master of Science in Chemistry in 1913 and Doctor of Philosophy in Chemistry in 1915.

While obtaining his degree formally under Dr. H. S. Grindley, Dr. Mitchell confessed to us on occasion that much of his learning and much stimulation toward the direction of his later research came from reading the works of Atwater, Armsby, Benedict, Thomas and other old masters of nutrition.

Thus, his scientific concepts were formed early in his career. The Division is still in possession of a provocative treatise of some 124 pages on the protein requirement of man, "proof read and corrected, October 29, 1909," as indicated in his handwriting on page one. He was among the first proponents of supplementation of a poor protein with another one which would supply the amino acid deficient in the first protein and this was the substance of his Ph.D. thesis.

He was first employed as an assistant analyst working with Dr. H. S. Grindley in the Laboratory of Physiological Chemistry of the Department of Chemistry in July of 1909. In 1911 Dr. Grindley transferred his laboratory to help establish the Laboratory of Physiological Chemistry in the Department of Animal Husbandry in the College of Agriculture. Mainly through Dr. Mitchell's efforts, the name of the laboratory was later changed to the Division of Animal Nutrition (now the Division of Nutritional Biochemistry). Dr. Mitchell started with a Bachelor's degree as Assistant Chemist in the Illinois Agricultural Experiment Station in 1909; with a masters degree, he was promoted to Associate in Animal Nutrition in 1913 and became Associate Professor in 1920, and Professor of Animal Nutrition in 1925. Dr. Grindley ceased active participation in University affairs in 1920 and in 1929 Dr. Mitchell was formally appointed Head of the Division of Animal Nutrition, a position he held until 1952 when he asked that he be relieved of his administrative duties to devote the remaining two years of his academic tenure to writing, particularly to initiating his two volumes on Comparative Nutrition.

Dr. T. S. Hamilton joined the Division upon his return from military service in 1920 having obtained his B.S. in Chemistry in 1917. He later obtained both M.S. (1922) and Ph.D. (1937) degrees in the Division under Dr. Mitchell. From 1920, until he accepted the appointment as Director of the Illinois Agricultural Experi-

ment Station in 1954, he collaborated closely with Dr. Mitchell in the design and management of the experimental projects and in the teaching and administrative duties of the Division.

These years of association with Dr. H. H. Mitchell were years of tremendous stimulation for all of us in the Nutrition Division. The late Dr. Harry Spector joined us in 1943, Dr. R. M. Forbes in 1949, Dr. S. P. Mistry, a postdoctorate of the Division, in 1952, and Dr. H. H. Draper, a student of the Division, in 1954. Dr. Mitchell's distinction and world-wide reputation brought to Illinois students and visitors who are now distinguished nutritionists in many parts of the world, among whom were such stimulating personalities as Dr. Kenneth Blaxter, Dr. Hamish Munro, Dr. Thor Homb, and Dr. John Moustegaard, to name only a few. All of us treasure the memory of this great individual.

Dr. Mitchell, as he was addressed by students and staff, was known behind his back as Mitch. He had a dignity and reserve that argued against using this more familiar term to his face. However, he was unpretentious and I'm sure welcomed the use of this nickname by those of his staff who had been associated with him from the earliest days of the Division. The rest of us used it invariably behind his back but always with respect and kindness.

He was human, and in the days when smoking was not permitted in his laboratories he was known to retire to his car in the parking lot to enjoy his pipe and a couple of innings of a ball game. During the World Series a small portable radio frequently appeared on his office desk where he was able to listen to at least a portion of the games. His devotion to his work, however, more often than not kept him at his desk for hours after the rest of the staff had gone home. Even then one would find him leaving for home laden with enough reports and reading material to keep him busy well into the night.

When planning a new project or before writing a report Dr. Mitchell was often seen sitting quietly at his desk for long periods of time, leaning back in his chair now and then in deep thought. Then he would walk slowly out of his office and down the hall with his head down and his hands clasped behind his back. Few of us disturbed him as he thus moved about the Division. He had a great gift of mental organization, and when he finally returned to his desk he was ready to express his ideas in writing, clearly, concisely and exceptionally well organized. His first attempt on paper — the first handwritten draft — required very little revising or editing.

Dr. Mitchell always welcomed a good argument; and many shall always remember particularly the lengthy and heated discussions he and Dr. Kenneth Blaxter used to have during the latter's year at Illinois. Surely both gained greatly from these exchanges as we all profited from our own discussions with Dr. Mitchell. He usually had more respect for the person who could defend his point of view logically and well, even though his concept might be wrong, than the one who could not. A favorite question of his was, "On what authority do you base your opinion?" He always leveled his criticism at the idea presented rather than at the person. As Dr. W. E. Carroll once stated, "I never knew a man who could think more truly to the kernel of difficult problems and be more generous in cases of differences of opinion than Dr. Mitchell." Unfortunately, not all scientists were willing to accept his pointed criticism in the completely impersonal and friendly manner in which they were offered. I (BCJ) well remember showing him a manuscript being sent to *The Journal of Nutrition*. He told me that if it came to him for review (he was Associate Editor at that time) he would turn it down, since I had used neither paired feeding nor statistics. Another similar anecdote — I (RMF) asked him to read a manuscript I had prepared. A few days later the paper was returned with a note which said, "Thank you for giving me the opportunity to read this very interesting but unconvincing manuscript."

Much of his early research was supported by Hatch funds. He was under contract with the Armed Forces for a number of years during and after World War II. Members of his staff were recipients of Federal grants. Nevertheless, he was never in sympathy with any "new deal"

or "new frontier." He vehemently opposed any intervention by the Federal government in the design or execution of academic research, in any issues which he thought the state or local officials could negotiate, or in any private enterprise that the individual could manage himself.

He did, however, have definite interest in national and international affairs and in local civic issues. His many letters to congressmen, his letters to the F.D.A. in support of national acceptance of fish flour as a wholesome source of protein, and those to the editors of the local papers during a campaign for fluoridation are evidence of his active participation in current national and local affairs. He carefully limited this participation, however, to areas of knowledge with which he was well versed.

Mathematics had been his first minor in his pursuit of a Ph.D. degree. From that early period on, the analytical and statistical viewpoint functioned in almost all facets of his everyday life. He had a hobby of reading mystery stories, the more complicated and difficult to solve, the better. He thoroughly enjoyed working puzzles, perhaps finding more satisfaction in the follow-up calculation of the probability of winning. Once given the high score on a national typing speed test he calculated the number of letters his typists should be able to finish in a day. Fortunately they were never held to this estimate.

It was difficult for him to engage in conversations unrelated to his research interests and he could not understand how others could have ideas different from his in such areas as social responsibility and social justice, war and peace and so on. His remark after a discussion was sometimes, "How can he think that way?" Many envied him the absolute sureness he had of the logic of his viewpoint.

Dr. Mitchell was handicapped by an inability to read at ease under ordinary lighting conditions, so that most of his achievements were accomplished while he was suffering from high blood pressure and very poor eyesight — severe myopia, glaucoma and developing cataracts. When he walked down the street his gait for many years was slow and cautious, with eyes cast upon the ground to watch each step. He crossed busy intersections in similar fashion; however, automobile drivers seemed to sense his difficulty and he was never hit. When acquaintances passed him, unless they spoke first he failed to recognize them. However, he always caught and identified a familiar voice.

His inability to see well, to read signs at any distance or to recognize familiar faces, and a pride that prevented him from asking small favors, were factors greatly responsible for his low attendance record at many professional meetings beyond those held on the University of Illinois campus. While his colleagues were at a meeting he busied himself at his desk; he had the abstracts before him, and with proper lighting and magnifying glasses at hand he carefully read and evaluated the contributions — even recalculated some of the data. It was often disconcerting, indeed astonishing, to his co-workers who, on returning from that particular meeting or symposium found that he could discuss a paper quite thoroughly. His personal physical barriers did not dampen his interests or prevent his scientific accomplishments. After his cataracts were removed in 1955 he commented, "My, what I could have accomplished these past 30 years with the eyes I now possess!"

His family and his work were his life. He was married to Ethel Opal Kilbury in Urbana on July 8, 1910. He was a devoted husband and father of four children, Donald Stanley, David Kilbury, Marguerite Evelyn and Robert Hanson. Robert, the youngest, lost his life serving in the Pacific early in World War II. Dr. Mitchell never failed to remember a birthday or important anniversary of any member of his family. Owning a home within a few blocks of the campus the Mitchells rented rooms to three or four men students after their own children had finished their education until the time of Dr. Mitchell's death. Many a student, according to the late Professor Sleeter Bull, waited patiently for the opportunity to become a part of that happy household. Once under that roof the student usually remained for as long as he was in residence at the University. Dr. Mitchell proved to be somewhat awkward in his attempts to handle simple household repairs. With a reassur-

ing comment from his wife, such as, "Now, Harold, go on to your office; the furnace will be working by the time you return," he would start slowly to his office. The student residents more often than not came to Mrs. Mitchell's assistance once her husband was out of sight.

Dr. Mitchell seldom used all of his vacation time in any one year, nor did he and his wife travel extensively. Instead, Mrs. Mitchell, a blithe and vivacious spirit, made the home a haven for joyous reunions. Their three children returned home frequently with their families or their friends. Numerous colleagues and students of Dr. Mitchell were also known to spend many a pleasant day at 909 West Nevada Street.

As is well known, Dr. Mitchell's research program extended far beyond the area of animal science. Early in the 1930's there were collaborative studies already in progress with Dr. W. A. Ruth of the Department of Horticulture on the potential toxicity of foods and feeds treated with spray residues containing lead, arsenic and fluorine or contaminated by absorption of these elements through the plant roots. Studies of the physiological balance and tissue deposition of these substances followed in logical sequence and in the early 1940's Dr. Mitchell served as a consultant in a legal suit concerning industrial fluorine intoxication of livestock.

His interest in fluorine turned also to the function of minute quantities in tooth formation and in skeletal development and composition. Dr. Isaac Schour of the University of Illinois College of Dentistry and Dr. F. J. McClure (Dr. Mitchell's first Ph.D. student) of the National Institutes of Health joined Dr. Mitchell in these early projects on the beneficial effects of minimal amounts of fluorine for both man and beast.

He also collaborated with Dr. Julia Outhouse, Dr. Gladys Kinsman, Dr. Janice Smith and others of the Department of Home Economics in human studies of the utilization of, and requirements for, calcium and various proteins. University students served as the experimental subjects. Concurrently Dr. Mitchell worked with Dr. F. R. Steggerda in the Department of Physiology, particularly on experiments concerned with the balance and utilization of, and requirements for calcium by adult man.

During the years of World War II and for a short period thereafter, Dr. Mitchell was awarded a contract with the Office of Scientific Research and Development of the Armed Forces; and, in cooperation with Dr. M. F. Fahnestock of the College of Engineering and Dr. R. W. Keeton and his colleagues in the College of Medicine, he directed experiments concerned with the role of nutrition in the reactions of man to some of the stresses of his physical environment. He also collaborated with the College of Medicine in studies on the significance of nutritional status in convalescence after surgery or disease.

An air-conditioned metabolic chamber built in Davenport Hall in 1929 and used in the Division for energy studies on large animals was later reconstructed to accommodate human beings and to simulate tropical and desert conditions. A similar one was built at the College of Medicine to simulate arctic conditions, and a low-pressure chamber was constructed in the College of Engineering to study the effects of altitude. From the composite results of these coordinated studies, all made with conscientious objectors as the experimental subjects, Dr. Mitchell developed his intense interest in the adaptive capacity of man to his physical environment and to different planes of nutrition.

Between the years 1930 when his first graduate student (Dr. F. J. McClure) obtained his Ph.D., and 1955 when Dr. Mark Bert obtained the last Ph.D. under Dr. Mitchell's direction, he gave 17 Ph.D. degrees. These students included such other leaders in nutrition as Dr. T. S. Hamilton, Dr. E. Wise Borroughs, Dr. E. P. Singsen, Dr. Lorin Harris, and Dr. W. H. Pfander, to name but a few.

However, Dr. Mitchell did much of his research not with students but with the help of a dedicated technical staff who were associated with the team of Mitchell and Hamilton for many years. Mr. William Toon Haines, Teenie to all of us, came to the Division in 1916. He carried out carefully and accurately the large animal, and later human experiments, designed by Dr. Mitchell and planned in de-

tail by Dr. Hamilton. For 44 years Teenie helped keep Dr. Mitchell supplied with data from which came the calculations of farm animal requirements for energy and protein and later the calculations of nutrient losses in man under tropic and desert conditions. It was Teenie who first showed me (BCJ) how to clean a sheep cage for the collection of urine and feces, in the days when Dr. Mitchell was studying the utilization of urea nitrogen by the ruminant. In a smaller way, for almost 30 years (1924–1950), Miss Jessie Beadles carried out the small animal research planned by Dr. Mitchell on calcium and phosphorus requirements, on iron metabolism and of course, the thousands of determinations of biological value of protein which made up a very considerable portion of Dr. Mitchell's research.

One of Dr. Mitchell's very important contributions to the experience of all graduate students, major and minor, in animal nutrition over a period of more than 25 years was his advanced nutrition course. This course met 5 days a week for one semester. As one of his students exclaimed upon returning to the laboratory for a visit, "It is the one true graduate course in the University." As the years went by the course continued to emphasize more and more the quantitative aspects of all nutrition. Dr. Mitchell insisted he was a nutritionist, not a biochemist, and as the complexities of intermediary metabolism became elucidated he continued to fight for his first love — nutrition — the summation of all the biochemical reactions in the living organism. This course culminated in the writing of his two volumes "Comparative Nutrition of Man and Domestic Animals." Another source of continuing stimulation to both staff and students was the Animal Nutrition Seminar which met for two hours each week and was attended not only by his own staff but also by Dr. H. E. Carter and Dr. Carl Vestling from Biochemistry, Dr. Robert E. Johnson from Physiology, Dr. Julia Outhouse Holmes and Dr. Wilhelmina Armstrong from Home Economics and many other distinguished members of the University of Illinois faculty.

It was in this seminar that many of Dr. Mitchell's ideas were most forcefully presented and discussed, pro and con, with great good will and enthusiasm. Many papers were reviewed and always his insistence on statistical treatment of the data and on controlled food intake was an important part of the discussion. Dr. Mitchell often said that there was no nutritional experiment in which food intake was not an important part of the data. We remember the chagrin, however, of one of his students who tried to determine the thiamine requirements of female rats for gestation using paired feeding!

Few investigators have had an influence on nutritional research and development of sound nutritional concepts equal to that of Dr. Mitchell. He excelled in the ability to correlate and integrate data and ideas from a variety of sources. This quality of keen perception and quick recognition of pertinent facts and his ability to pursue a problem to its logical solution, is largely responsible for his many achievements and contributions to science. His research program was characterized by logical planning and minute attention to the control of experimental conditions. He was an early and very strong advocate of the application of statistical treatment of experimental data.

His influence on nutritional science was international. In 1937 he was one of eleven scientists, and the only one from America, to be invited to present a paper at the Volta Congress sponsored by the Royal Academy of Italy. His professional correspondence, both national and international, was voluminous. He served as Corresponding Editor of *Nutrition Abstracts and Reviews* during World War II, sending to the home office in Aberdeen abstracts taken from journals that were not then available from the European continent and also choosing authors for some of the major review articles for the publishers.

He was a member of the original 1928 Editorial Board of the *Journal of Nutrition*. He was appointed to the Board on two other occasions, 1939 and 1948, and he served as associate editor from 1941 to 1944. This position he accepted despite the fact that throughout this period he read everything through a large magnifying glass on a stand on his desk. He took his editorial responsibilities very seriously

and we believe his critical hand contributed to the present excellence of our journal.

Among his many contributions to nutritional science the following are outstanding:

1) Development of the Biological Value method of determination of the nutritional value of proteins.

2) Demonstration of the correlation between the nutritive value of a protein and its content of essential amino acids as determined by animal experimentation.

3) Use of controlled feed intake in nutrition studies, and the use of proper controls and consideration of *all* excretory pathways in the accurate estimation of nutrient requirements of man and animals; and for studies of energy, mineral, nitrogen and water balances.

4) Innovation of the factorial approach of the determination of nutrient requirements.

5) Demonstration of the value of nutrient balance in rations.

6) Studies of the protein requirement of man and domestic animals.

7) Studies of the energy requirements of domestic animals and man.

8) Studies of the mineral requirements, particularly calcium, of animals and man.

9) Analysis of the composition of a complete human body for various minerals and nitrogen (the first analysis of its kind).

10) Studies of adaptation to different planes of nutrition.

11) Effect of environmental temperature (particularly hot humid versus hot dry) on nutrient requirements of man.

Not long before he became professionally inactive I (ME) asked Dr. Mitchell what he considered to be the best of his contributions to the field of nutrition. With little hesitation he enumerated these in the following order: 1) the biological value of proteins; 2) the amino acid index in evaluation of proteins; 3) the "validity" of Folin's concept of dichotomy of protein metabolism; 4) composition of the entire human body; 5) dermal loss of nutrients and its effect upon balance studies; 6) demonstration of nutrient requirements for adult growth, i.e., for hair, skin, nails; and 7) physiological adaptation to nutritional levels and adaptation of the organism to environment and to conditions in general.

Dr. Mitchell's research was profound and lasting. His long-time associate, Dr. Hamilton, has said that each of his approximately 300 publications is characterized by a thoroughness of scientific approach seldom seen in biological experimentation. His researches were planned precisely, the plans were followed with exactness, and his data were critically evaluated so that his research represents the most permanent type of scientific endeavor. His ability as a fair and just critic has been directly and indirectly responsible for many noteworthy contributions from other laboratories.

Together with accomplishments in research Dr. Mitchell was known for his meticulous and astute approach to the scientific literature and for his great ability to summarize and evaluate critically the nutritional science issues of his day. He was firm in his opinions, but always a gentleman in their defense.

Although he was not considered an impressive lecturer, attendance in his classes was a rewarding experience. His course outlines were scrupulously organized and correct in detail; the material was comprehensive and all inclusive. By presenting individual papers directly to the class he stimulated the students to analyze and evaluate the data and to defend their own conclusions. With his breadth and depth of knowledge, his keen perception of values, and his varied and diligent scrutiny of the literature, the courses Dr. Mitchell taught were always open and direct avenues to research. He never failed to correlate teaching with research.

During a 10-year period after retirement Dr. Mitchell continued his writing, publishing some 18 papers. As a culmination of his long teaching and research experience he devoted the greater portion of these retirement years to writing his two-volume treatise on Comparative Nutrition of Man and Domestic Animals.

He claimed always that books were his teachers. This opinion was reflected in the extensive library he maintained, first in his office and later in the Divisional reading room. He purchased numerous texts and subscribed to the leading periodicals

in his field of interest with money out of his own pocket, making this material available to his students and expecting them to use it. He continued to add to his collection as long as he was professionally active — as late as 1964. The major portion of this library still remains in the Division, as well as an extensive file of abstracts and reprints which he instigated very early in his career and for which Miss Helen Keith, Mrs. Elizabeth Curzon and Miss Marjorie Edman were successively responsible. Throughout his career he depended heavily on the bibliographic and editorial services of these co-workers.

Dr. Mitchell was a member of the Advisory Committee of the United States Department of Agriculture Soil, Plant and Nutrition Laboratory located in Ithaca, New York, during its early and formative years. He served on the Committee of Animal Nutrition of the Agricultural Board, National Research Council — National Academy of Sciences, from 1925 to 1945.

Other scientific associations of which he was a member are: Sigma Xi, serving as President of the Illinois Chapter in 1942; Phi Lambda Upsilon; Phi Kappa Phi; Society for Experimental Biology and Medicine; American Society of Biological Chemists; American Dietetic Association; American Chemical Society; American Association of University Professors; and American Association for the Advancement of Science.

Important awards which he received include: the Borden Award, 1945 (administered by the American Institute of Nutrition) for investigations related to human calcium requirement and the nutritive value of milk; the Morrison Award, 1960 (administered by the American Society of Animal Production and one of the highest tributes in the field of agriculture) for his outstanding contributions in the knowledge of proteins, their value and animal requirements; and the Osborne and Mendel Award, 1966 (administered by the American Institute of Nutrition). The latter award citation reads, "For his preeminent studies in protein, mineral and energy metabolism, culminating in his authorship of the two-volume compendium, Comparative Nutrition of Man and Domestic Animals, for his skill and precision in research and his capacity to interpret and correlate physiological phenomena which have endowed us with a rich heritage of basic principles in nutrition."

In 1959 he acted as honorary chairman of the program dedicating Burrill Hall, the new biology building on the University of Illinois campus. In the same year he was one of the four scientists honored by the American Association of Heating and Air-Conditioning Engineers at the Arthur Cutts Willard commemorative dinner. This occasion was in recognition of the contributions made by President Willard and four other University of Illinois scientists, Raymond B. Allen, Robert W. Keeton, A. P. Kratz, and H. H. Mitchell, for their respective contributions to means of coping with some of the problems imposed upon man by his immediate environment.

He again was honored in company with Professors T. S. Hamilton and W. C. Rose at a symposium on Protein Nutrition and Metabolism held in 1962 at the University of Illinois and dedicated to these three men for their contributions to this area of research.

His circle of scientific admirers is wide and he had a close circle of intimate friends. Those who knew him well found themselves in the presence of a warm and generous character with a boundless curiosity and a genuine modesty. All nutritionists are his beneficiaries.

MARJORIE EDMAN

R. M. FORBES
Division of Nutritional Biochemistry
Department of Animal Science
University of Illinois
Urbana, Illinois

B. CONNOR JOHNSON
Department of Biochemistry
University of Oklahoma Medical Center
Oklahoma City, Oklahoma

Choline Biosynthesis in Germfree Rats [1]

EVA KWONG, GRACE FIALA, RICHARD H. BARNES, DORINNE KAN AND STANLEY M. LEVENSON

Graduate School of Nutrition, Cornell University, Ithaca, New York, and Department of Surgery, Albert Einstein College of Medicine, Yeshiva University, Bronx, New York

ABSTRACT Liver choline biosynthesis was studied in germfree rats receiving a choline-deficient diet. Weanling conventional open-animal-room and ex-germfree conventionalized rats fed the choline-deficient diet for 2 weeks had elevated liver fat and decreased specific activities of liver choline following injection of 2 $\mu Ci^{14}CH_3$-labeled methionine into the portal vein. On the other hand, in germfree rats fed the choline-deficient diet there was no significant increase in liver fat nor was there a decrease in the rate of liver choline biosynthesis as measured by labeled methyl transfer. In these respects germfree rats fed a choline-deficient diet appeared to be the same as control rats fed a diet with adequate choline. These findings are consistent with a previous observation of a lessened nephropathy in germfree rats fed a choline-deficient diet, and also are similar to results obtained with conventional rats in which coprophagy had been prevented.

In a previous study (1), it was proposed that prevention of coprophagy protects young rats from choline deficiency by maintaining choline biosynthesis in the liver. Since prevention of coprophagy may alter the relative numbers of various intestinal bacterial species (2–4), this protection and the maintenance of choline biosynthesis was postulated to be related to the blocking of microbial reinoculation of the gut that results from the ingestion of fresh feces (1). Further, it was known that germfree rats are much more resistant to acute choline deficiency nephropathy than open-animal-room or conventionalized rats.[2] To study the protective mechanisms of coprophagy prevention and the germfree state further, a collaborative study between the Germfree Laboratory of S. M. Levenson, Albert Einstein College of Medicine, and the Graduate School of Nutrition Laboratory of R. H. Barnes, Cornell University, was initiated to investigate the rate of choline synthesis in the liver of normal and choline-deficient young male germfree rats.

EXPERIMENTAL

Male weanling rats of the Fischer strain (21–23 days old) were used in this study. The basic composition of the test diet is shown in table 1. It was autoclaved at 260° for 25 minutes. One and one-half milligrams of choline per milliliter of drinking water were given to the 3 control groups of open-animal-room (OAR), conventionalized (ex-germfree-CONV),[3] and germfree rats (average 16.5 to 18 mg choline/day). To prevent the high mortality that results from feeding a choline-free diet to weanling rats, experimental groups were given water containing 0.36 mg choline/ml of water for 6 days (average 3.6 to 4.3 mg choline/day). Thereafter, the experimental rats were given water without choline. Both food and water were provided ad libitum. Water consumption was measured every other day, but food intake was not determined. Rats were weighed at the start of the study and twice a week thereafter. Six rats in each group were housed in plastic

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[1] At Cornell University this research was supported in part by funds provided through the State University of New York and a research grant from the National Science Foundation. At Albert Einstein College of Medicine, Yeshiva University, this research was supported in part by National Institutes of Health Grants no. 5P01 AM 05664 AMP and 5-K5-GM-14,208-05 (Career Research Award to Dr. Stanley M. Levenson).

[2] Levenson, S. M., A. L. Nagler, E. Seifter and E. F. Geever 1967 Influence of microorganisms, aureomycin, neomycin and vitamin B_{12} on acute choline deficiency in Fischer rats. Federation Proc., 26: 526 (abstract).

[3] Charles River Breeding Laboratories, Wilmington, Massachusetts. Conventionalized rats were littermates of the germfree rats contaminated with cecal contents of open-animal-room rats on the day after weaning, the day the experiments were started, and maintained in the same sort of isolators as the germfree. The term "germfree" as used in this paper refers to rats free from viable bacteria, parasites, or fungi as determined by methods published elsewhere (5).

TABLE 1

Composition of choline-deficient diet

	g
Casein [1]	10
Dextrin	60
Hydrogenated vegetable oil [2]	20
Salts [3]	4
B-vitamins	4
Fat-soluble vitamins in corn oil	2
Total	100

B-vitamins in 4.0 g dextrin

	mg
Thiamine·HCl	0.80
Riboflavin	1.60
Pyridoxine·HCl	0.80
Ca pantothenate	8.00
Niacin	8.00
Inositol	40.00
Biotin	0.04
Folic acid	0.40
Vitamin B_{12}	0.006
Menadione	2.00

Fat-soluble vitamins in 2.0 g corn oil

	mg
Vitamin A acetate	0.62
Vitamin D (calciferol)	0.009
α-Tocopherol	10.00

[1] Casein, extract, vitamin tested, General Biochemicals, Chagrin Falls, Ohio.
[2] Primex, Procter and Gamble Company, Cincinnati.
[3] Hubbell, R. B., L. B. Mendel and A. J. Wakeman 1937 A new salt mixture for use in experimental diets. J. Nutr., 14: 273.

isolators immediately upon arrival at the laboratory. The husbandry techniques used and the microbiologic testing have been described elsewhere (5).

After receiving the experimental diet for 2 weeks, the rats were anesthetized with ether and 2 μCi L-methionine methyl-^{14}C were injected into the portal vein. Fifteen minutes later the liver was excised and an aliquot was homogenized with an equal volume of 10% TCA and 2.5% $MgCl_2$. Phospholipid was extracted and hydrolyzed and the free choline was determined quantitatively by precipitation as the Reinecke salt and then dissolved in acetone and assayed colorimetrically (1). Radioactivity was determined in a Packard liquid-scintillation counter. Liver fat was measured in an aliquot of fresh liver which was weighed, ground with anhydrous sodium sulfate and extracted with chloroform in a Soxhlet extractor for 4 hours. The chloroform extract was dried to constant weight. The kidneys were examined histologically after fixation in 10% buffered formalin. Hematocrit, plasma total solids concentration, plasma urea nitrogen concentration and liver cholesterol (6) were determined by standard procedures.

RESULTS AND DISCUSSION

Results in table 2 indicate that there was no growth difference between control and experimental rats in all 3 groups; however, the germfree rats were slightly heavier than the OAR and CONV rats. Germfree rats have enlarged ceca which are much larger than those of conventional rats (7). Hence, the 5 to 8 g difference in body weight may be accounted for by the increased cecal contents in the germfree rats.

Since a small amount of choline was added at the beginning of the experiment, none of the experimental animals had abnormal plasma urea nitrogen concentrations at killing and none had significant changes in kidney morphology, nor was there significant difference in plasma total solids concentrations. The hematocrit was slightly lower in the choline-deficient conventionalized rats but the significance of this observation is not understood. Anemia in acute choline deficiency is seen only in rats with severe hemorrhagic nephropathy.

Choline biosynthesis in the liver as measured by the specific activity of liver choline following the injection of methyl-labeled methionine into the portal vein is detailed in table 3. The findings in the 3 control groups were almost identical. In the OAR and CONV choline-deficient rats, however, the higher levels of liver fat were associated with lower specific activities of liver choline. In the germfree rats, the liver fat did not increase significantly when they were fed the choline-deficient diet and, at the same time, liver choline specific activity did not decrease. The decreased specific activity of liver choline in the OAR and CONV choline-deficient rats was accompanied by slightly lower liver choline content and the lower total choline counts rule out the possibility that the lowered specific activity was due to dilution by nonlabeled choline. These results are in agreement with the studies utilizing coprophagy prevention in rats (1).

TABLE 2

Body, liver, and kidney weights, hematocrits, plasma total solids, and plasma urea nitrogen in germfree, conventionalized and open-animal-room rats

	Open-animal-room		Conventionalized		Germfree	
	Control (A)	Experimental (B)	Control (C)	Experimental (D)	Control (E)	Experimental (F)
Body wt, g	52.8 ± 1.56 [1]	52.8 ± 2.15	53.8 ± 1.14	55.8 ± 3.65	60.8 ± 2.58	60.3 ± 3.48
Liver wt, g	2.02 ± 0.08	2.21 ± 0.11	2.04 ± 0.10	2.07 ± 0.17	2.05 ± 0.09	2.32 ± 0.14
Kidney wt, g	0.52 ± 0.01	0.53 ± 0.02	0.56 ± 0.03	0.54 ± 0.02	0.56 ± 0.03	0.57 ± 0.03
Kidney wt, % body wt	0.98	1.01	1.05	0.97	0.93	0.95
Hematocrit, %	44.8 ± 1.2	43.3 ± 1.8	42.6 ± 1.0	38.3 ± 1.0 [2]	40.8 ± 1.6	43.2 ± 1.3
Total solids in plasma, % [3]	6.47 ± 0.36	6.43 ± 0.30	5.93 ± 0.15	5.65 ± 0.15	6.14 ± 0.14	6.16 ± 0.19
Plasma urea nitrogen, mg/100 ml	19 ± 1	20 ± 1	18 ± 2	19 ± 1	18 ± 1	19 ±2

[1] SE of mean.
[2] Control and experimental value different ($P < 0.025$).
[3] Determined by refractometry.

In the study of Levenson et al.[2] germfree rats showed less nephropathy and lower mortality than OAR or CONV rats, but accumulation of liver fat was enhanced when they were fed diets deficient in choline and vitamin B_{12} but supplemented with 5% cholesterol and 0.4% cystine. The difference between their finding increased liver fat in the germfree rat and the present finding of no increase may possibly be explained by the enhanced degree of choline deficiency produced in their study by feeding cholesterol and cystine.[2] In support of this view is their finding less liver fat in germfree rats than in conventionalized or OAR rats when cholesterol and cystine were not added, so that the diet induced a milder choline deficiency more nearly analogous to that used in our present experiments.

The fact that germfree rats do not become choline-deficient as rapidly as OAR and CONV rats may be a result of the lower basal metabolic rate of germfree rats.[4] The absence in germfree rats of choline destruction by its conversion to trimethylamine (8) and the absence of bacterial utilization of methionine could also result in a reduction in their choline requirement. From the results of the present study, it is certainly clear that the germfree rat retains the ability to synthesize liver choline to a greater extent than the OAR and CONV rats when subjected to a mildly choline-deficient diet. The mechanisms by which decreased choline destruction and increased choline synthesis are related and how these two phenomena contribute to the choline economy and protect against acute choline deficiency are uncertain both for germfree rats and in the case of OAR rats under conditions of coprophagy prevention. It is believed by Kwong and Barnes that coprophagy prevention exerts its effect in increasing hepatic choline biosynthesis and in protecting such rats from the early effects of choline deficiency (liver fat accumulation and nephropathy) by altering the intestinal

[4] Levenson, S. M., D. Kan, M. Lev and F. S. Doft 1968 Influence of microorganisms on mammalian metabolism and nutrition with specific reference to oxygen consumption, carbon dioxide production and colonic temperatures. In press.

TABLE 3

Liver cholesterol, fat, and synthesis of choline in germfree rats [1]

	Open-animal-room		Conventional		Germfree	
	Control (A)	Experimental (B)	Control (C)	Experimental (D)	Control (E)	Experimental (F)
Liver cholesterol, mg/g wet wt	1.64 ± 0.26 [2]	1.87 ± 0.29	1.52 ± 0.22	1.65 ± 0.10	1.49 ± 0.08	1.47 ± 0.09
Total cholesterol in liver, mg	3.28 ± 0.51	4.15 ± 0.67	3.11 ± 0.51	3.39 ± 0.35	3.05 ± 0.19	3.41 ± 0.29
Total fat, % in liver	4.47 ± 0.36	6.92 ± 0.20 [3]	4.32 ± 0.15	6.35 ± 0.89 [4]	4.30 ± 0.28	5.15 ± 0.67
P value	(A)vs.(E):ns	(B)vs.(F):0.05	(C)vs. (E):ns	(D)vs.(F):ns		
Liver choline, mg/g wet wt	0.38 ± 0.06	0.24 ± 0.05	0.20 ± 0.07	0.15 ± 0.04	0.25 ± 0.04	0.19 ± 0.03
Total choline in liver, mg	0.78 ± 0.14	0.51 ± 0.10	0.42 ± 0.14	0.33 ± 0.10	0.52 ± 0.09	0.43 ± 0.07
^{14}C-choline, cpm/mg choline	25,781 ± 1,975	16,688 ± 3,539 [4]	26,536 ± 2,519	15,755 ± 2,736 [5]	24,694 ± 2,759	23,661 ± 974
Total ^{14}C-choline	19,782 ± 3,451	8,768 ± 3,219 [4]	12,033 ± 4,838	6,241 ± 2,756	12,814 ± 2,475	10,109 ± 1,630

[1] Male rats of the Fischer strain 36 days old when killed (phospholipid choline and free choline were not measured).
[2] SE of mean.
[3] Control and experimental value different ($P < 0.001$).
[4] Control and experimental value different ($P < 0.05$).
[5] Control and experimental value different ($P < 0.025$).

microflora. Evidence for this is their finding a decrease in trimethylamine formation from choline when coprophagy is prevented.[5] The greater resistance to choline deficiency of germfree rats or OAR rats prevented from practicing coprophagy may enable them to retain the capacity for hepatic synthesis of choline longer than OAR and CONV rats which practice coprophagy. On the other hand, the opposite explanation may hold and germfree or OAR rats prevented from practicing coprophagy may be resistant to nutritional choline deficiency because they retain the ability to synthesize choline in their livers. In the first case, resistance to choline deficiency might be due directly to a conservation of body choline through decreased requirement and decreased bacterial destruction. In the second case, protection would be due primarily to an increased synthesis of choline. Obviously both mechanisms could be contributing to the total choline economy of the body.

Even though the transmethylation measurement has been referred to as choline synthesis, this process actually involves the methylation of a phospholipid ethanolamine moiety. Therefore, the amount of this precursor available for methylation could have a marked effect upon the specific activity of the phospholipid choline that was recovered.

ACKNOWLEDGMENT

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[5] Chen, R., E. Kwong and R. H. Barnes 1967 Urinary trimethylamine following choline administration in the study of intestinal microflora of the rat. Federation Proc., *26:* 526 (abstract).

Effect of *N*-(α-Methylbenzyl) Linoleamide on Lipid Levels of Plasma and Liver in Cholesterol-fed Rats

H. FUKUSHIMA, S. AONO AND H. NAKATANI
Research Department, Pharmaceuticals Division, Sumitomo Chemical Company, Ltd., Osaka, Japan

ABSTRACT Cholesterol-lowering effect of *N*-(α-methylbenzyl) linoleamide (MBLA) was studied in rats fed cholesterol for 8 weeks and compared with that of *N*-cyclohexyl linoleamide (CHLA). By addition of 0.2, 0.1 or 0.05% of MBLA or 0.2% of CHLA to the cholesterol diet, the elevation of plasma cholesterol levels was markedly depressed and the deposition of lipids in the liver was also depressed. The estimated potency of MBLA was about 6.5-fold of that of CHLA with respect to plasma-liver cholesterol pools. The mixture of linoleic acid and α-methyl benzylamine (1:1, by molecular weight) did not show any significant effect on plasma and liver lipid levels.

Previously it was reported that *N*-cyclohexyl linoleamide (CHLA)[1] showed a marked cholesterol-lowering and anti-atherogenic effect in rabbits fed cholesterol (1, 2); its cholesterol-lowering effect was thought to be caused by interference with cholesterol absorption from the intestinal tract as with sitosterols (3). From further studies on various amide derivatives of fatty acids synthesized in this laboratory, *N*-(α-methylbenzyl) linoleamide (MBLA)[2] was found to have a more pronounced cholesterol-lowering effect on rabbits fed cholesterol (4).

The present study was conducted to determine the influence of MBLA and CHLA on plasma cholesterol levels and liver lipid levels in rats fed cholesterol.

MATERIALS AND METHODS

Test compounds. *N*-(α-Methylbenzyl) linoleamide (MBLA) and *N*-cyclohexyl linoleamide (CHLA) were prepared from purified linoleic acid and DL-α-methyl benzylamine or cyclohexylamine. The structures of MBLA and CHLA are as follows:

$$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_7CONHCH(CH_3)C_6H_5$$

MBLA

$$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_7CONHC_6H_{11}$$

CHLA

The physical constants and analytical data were the same as those reported previously (4).

Diets. The composition of the high cholesterol diet, in grams per 100 g, was as follows: casein, 20; hydrogenated coconut oil, 10; sucrose, 62.3; agar, 2; cholesterol, 1; ox-bile extract, 0.5; salt mixture (5), 4; and an adequate amount of vitamins.[3] The test compound was added to the high cholesterol diet at the expense of sucrose.

Animals and feeding. Male rats of the Wistar strain, approximately 150 g, were maintained on a commercial diet[4] for 1 week and those which showed a normal increase in body weight were used for the experiment. In experiment 1, 68 animals were divided into 7 groups so that the distribution of body weight was comparable among the groups. They were housed 3 or 4 in a cage and the diet and water were supplied ad libitum. Group 1 was given the high cholesterol diet, as a control, and other groups received the high cholesterol diet supplemented with the test compounds shown in the upper part of table 1.

In experiment 2, pair-fed experiment, rats were housed in screen-bottom cages. Group 8 (11 rats) was given the high cholesterol diet as control and group 9 (10

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[1] Clinolamide, international non-proprietary name. Linolexamide was one of the suggested international non-proprietary names.

[2] Code number of this compound is AC-223.

[3] Vitamins: (mg/kg diet) choline chloride, 1500; inositol, 1000; niacinamide, 80; Ca pantothenate, 50; p-aminobenzoic acid, 20; *dl*-α-tocopherol acetate, 10; vitamin A acetate, 8.62; thiamine·HCl, 5; riboflavin, 5; menadione, 5; pyridoxine·HCl, 2.5; folic acid, 2; biotin, 0.2; calciferol, 0.063; and cyanocobalamin, 0.02.

[4] CLEA Mouse/Rat Chow (CE-2), Japan CLEA Company, Tokyo, Japan.

TABLE 1
Experimental design

Group	Treatment	No. of animals
	Experiment 1	
1	None (high cholesterol diet)	10
2	MBLA,[1] 0.2% in high cholesterol diet	11
3	MBLA, 0.1% in high cholesterol diet	10
4	MBLA, 0.05% in high cholesterol diet	11
5	CHLA,[2] 0.2% in high cholesterol diet	6
6	CHLA, 0.1% in high cholesterol diet	7
7	LA + α-MB,[3] 0.2% in high cholesterol diet	8
	Experiment 2[4]	
8	None (high cholesterol diet)	11
9	MBLA, 0.1% in high cholesterol diet	10

[1] MBLA indicates *N*-(α-methylbenzyl) linoleamide.
[2] CHLA indicates *N*-cyclohexyl linoleamide.
[3] The mixture of linoleic acid (LA) and α-methyl benzylamine (α-MB) (1:1, by molecular weight).
[4] Pair-fed.

rats), the high cholesterol diet supplemented with 0.1% of MBLA. Each animal in both groups was given a weighed amount of the diet, 6 to 8 g/day, so that daily food intake was the same. Water was supplied ad libitum.

During the experimental period of 8 weeks, body weight was recorded twice a week, and at 2-week intervals blood samples were taken from the tail vein through a heparinized syringe for analysis of plasma cholesterol. At the end of the experimental period, animals were exsanguinated by heart puncture under ether anesthesia. The livers, kidneys, and adrenals were removed and weighed. A portion of the liver was extracted immediately for lipid analysis.

Analysis. Total cholesterol was determined by the method of Herrmann (6), free cholesterol by the method of Brown et al. (7), and esterified cholesterol was calculated from the amounts of total cholesterol and free cholesterol. Lipid phosphorus was determined by King's molybdenum blue method (8) after incineration in the presence of perchloric acid. Phospholipid values are expressed as 25 multiples of lipid phosphorus. Liver lipids were extracted as follows: 5 g of fresh liver were ground with 25 g of anhydrous sodium sulfate and extracted with chloroform. Aliquots of the chloroform extract were used for determination of lipid components. Total lipids were estimated gravimetrically.

RESULTS

Experiment 1. Figure 1 shows changes of mean plasma cholesterol levels of the groups in experiment 1. Mean plasma cholesterol levels of the control group elevated rapidly to about 320 mg/100 ml in 2

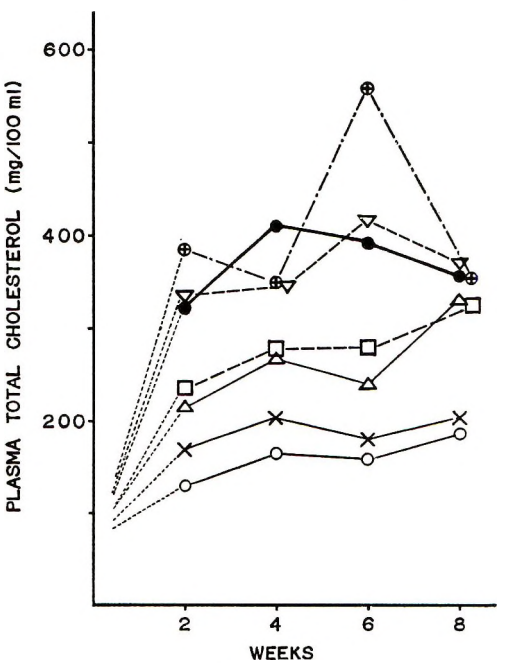

Fig. 1 Changes of mean plasma cholesterol levels of rats of experiment 1: ●———●, group 1; ○———○, group 2; ×———×, group 3; △———△, group 4; □— — —□, group 5; ▽— — —▽, group 6; and ⊕—— - ——⊕, group 7. See table 1 for description of groups.

TABLE 2

Mean body weight gain and organ weight of rats at end of experimental period

Group [1]	Body wt		Organ weight/Body wt [2]		
	Final	Gain/8 weeks	Liver	Kidneys	Adrenals
	g	*g*	*g/100 g*	*g/100 g*	*mg/100 g*
			Experiment 1		
1	263	126 ± 5 [3]	4.74 ± 0.12	0.65 ± 0.02	18 ± 1
2	188	42 ± 6 **	4.23 ± 0.08 **	0.63 ± 0.01	22 ± 1
3	207	59 ± 7 **	4.26 ± 0.28	0.63 ± 0.03	19 ± 2
4	225	79 ± 13 **	4.16 ± 0.11 **	0.64 ± 0.01	19 ± 1
5	243	93 ± 9 **	4.20 ± 0.11 *	0.63 ± 0.01	21 ± 2
6	247	99 ± 14	4.82 ± 0.34	0.65 ± 0.01	19 ± 2
7	277	128 ± 11	4.58 ± 0.16	0.65 ± 0.02	19 ± 3
			Experiment 2 [4]		
8	157	9.7 ± 2.5	4.33 ± 0.09	0.73 ± 0.01	19 ± 1
9	154	8.1 ± 2.5	4.23 ± 0.10	0.74 ± 0.01	17 ± 1

[1] See table 1 for description of groups.
[2] The values for rats fed commercial diet [5] obtained from another experiment were as follows (per 100 g of body weight): liver, 4.2 g; kidneys, 0.65 g; adrenals, 15 mg.
[3] Mean ± SE.
[4] Pair-fed.
[5] See footnote 4 of text.
* Significantly different from corresponding control ($P < 0.05$).
** Significantly different from corresponding control ($P < 0.01$).

weeks and maintained higher levels (350 to 425 mg/100 ml) for the following 6 weeks. With MBLA supplementation, the elevation of plasma cholesterol levels was markedly depressed and the response was dose-dependent. In the group treated with 0.2% of CHLA, the elevation was also depressed, but CHLA was less effective than MBLA. Mean plasma cholesterol level of the group treated with 0.2% of the mixture of linoleic acid and α-methyl benzylamine showed an increase similar to that of the control group. No animal in the groups treated with MBLA and CHLA showed any signs of illness, though the suppression of body weight gain was observed (table 2). Average food intake of these groups was less than that of the control as shown in table 3. Liver weight (grams per 100 g of body weight) was increased by feeding the high cholesterol diet. These increases were prevented by administration of MBLA or high levels of CHLA. There was no significant difference in weights of kidneys and adrenals. Levels of total cholesterol, free cholesterol, esterified cholesterol, and phospholipids of plasma at the end of the experimental period are shown in table 4 and those of liver are shown in table 5. Administration of MBLA depressed the elevation of total cholesterol, free cholesterol, esterified cholesterol, and phospholipid levels of plasma. The CHLA and the mixture of linoleic acid and α-methyl benzylamine did not show any significant effect on plasma lipid levels at the end of the experimental period. As to liver lipid levels, the animals receiving MBLA or CHLA showed markedly lower levels of total lipids and cholesterol than those of the control group. Liver phospholipid levels (grams per 100 g of liver) were rather increased by administration of MBLA or CHLA, but the net amount of liver phospholipids per animal was not modified significantly between the groups, because liver weight of the control group was larger than those of the treated groups (table 2). Plasma-liver cholesterol pools (9) of rats receiving MBLA or CHLA were

TABLE 3

Average food intake of rats at days 14 and 28 (exp. 1)

Group [1]	Food intake	
	Day 14	Day 28
	g/animal/day	
1	11.1	15.0
2	6.5	10.4
3	7.6	12.1
4	8.4	11.4
5	8.1	13.3
6	10.4	12.7
7	12.7	14.4

[1] See table 1 for description of groups.

TABLE 4

Plasma lipid levels of rats at end of experimental period

Group [1]	Cholesterol			Phospholipids [2]
	Total	Free	Ester	
	mg/100 ml	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*
		Experiment 1		
1	357 ± 25 [3]	63 ± 6	295 ± 19	170 ± 5
2	184 ± 17**	41 ± 6*	144 ± 12**	118 ± 7**
3	206 ± 28**	46 ± 7	160 ± 21**	118 ± 7**
4	331 ± 32	77 ± 8	254 ± 8	141 ± 8**
5	325 ± 46	66 ± 11	259 ± 35	152 ± 11
6	373 ± 44	75 ± 12	298 ± 32	193 ± 14
7	354 ± 43	65 ± 10	289 ± 33	194 ± 12
		Experiment 2 [4]		
8	235 ± 18	37 ± 3	198 ± 15	156 ± 8
9	136 ± 18**	22 ± 4**	114 ± 14**	134 ± 8

[1] See table 1 for description of groups.
[2] Phospholipids value represents lipid P × 25.
[3] Mean ± SE.
[4] Pair-fed.
* Significantly different from corresponding control ($P < 0.05$).
** Significantly different from corresponding control ($P < 0.01$).

TABLE 5

Liver lipid levels of rats at end of experimental period

Group [1]	Total lipids	Cholesterol			Phospholipids [2]
		Total	Free	Ester	
	g/100 g	*mg/100 g*	*mg/100 g*	*mg/100 g*	*g/100 g*
			Experiment 1		
1	10.24 ± 0.52 [3]	4035 ± 334	346 ± 9	3689 ± 326	2.67 ± 0.05
2	4.79 ± 0.20**	744 ± 85**	255 ± 6**	489 ± 80**	3.07 ± 0.05**
3	5.19 ± 0.40**	1096 ± 247**	258 ± 9**	838 ± 240**	2.91 ± 0.09*
4	6.05 ± 0.25**	1593 ± 161**	293 ± 7*	1301 ± 156**	2.91 ± 0.05*
5	7.24 ± 0.88**	2132 ± 473**	302 ± 16*	1830 ± 459**	3.05 ± 0.06**
6	8.00 ± 0.66*	2506 ± 320**	318 ± 9*	2188 ± 311**	2.89 ± 0.07
7	12.38 ± 1.20	3916 ± 240	348 ± 14	3569 ± 228	2.76 ± 0.08
			Experiment 2 [4]		
8	7.48 ± 0.69	2306 ± 351	306 ± 14	2000 ± 340	2.44 ± 0.06
9	5.16 ± 0.30**	918 ± 195**	252 ± 7**	666 ± 189**	2.51 ± 0.07

[1] See table 1 for description of groups.
[2] Phospholipids value represents lipid P × 25.
[3] Mean ± SE.
[4] Pair-fed.
* Significantly different from corresponding control ($P < 0.05$).
** Significantly different from corresponding control ($P < 0.01$).

markedly lower than those of the control group. Figure 2 shows the dose-response curve of experiment 1 on plasma-liver cholesterol pools at the end of the experimental period. Estimated potency of MBLA was about 6.5-fold of that of CHLA.

Experiment 2. The lower part of table 2 shows the mean body weight gain and organ weights of both groups. There was no significant difference in body weight gain or organ weight between two groups. Plasma cholesterol levels of the control group showed rapid elevation with MBLA markedly depressing the elevation of plasma cholesterol levels as shown in figure 3 and table 4. Plasma cholesterol levels of the 0.1% MBLA-treated group showed only about half the value of the control group. Similar decreases in liver total lipids and cholesterol were also observed by administration of MBLA as shown in table 5. The decrease of esterified choles-

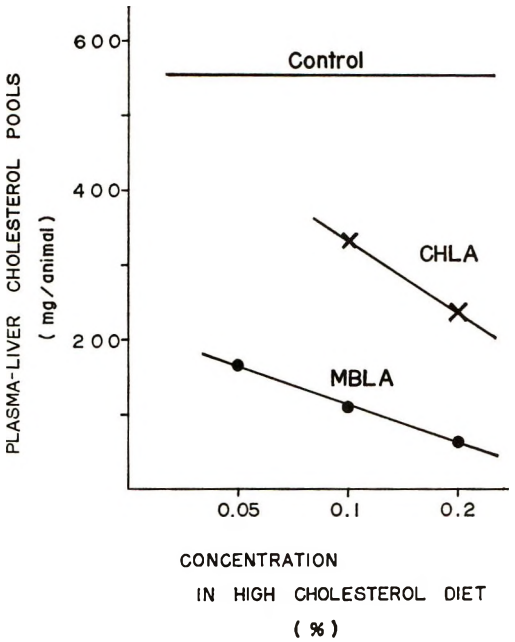

Fig. 2 Dose-response curve of experiment 1 on plasma-liver cholesterol pools (9) at the end of the experimental period. The line for controls represents the mean value of rats fed the high cholesterol diet. MBLA = *N*-(α-methylbenzyl) linoleamide. CHLA = *N*-cyclohexyl linoleamide.

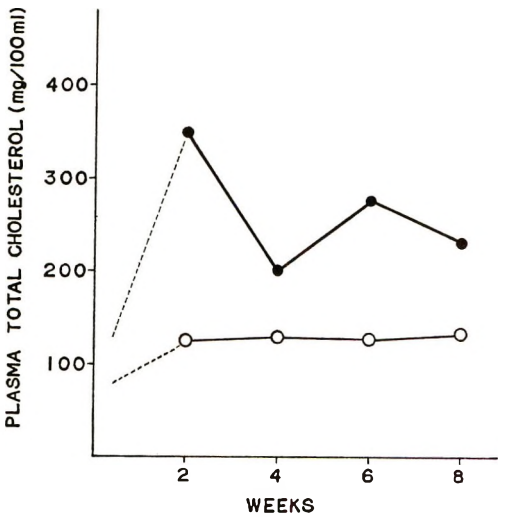

Fig. 3 Changes of mean plasma cholesterol levels of rats of experiment 2: ●——●, group 8; ○——○, group 9. See table 1 for description of groups.

terol was more marked than that of free cholesterol, as seen in the table. The plasma-liver cholesterol pools of the 0.1% MBLA-treated group and of the control group were 66.8 ± 14.4 and 169.3 ± 27.2 mg/animal, respectively.

DISCUSSION

The rat is generally resistant to elevation of plasma cholesterol levels by cholesterol feeding. It was observed by various investigators (10, 11) that, after plasma cholesterol levels reached a peak in rats during weeks 4 to 8 of cholesterol feeding, they declined despite continued cholesterol feeding. In the present experiment, the plasma cholesterol levels of the control group elevated rapidly and reached a peak at week 4; thereafter they declined gradually against cholesterol feeding. With MBLA, plasma cholesterol was markedly depressed in the early stage, but later was observed to increase progressively. At the end of the experimental period (8 weeks), however, MBLA-treated groups showed lower cholesterol levels than the control group.

With administration of MBLA or CHLA, body weight gain was depressed compared with that of the control. One cause suggested was that the rat avoided the diet containing MBLA or CHLA. It was doubted, however, that the cholesterol-lowering effect was due to lesser amounts of food. In pair-fed animals (exp. 2), it was shown that the effect of MBLA was entirely independent of the amount of food intake because the plasma cholesterol levels of the control group rapidly elevated and administration of MBLA depressed the elevation of plasma cholesterol levels similar to values in experiment 1.

Ridout et al. (12) reported that, in rats, the absorption of excess cholesterol produced the increase of cholesterol deposition in the liver, especially in the form of esterified cholesterol. In the present experiment, the same results were demonstrated. In the control group, liver cholesterol levels markedly increased in esterified form but free cholesterol levels showed only a slight increase. The MBLA decreased cholesterol deposition in the liver, especially in esterified form.

It is known that the percentage of phospholipids in the liver is decreased by feeding cholesterol or a fat-rich diet though the net amount is unchanged (13). In the present experiment, the net amount of liver phospholipids was shown not to be modified significantly.

The mixture of linoleic acid and α-methyl benzylamine (1:1, by molecular weight) showed no significant effect on lipid levels of plasma and liver. It can be considered that the effect of MBLA was not due to the effect of linoleic acid and α-methyl benzylamine, either singly or in combination, which might be produced by hydrolysis of MBLA. The cholesterol-lowering mechanism of CHLA was thought to result from interference with absorption of cholesterol from the intestine (3). The MBLA is considered to have the same mode of action as CHLA.

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Effects of Altering the Proportions of Essential to Nonessential Amino Acids on Growth and Plasma Amino Acid Levels in the Rat [1,2,3]

VERNON R. YOUNG AND JESUS ZAMORA [4]
Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts

ABSTRACT The quantitative relationship between essential and nonessential amino acids was studied in weanling rats fed L-amino acid diets supplying either 2.23 or 2.90% dietary nitrogen for 14 days. The proportion of total essential to total nonessential amino acids was varied, and weight gains, food consumption, and free amino acids in plasma after a 15- to 18-hour fast were measured. For the 2.23% dietary series, maximum growth was achieved when the ratio of total essential amino acids per gram total dietary nitrogen (E/T_N ratio) was between 3.37 and 4.71; for the 2.90% N series, it was achieved when the E/T_N ratio was between 3.03 and 4.04. Increased proportions of dietary total essential amino acids resulted in increases in plasma threonine, lysine, phenylalanine, and valine as maximum growth rate was obtained. At the highest essential amino acid intake, growth rate declined, and the levels of all these plasma amino acids except threonine decreased.

Although the role of nonessential amino acids and total nitrogen component in fulfilling the protein requirement has been studied in experimental animals (1–3) and in man (4–7), the quantitative relationship between the essential and nonessential amino acids has received limited study. Using DL-amino acids, Stucki and Harper (8) found that the chick is sensitive to changes in the ratio of essential to nonessential amino acids. A subsequent study on rats by these workers (9) showed that the rat was less sensitive than the chick to the dietary ratio of these two groups of amino acids.

The relationship between plasma amino acids and protein status has also been reviewed (10, 11). With protein depletion, the ratio of plasma essential to nonessential amino acids is low (12); the plasma amino acid ratio has been proposed as a possible means of assessing protein status under both experimental (13) and field (14) conditions. Whitehead and Dean (15) noted the lack of information on the effect of the ratio of dietary essential to nonessential amino acids on plasma amino acid levels. Harker et al. (16) recently pointed to the need for studies on the effect of administering well-balanced mixtures of amino acids and different levels of amino acids fed in the same proportions on plasma and tissue amino acid levels.

Development of L-amino acid diets, which support maximal growth in rats (17), has facilitated the study of amino acid interrelationships. Many previous studies have used either DL-amino acid mixtures or diets which failed to support maximal growth rates. We have conducted a series of experiments to evaluate the relationship between dietary essential and nonessential amino acids in rats using L-amino acid diets (in agar-gel) that were capable of supporting maximum growth. Our study also included an investigation of plasma amino acid levels in fasted rats previously given isonitrogenous diets containing varying proportions of essential to nonessential amino acids.

EXPERIMENTAL

Male weanling rats of the Sprague-Dawley strain [5] were fed a 10% casein diet for a 2-day adjustment period before the beginning of the experiment. The rats were

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[3] Contribution no. 1194 from the Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139.
[4] Present address: Universidad Agraria La Molina, Lima, Peru.
[5] Charles River Breeding Laboratories, Wilmington, Massachusetts.

housed in individual galvanized cages with wire-mesh bottoms and maintained in animal quarters at approximately 24° with relative humidity about 50%. The mean body weight of the rats in each group differed by less than 2 g. Water and food were given ad libitum and food consumption, determined as dry weight, was measured. Animals were weighed on days 2, 5, 7, 10 and 14 of the 2-week experimental period.

The L-amino acid mixtures [6] used in these experiments are listed in tables 1 and 2 and were based upon the amino acid mixtures used by Rogers and Harper (17) and Chen.[7] The total N content of the diets was either 2.23 or 2.90%, corresponding to protein contents (× 6.25) of 13.9 and 18.1%, respectively. The essential amino acid mixture (mixture A) was varied from 2.05 to 13.52% of the diet in the 2.23% dietary N series and from 2.66 to 17.56% in the 2.90% dietary N series.

The composition of the diets (per 100 g) was as follows: corn oil, 10.0; salt mixture (17), 5.0; vitamin mixture (17), 0.5; choline chloride, 0.2; sodium acetate, 1.80; and dextrin and sucrose (2:1 weight ratio) to make 100%, depending upon the total weight of amino acids included in the diet. The amino acid mixtures were first mixed in a ball-mill for 8 hours. The diets were made into agar-gel form by the method of Rogers and Harper (17) and stored at 4° in covered containers until used.

At the end of the 14-day experimental period, the rats were fasted overnight (15 to 18 hours), anesthetized with pentobarbital sodium [8] (5 mg/100 g body weight), and blood was withdrawn from the posterior vena cava into a heparinized syringe. The blood sample was centrifuged at 4° and plasma was separated. The samples were pooled for the various dietary groups studied and the concentration of free amino acids was determined on an ultrafiltrate using a Technicon amino acid analyzer.

TABLE 1

Composition of essential amino acid mixture (mixture A)

Amino acid	Relative proportion [1]
L-Arginine [2]	6.59
L-Histidine [2]	1.41
L-Isoleucine	3.82
L-Leucine	5.00
L-Lysine [2]	6.35
L-Methionine	3.53
L-Cystine	2.06
L-Phenylalanine	5.00
L-Threonine	3.53
L-Tyrosine	2.06
L-Tryptophan	1.00
L-Valine	3.82

[1] The weight of amino acid in the mixture relative to the weight of L-tryptophan. The amino acid mixture supplies 15.4 g N/100 g of mixture.
[2] Added to the diets as the hydrochlorides.

TABLE 2

Composition of nonessential amino acid mixture (mixture B)

Amino acid	Relative proportion [1]
L-Serine	0.35
L-Proline	1.00
Glycine	1.40
L-Glutamic acid	4.50
L-Aspartic acid	0.35
L-Alanine	0.35

[1] The weight of amino acid in the mixture relative to the weight of L-proline. The amino acid mixture supplies 12.6 g N/100 g of mixture.

RESULTS

The total gains in weight obtained with the various L-amino acid diets during the 14-day experimental period are summarized in table 3. For both levels of dietary nitrogen, 2.23 and 2.90%, the groups fed the lowest levels of mixture A (essential amino acids) showed the lowest body weight gains. Maximum growth rates were obtained when the weight ratio of essential to nonessential amino acids (E/N) was 0.88 to 2.16 (groups 7–10) for the 2.23% N dietary series, and 0.71 to 1.34 (groups 18–21) for the 2.90% N series. Growth rates at the same E/T_N (fig. 1) or I/D (nonessential amino acid N) (fig. 2) ratios, however, were higher in rats given the higher content of dietary nitrogen. A plot of body weight gain against intake of total essential amino acids (fig. 3) shows that the intake of this mixture (mixture A) was the critical dietary variable influencing maximum growth rates with most of the diets. Too high a proportion of essential amino acids, however, limited growth.

[6] Purchased from General Biochemicals, Inc., Chagrin Falls, Ohio.
[7] Chen, D. 1966 M.S. Thesis, Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts.
[8] Nembutal.

TABLE 3

Effect of changing the ratio of essential to nonessential amino acids at two levels of N on performance of rats fed L-amino acid diets

Group no.	Total N	Mix A	Mix B	Weight gain [1]	Food intake	PER [2]
		% of diet			*g/2 weeks*	
1	2.23	2.05	15.21	10.0±4	189	0.44
2	2.23	4.19	12.70	45.5±3	190	1.72
3	2.23	6.01	10.36	63.7±3	181	2.50
4	2.23	6.38	9.94	72.5±4	176	2.95
5	2.23	6.76	9.47	75.9±3	161	3.37
6	2.23	7.13	9.01	83.4±4	178	3.37
7	2.23	7.51	8.55	88.6±4	164	3.88
8	2.23	8.27	7.55	91.4±4	170	3.85
9	2.23	9.00	6.71	89.1±4	165	3.87
10	2.23	10.51	4.87	92.0±6	192	3.44
11	2.23	12.01	3.04	68.3±5	224	2.27
12	2.23	13.52	1.19	53.8±3	165	2.34
13	2.90	2.66	19.80	19.8±3	177	0.64
14	2.90	5.42	16.43	56.0±3	172	1.80
15	2.90	6.83	14.70	76.5±3	173	2.43
16	2.90	7.51	13.80	90.3±4	168	2.96
17	2.90	7.80	13.50	91.4±4	163	3.09
18	2.90	8.78	12.30	94.4±4	151	3.45
19	2.90	9.76	11.11	96.0±4	160	3.30
20	2.90	10.74	9.99	103.6±4	179	3.19
21	2.90	11.71	8.72	102.7±2	171	3.31
22	2.90	13.66	6.37	86.7±4	166	2.89
23	2.90	15.62	3.94	67.6±4	170	2.20
24	2.90	17.56	1.56	42.2±2	173	1.35

[1] Mean ± SD for 6 rats.
[2] PER (protein efficiency ratio) = weight gain (g) per gram total protein intake.

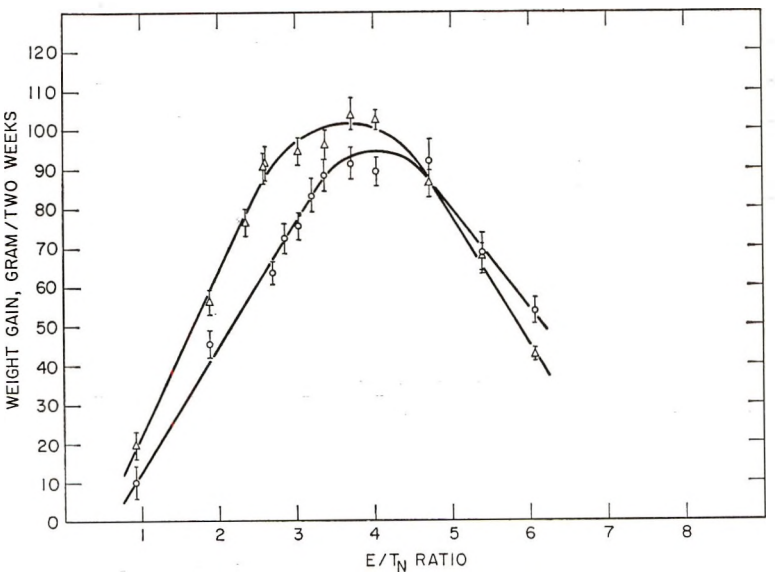

Fig. 1 Total weight gain (in 14 days of experimental period) of rats given L-amino acid diets containing varying ratios of total essential amino acids (g) per gram dietary N (E/T_N ratio). o, 2.23% dietary N; Δ, 2.90% dietary N. Vertical lines represent plus or minus one standard deviation for each group of 6 rats.

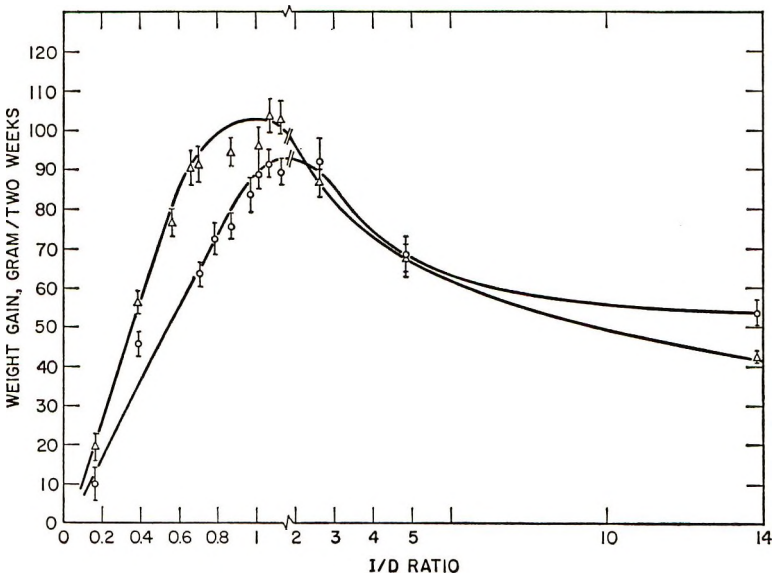

Fig. 2 Total weight gain (14 days) of rats given L-amino acid diets containing varying ratios of (g) essential to (g) nonessential amino acid N (I/D ratio). o, 2.23 or △ 2.90% dietary N, respectively. See figure 1 for further details.

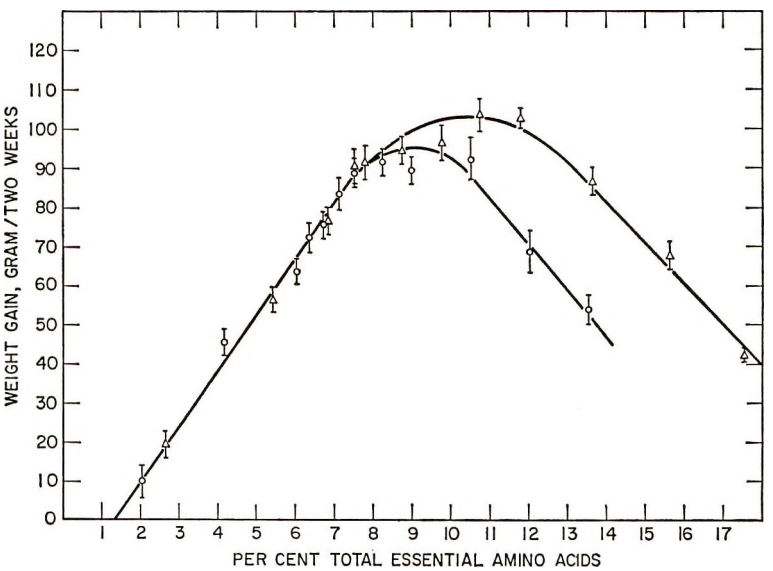

Fig. 3 Total weight gain (14 days) of rats given L-amino acid diets containing either 2.23 % (o) or 2.90% (△) dietary N and varying in concentration of total essential amino acids. Further details are given in figure 1.

Total dietary nitrogen appeared to have little effect on growth when the intake of the essential amino acid mixture was low, although growth rate was related to the intake of these amino acids (fig. 3). At relatively high intakes of the essential amino acid mixture, above about 10% of total dietary essential amino acids, performance was better with the higher level of dietary nitrogen.

Table 3 also summarizes food intake and protein efficiency ratio (PER). Food intake results did not reveal any significant trends with the varying ratios of essential and nonessential amino acid mixtures. Within each dietary nitrogen level, PER values were lowest when obtained at the lowest intakes of essential amino acids and highest at intermediate ratios of essential to nonessential amino acids. As would be expected, PER values were higher for the groups showing maximum growth in the dietary series with the lower nitrogen content.

The concentrations of plasma amino acids obtained from fasted rats previously fed diets of varying proportions of essential to nonessential amino acids are summarized in table 4. Increased proportions of total essential amino acids in the diet resulted in increases in threonine, lysine, and phenylalanine. Valine also showed a tendency to increase with higher intakes of the essential amino acid mixture. At the highest levels of essential amino acid intakes (groups 11, 12, 23 and 24), the concentration of lysine and phenylalanine decreased, but the concentration of threonine did not. The concentrations of individual nonessential amino acids were highly variable between groups and showed no consistent changes; therefore, only the total concentration of these amino acids is given in table 4.

DISCUSSION

The present studies utilized a well-balanced L-amino acid diet which supported a growth rate equal to that obtained with a good quality intact protein (17). Our findings support those of earlier workers (1–3, 8, 9, 18–20) indicating that too high a proportion of essential amino acids is inhibitory to growth, regardless of the dietary nitrogen level. The best groups (numbers 20 and 21) gained at a rate of 7.4 g/day. The range of I/D ratios (fig. 2) in which rats grew best in the present studies was from about 1.0 to 2.66 at the levels of dietary N utilized; this compares favorably with the range of 4.0 to less than 1.0 suggested by Stucki and Harper (9) in their studies with rats. The higher maximum growth rates obtained in the present

TABLE 4

Concentrations of free amino acids (μmoles/100 ml) in plasma [1] of rats 14 days after feeding diets containing varying proportions of essential and nonessential amino acids

Group no.	Amino acid								Total of essential	Total of nonessential [2]
	Histidine	Isoleucine	Leucine	Lysine	Threonine	Tyrosine	Phenylalanine	Valine		
1	4	6	9	21	10	5	4	10	69	128
2	4	6	9	22	11	5	5	11	73	128
3	4	6	10	25	12	8	6	12	83	167
5	5	5	11	32	21	6	6	18	104	125
7	5	7	11	40	—	31	13	13	—	117
8	6	9	12	50	39	18	11	20	165	170
9	7	9	13	46	45	9	9	16	154	178
10	16	17	23	119	—	20	14	34	—	—
11	8	11	14	40	151	9	9	19	261	179
12	6	5	10	28	115	4	5	9	182	—
13	4	6	9	22	10	5	5	10	71	130
14	4	6	10	23	12	7	6	12	80	156
15	4	6	10	28	10	11	6	12	87	150
19	8	8	13	56	25	11	8	15	144	160
20	13	17	21	93	55	13	14	29	255	—
21	13	20	25	129	96	16	15	35	349	237
22	18	15	21	94	—	12	12	27	—	—
23	7	9	13	45	97	7	8	17	203	156
24	7	8	12	41	156	9	8	16	257	140

[1] Analysis was performed on a pooled plasma sample from 6 rats in each dietary group.

[2] Includes aspartic, glutamic, glycine, alanine, and serine plus glutamine. The concentrations (mean ± SD) of the amino acids for 6 rats at the beginning of the experimental period were as follows: (μmoles/100 ml) histidine, 2.9 ± 0.3; isoleucine, 1.9 ± 0.1; leucine, 5.6 ± 0.4; lysine, 20.3 ± 0.7; threonine, 13.0 ± 0.1; tyrosine, 3.0 ± 0.7; phenylalanine, 2.0 ± 0.2; valine, 7.3 ± 0.3; aspartic, 1.9 ± 0.2; serine plus glutamine, 1.5 ± 1.5; glutamic, 16.5 ± 0.9; glycine, 1.5 ± 2.3; and alanine, 12.8 ± 0.3.

experiments may allow a more sensitive test of the influence of changes in the ratio of essential to nonessential amino acid nitrogen on performance, and this probably explains the quantitative difference between our results and those of Stucki and Harper (9). The present studies also demonstrate that the optimal ratio of essential to nonessential amino acids varies with the dietary nitrogen content and that the proportion of essential amino acid nitrogen required for maximal growth decreases as the nitrogen content of the diet is increased. Maximum growth was achieved in the 2.23% N series with dietary E/T_N ratios approximately between 3.37 and 4.71, whereas the range of ratios associated with best performance in the 2.90% N dietary series was approximately between 3.03 and 4.04.

For the free essential amino acids studied, a relatively constant level was obtained in plasma at low intakes of the total essential amino acids for each dietary N series. The concentrations of lysine, threonine, phenylalanine and valine appeared to increase with diets supplying the intakes of total essential amino acids which supported maximum growth rates. Except for threonine, which seemed to increase still further, the concentrations of the amino acids appeared to decline as the proportion of essential to nonessential amino acids was increased beyond the point of maximum weight gains. These results agree with those of Harker et al. (16) who found that the lysine content of plasma obtained after a 6-hour fast in rats fed four levels of amino acids in the same proportions was directly related to intake. These workers, however, also found that the plasma concentration of threonine was only related to intakes of the amino acid when fed below the minimal requirement.

Morrison et al. (21) found that plasma lysine rose rapidly in response to added dietary lysine. They also showed that until lysine ceased to be a factor limiting growth, plasma-free lysine levels were much less influenced by dietary lysine content than were weight gains. A similar trend was observed in the present experiments for plasma lysine. Although Morrison and co-workers observed a reciprocal relationship between lysine and threonine, this relationship was not clearly apparent in our studies, except at the highest intakes of the essential amino acid mixture. The probable reason is that the intake of total essential amino acids was varied in our studies, whereas lysine intake alone was varied in the studies of Morrison et al.

In 7-day assays with chicks, Zimmerman and Scott (22) showed that the first limiting amino acid in the diet remained at a low constant level in the blood irrespective of the degree of dietary deficiency and that dietary increments in excess of the amount needed to maximize weight gain resulted in a rapid accumulation of the amino acid in the blood. In their studies the chicks were not fasted, whereas our studies were based on plasma samples from fasting rats. A decrease in the level of plasma-free amino acids in the present studies occurred when the dietary proportion of essential to nonessential amino acids was greater than that which supported maximum growth. These results may, therefore, reflect changes in protein status of the experimental animals more closely than changes in the dietary content of the essential amino acids.

McLaughlan and Illman (23) reported an almost linear plasma response with dietary levels of lysine, tryptophan, leucine, histidine, and isoleucine 6 hours after feeding, and they have utilized the plasma response curve for estimating amino acid requirements. In our studies on plasma samples from fasting animals, the lysine content may more closely be characterized by an exponential relationship with varying dietary intakes of the essential amino acid mixture (table 4) up to the point at which growth rate was reduced.

Zimmerman and Scott (24) have shown that the plasma amino acid pattern in fasting chicks is influenced by the length of fast, and they suggest that the plasma amino acid pattern has limitations which detract from its usefulness as a reference point in plasma amino acid studies. The present findings are consistent with this view and suggest a positive correlation between the level of amino acids in plasma and the growth response. In utilizing plasma amino acid levels in fasted animals as a reference in assessing amino acid requirements, it also appears critical to de-

termine the lowest plasma concentration of the amino acid that is consistent with maximum growth performance. Furthermore, Harker et al. (16) emphasized the many factors which affect the levels of amino acids in plasma, and from the present results, it appears that further studies will be required before the relationship between dietary amino acid intake and plasma amino acid levels can be described adequately.

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Effect of Hypophysectomy on Pathologic Changes in Rats Force-fed a Threonine-devoid Diet [1]

HERSCHEL SIDRANSKY AND ETHEL VERNEY
Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania

ABSTRACT This study was conducted to determine whether the morphologic and biochemical changes previously observed in intact rats force-fed a purified diet devoid of threonine could have been influenced by hypophyseal hormones, particularly growth hormone. Young hypophysectomized rats with or without low doses of cortisone acetate were force-fed a purified diet devoid of threonine for 1 or 2 days. These animals developed an enlarged fatty liver with a periportal distribution of lipid and an increase in hepatic glycogen, and atrophy of the pancreas, stomach and spleen. The animals force-fed the threonine-devoid diet showed increased incorporation of ^{14}C-leucine into hepatic protein with decreased incorporation into gastrocnemius and splenic proteins in comparison with control animals force-fed a complete diet. These changes were similar to those reported earlier in similar experiments with normal rats. Although some of the hepatic changes in rats force-fed the threonine-free diet are similar to those obtained in normal or hypophysectomized rats treated with growth hormone, the present results indicate that the pathologic changes found in animals with threonine deficiency are not due to increased pituitary gland secretion.

In earlier studies (1–3) young rats force-fed for 3 to 8 days a purified diet devoid of a single essential amino acid, such as threonine, were found to develop pathologic changes that closely resemble many of the changes found in infants with kwashiorkor (4). The changes observed in the animals force-fed a threonine-devoid diet, but not in those fed the complete diet, consist of fatty liver with increased glycogen and atrophy of the pancreas, submaxillary gland, stomach, thymus and spleen (1).

In other studies (5–9) the administration of hormones, such as cortisone and growth hormone, to normal animals has been reported to produce morphologic and chemical changes in the liver similar to those observed in the amino acid deficiency experiments (1,10). In an earlier study (11) adrenalectomized rats maintained on low doses of cortisone were found to react similarly to intact rats when they were force-fed a threonine-devoid diet. These results indicated that the pathologic changes in animals force-fed the deficient diet were not due to increased adrenal hormone production. In the present study young hypophysectomized rats were force-fed the threonine-devoid diet to determine whether or not hypophyseal hormones, particularly growth hormone, influenced the induction of pathologic changes. The results indicate that hypophysectomized animals responded in a manner similar to that of intact animals.

METHODS

Hypophysectomized female [2] and male [3] rats were used. During the immediate post-hypophysectomy period the animals were fed milk, bread and oranges or 5% sucrose in drinking water in addition to the commercial diet.[4] In all experiments groups of animals, each of the same sex, age and weight were used.

The hypophysectomized rats were kept postoperatively the following number of days in each experiment: experiment 317, 9 days; experiment 326, 9 days; experiment 343, 8 days; experiment 423, 5 days; experiment 433, 30 days; and experiment 446, 13 days. During this time the animals did not gain weight. The animals were then force-fed the complete diet for 3 to 6 days before they were divided into 2 groups: the control group continued to

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[2] Hormone Assay Company, Chicago.
[3] Zivic-Miller, Pittsburgh.
[4] Wayne Lab-Blox Allied Mills, Inc., Chicago.

receive the complete diet and the experimental group received the threonine-devoid diet. In a few preliminary experiments, hypophysectomized rats were force-fed the complete diet for several days but most of the animals died. Correspondingly early deaths have been found in adrenalectomized rats force-fed purified diets (11). Therefore, in all but one of the subsequent experiments, animals were given low doses of cortisone acetate intramuscularly every day. At the start of the force-feeding, the animals received 0.2 mg cortisone acetate daily in exp. 317, 326, and 343; 0.4 mg cortisone acetate daily in exp. 433 and 446; and no cortisone in exp. 423. When rats were started on the control or experimental diet, they weighed on the average in each experiment as follows: experiment 317, 69 g; experiment 326, 81 g; experiment 343, 74 g; experiment 423, 138 g; experiment 433, 160 g; and experiment 446, 132 g. Intact rats force-fed the complete or threonine-devoid diet were not used simultaneously in this experimental study since data in regard to such animals have been reported earlier in several studies (1,11–13).

The purified diet was similar to that used in our earlier studies (1–3, 10–13). The percentage composition was as follows: essential amino acids, 9.2; nonessential amino acids, 8.1; salt mixture, 4; vitamin-sucrose mixture, 5; corn oil [5], 5; cod liver oil, 1.5; and dextrin, 67.2. Dextrin was substituted for the threonine in the threonine-devoid diet. The diets were blended with distilled water so that each milliliter of diet mixture contained 0.5 g of diet and was in a suitable form for administration by stomach tube. Rats were force-fed with plastic tubes 3 times daily, at 8:30 AM, 1 PM and 7 PM. During the period of 1 or 2 days when they were force-fed the complete or threonine-devoid diet, the animals received an average daily feeding of from 0.7 to 1.1 g of diet/10 g of initial body weight. Rats had access to water. They were housed in individual wire cages with raised bottoms and kept in an air-conditioned room.

Rats were weighed at the beginning and end of each experiment. They were anesthetized with ether and exsanguinated approximately 18 hours after the last tube-feeding. Each animal received an intraperitoneal injection of an aqueous solution of 2.5 μCi/100 g body weight of ^{14}C-leucine (10.0 μCi/μmole), uniformly labeled, 1 hour before killing. The organs were weighed fresh. In paired organs, the right organ was weighed and used. Pieces of tissue from selected organs were fixed in Zenker-formol solution and in 10% formalin. Paraffin sections were routinely stained with hematoxylin and eosin. Frozen sections of formalin-fixed liver were stained with oil red O. The methods used for chemical analyses have been described in detail in earlier studies (3, 10, 12, 13). Radioactivity in protein was measured using a Packard Tri-Carb liquid scintillation spectrometer. In a few experiments the level of free amino acids of pooled livers and in one experiment of pooled plasmas of control and experimental animals was determined in a Spinco amino acid analyzer, model 120B.

RESULTS

Changes in body and organ weights. Table 1 summarizes changes in the weight of the whole body and the liver, kidney, spleen and gastrocnemius muscle in rats of the different groups of six experiments force-fed for 1 or 2 days. The control and experimental animals essentially maintained their initial body weights. The livers were significantly heavier in the experimental than in the control animals in the 1 and 2 day experiments. The spleens weighed less after 1 day in the animals fed the threonine-devoid diet than in those fed the complete diet. The kidneys and gastrocnemius muscle weighed essentially the same in both groups.

Morphologic changes. The observations in the hypophysectomized animals force-fed the complete and threonine-devoid diets were identical to those described in earlier reports (1, 11, 13). In brief, while no pathologic changes were observed in the animals fed the complete diet, the animals fed the threonine-devoid diet for 2 days developed periportal fatty liver, excess hepatic glycogen, hepatic nucleolar enlargement, and a mild degree of atrophy of pancreas, stomach and spleen.

Biochemical changes. The values for hepatic lipid, glycogen and protein, and

[5] Mazola, Corn Products Company, New York.

TABLE 1

Body weight changes and organ weights of hypophysectomized rats force-fed complete or threonine-devoid diet

Exp. no.[1]	Group [2]	Sex	No. of rats	Duration	Changes in body wt [3]	Liver	Kidney	Spleen	Gastrocnemius muscle
				days	*g*	*g/100 g body wt*	*g/100 g body wt*	*g/100 g body wt*	*g/100 g body wt*
317	C	F	2	1	−0.5	3.68	0.41	0.33	0.48
	TD	F	3	1	+0.3	5.97	0.40	0.18	0.43
326	C	F	4	1	−1.5	4.71	0.46	0.29	0.55
	TD	F	6	1	+0.9	6.64	0.41	0.24	0.50
Summary	C	F	6	1	−1.2	4.37 ± 0.31 [4]	0.44 ± 0.02 [4]	0.30 ± 0.02 [4]	0.53 ± 0.02 [4]
	TD	F	9	1	+0.7	6.42 ± 0.38 [5]	0.41 ± 0.01	0.22 ± 0.01 [5]	0.48 ± 0.01
343	C	F	4	2	+1.5	4.08	0.42	0.21	0.55
	TD	F	4	2	−2.0	6.00	0.40	0.18	0.51
423	C	M	5	2	−0.2	3.60	0.37	0.22	0.54
	TD	M	5	2	−1.8	5.54	0.39	0.20	0.51
433	C	M	2	2	−2.0	5.04	0.39	0.19	0.52
	TD	M	2	2	−1.0	6.54	0.33	0.13	0.54
446	C	M	4	2	+1.0	5.00	0.36	0.19	0.50
	TD	M	4	2	−1.0	8.11	0.38	0.22	0.48
Summary	C	F and M	15	2	+0.3	4.29 ± 0.25	0.38 ± 0.01	0.21 ± 0.02	0.53 ± 0.01
	TD	F and M	15	2	−1.5	6.48 ± 0.42 [5]	0.38 ± 0.01	0.19 ± 0.01	0.51 ± 0.01

[1] Rats received cortisone acetate daily: 0.2 mg in experiments no. 317, 326 and 343; 0.4 mg in experiments no. 433 and 446; and none in experiment no. 423.

[2] C = complete diet; TD = threonine-devoid diet.

[3] Initial body weights ranged from 63 to 83 g in experiments no. 317, 326 and 343, and 130 to 160 g in experiments no. 423, 433 and 446.

[4] Mean ± SE of mean.

[5] $P < 0.01$.

protein of kidney, spleen and gastrocnemius muscle in groups of hypophysectomized rats force-fed the two diets for 1 and 2 days are presented in table 2. Liver lipid, glycogen and protein were greater in the experimental than in the control animals. Kidney and splenic protein were decreased in the experimental animals in the 1-day experiments but not in the 2-day experiments. Muscle protein was similar in both groups of animals.

Table 3 shows the results of ^{14}C-leucine incorporation into protein of liver, plasma, kidney, spleen and gastrocnemius muscle of animals force-fed the control and experimental diets. The results are expressed as specific activity for organ and plasma protein. In addition, they are expressed as radioactivity per total organ protein corrected per 100 g body weight and are therefore corrected for any differences in size of the nonradioactive-protein pool diluting the radioactive proteins.

Protein synthesis in the liver, as measured by ^{14}C-leucine incorporation into hepatic protein, was significantly increased in rats force-fed the threonine-devoid diet in comparison with those fed the complete diet. In several experiments the hepatic acid-soluble counts were also measured and revealed increases in the experimental animals in comparison with controls. These increases, which were generally less than in the acid-precipitable counts, were probably related to the increase in the hepatic free leucine pool of the experimental animals (table 4). Plasma protein (only in the 1-day experiments) and splenic protein incorporation were decreased while kidney protein incorporation was unchanged in experimental animals compared with controls. Gastrocnemius muscle protein incorporation was decreased in animals fed the experimental diet.

The results of free amino acids in the liver and plasma of animals force-fed the complete or threonine-devoid diet for 2 days are presented in table 4. In 3 experiments where the hypophysectomized rats received cortisone acetate daily the rats force-fed the threonine-devoid diet had increases in total hepatic levels of leucine, methionine, valine, alanine, aspartic acid, cystine, glutamic acid, glycine, proline and serine, and had a marked decrease in threonine in comparison with animals fed the complete diet. In these animals the plasma changes in the two groups were not marked except for threonine which was greatly decreased in the experimental animals. In one experiment where the hypophysectomized rats did not receive cortisone the rats fed the complete diet had lower levels of liver and plasma amino acids than in the previous control groups and the animals fed the threonine-devoid diet showed differences similar to those described previously. Since the experimental rats had a 50 to 70% increase in total hepatic free leucine over that in control rats at the time of killing, it is noteworthy that the ^{14}C-leucine incorporation into hepatic protein was increased in the experimental animals over that in the control animals (table 3) even though the free leucine pool of the liver was greater in the experimental animals.

DISCUSSION

This paper reports that morphologic and biochemical changes previously observed in intact rats force-fed a purified diet devoid of threonine may also be found in hypophysectomized rats with or without a low dose of cortisone and force-fed the threonine-free diet. Although some of the biochemical changes, such as enhanced hepatic protein and RNA synthesis, in animals fed the threonine-devoid diet are similar to those obtained in normal or hypophysectomized rats treated with growth hormone, our present results indicate that these changes with threonine deficiency are not due to increased hypophyseal secretion of growth hormone. In earlier studies the possible influential effect of increased adrenocortical secretion of corticosterone on the pathologic changes in animals force-fed a threonine-devoid diet had been ruled out (11). Hence, it is now established that alterations in the secretory activity of two vital endocrine glands, the adrenals and hypophysis, are not involved in the pathogenesis of the lesions in our experimental kwashiorkor-like model.

Earlier studies in vivo and in vitro (8, 14, 15) have demonstrated that the livers of hypophysectomized rats incorporate less radioactive amino acids into hepatic proteins than livers from normal rats or from

TABLE 2

Analyses of liver, kidney, spleen and gastrocnemius muscle of hypophysectomized rats force-fed complete or threonine-devoid diet

Exp. no.	Group [1]	No. of rats	Duration	Liver			Kidney protein	Spleen protein	Gastrocnemius muscle protein
				Total lipid	Glycogen	Protein			
			days	*mg/liver/100 g body wt*			*mg/organ/100 g body wt*		
317	C	2	1	175	51	868	77	63	72
	TD	3	1	252	709	1579	74	37	85
326	C	4	1	208	268	1061	81	52	55
	TD	6	1	297	842	1653	69	43	73
Summary	C	6	1	197 ± 11 [2]	181 ± 74 [2]	997 ± 68 [2]	80 ± 1 [2]	56 ± 1 [2]	61 ± 2 [2]
	TD	9	1	282 ± 20 [3]	798 ± 86 [3]	1628 ± 106 [3]	70 ± 4 [4]	41 ± 1 [3]	77 ± 8
343	C	4	2	192	89	927	79	39	88
	TD	4	2	235	594	1548	78	28	84
423	C	5	2	162	29	887	80	42	116
	TD	5	2	237	455	1417	79	39	118
433	C	2	2	224	504	1449	80	38	99
	TD	2	2	245	846	1748	63	25	87
446	C	4	2	411	103	1172	64	33	82
	TD	4	2	522	1061	1809	81	37	102
Summary	C	15	2	245 ± 36	128 ± 64	1049 ± 55	76 ± 2	38 ± 2	97 ± 4
	TD	15	2	314 ± 39	706 ± 117 [3]	1601 ± 122 [3]	77 ± 2	34 ± 2	101 ± 3

[1] C = complete diet; TD = threonine-devoid diet.
[2] Mean ± SE of mean.
[3] $P < 0.01$.
[4] $0.05 > P > 0.01$.

TABLE 3

Incorporation of ^{14}C-leucine into protein of liver, plasma, kidney, spleen and gastrocnemius muscle of hypophysectomized rats force-fed complete or threonine-devoid diet

Exp. no.	Group [1]	No. of rats	Duration	Liver protein		Plasma protein	Kidney protein [2]		Spleen protein [2]		Gastrocnemius muscle protein [2]	
				Specific activity	Total radio-activity	Specific activity	Specific activity	Total radio-activity	Specific activity	Total radio-activity	Specific activity	Total radio-activity
			days	*cpm/mg protein*	*cpm × 10^{-3}/ liver/100 g body wt*	*cpm/mg protein*	*cpm/mg protein*	*cpm × 10^{-3}/ kidney/100 g body wt*	*cpm/mg protein*	*cpm × 10^{-3}/ spleen/100 g body wt*	*cpm/mg protein*	*cpm × 10^{-3}/ muscle/100 g body wt*
317	C	2	1	361	316	715	269	20.6	483	30.4	64	4.58
	TD	3	1	418	669	430	238	17.7	399	14.9	35	2.97
326	C	4	1	406	431	555	309	24.9	578	29.8	135	7.41
	TD	6	1	397	661	371	293	20.2	346	14.9	34	2.53
Summary	C	6	1	391 ± 48 [3]	393 ± 63 [3]	608 ± 70 [3]	289 ± 20 [3]	22.8 ± 1.5 [3]	531 ± 42 [3]	30.1 ± 0.3 [3]	100 ± 35 [3]	6.00 ± 1.41 [3]
	TD	9	1	404 ± 41	664 ± 74 [4]	386 ± 82	266 ± 22	19.0 ± 1.3	373 ± 18 [5]	14.9 ± 0 [5]	35 ± 1	2.75 ± 0.22 [4]
343	C	3	2	652	600	672	174	13.7	532	21.0	27	2.39
	TD	3	2	887	1345	526	328	25.4	285	8.1	22	1.81
423	C	3	2	582	555	483						
	TD	5	2	802	1075	966						
433	C	2	2	666	975	647	387	30.8	536	20.4	41	4.06
	TD	2	2	1075	1775	776	515	32.3	657	16.6	44	3.84
446	C	3	2	798	849	720	392	25.1	587	19.2	46	3.73
	TD	2	2	1256	2963	716	224	18.1	246	9.1	18	1.82
Summary	C	11	2	675 ± 70	724 ± 88	629 ± 55	318 ± 71	23.2 ± 5.0	552 ± 11	20.2 ± 0.5	38 ± 6	3.39 ± 0.52
	TD	12	2	944 ± 85 [4]	1574 ± 244 [5]	743 ± 93	356 ± 85	25.3 ± 4.0	396 ± 132	11.3 ± 2.6 [4]	28 ± 8	2.49 ± 0.68

[1] C = complete diet; TD = threonine-devoid diet.
[2] Organs of each group were pooled in each experiment.
[3] Mean ± SE of mean.
[4] $0.05 > P > 0.01$.
[5] $P < 0.01$.

TABLE 4

Analyses of free amino acids of liver and plasma of hypophysectomized rats force-fed complete or threonine-devoid diet for 2 days

	Liver free amino acids [1]								Plasma free amino acids [1]			
Group [2]	C		TD		C		TD		C	TD	C	TD
Cortisone treated [3]	+		+		−		−		+	+	−	−
No. of experiments	3		3		1		1		1	1	1	1
	μmoles/g liver	*μmoles/ liver/ 100 g body wt*	*μmoles/g liver*	*μmoles/ liver/ 100 g body wt*	*μmoles/g liver*	*μmoles/ liver/ 100 g body wt*	*μmoles/g liver*	*μmoles/ liver/ 100 g body wt*	*μmoles/ml plasma*			
Essential amino acids												
Isoleucine	0.08	0.39	0.05	0.37	0.06	0.23	0.05	0.21	0.05	0.04	0.04	0.02
Leucine	0.20	0.93	0.20	1.39	0.13	0.48	0.15	0.82	0.15	0.13	0.07	0.09
Methionine	0.06	0.12	0.06	0.38	0.01	0.04	0.03	0.16	0.06	0.04	0.02	0.02
Phenylalanine	0.08	0.39	0.07	0.45	—	—	0.05	0.25	0.08	0.08	0.03	0.04
Threonine	0.43	2.16	0.12	0.98	0.35	1.26	0.03	0.14	0.36	0.01	0.14	0.03
Valine	0.16	0.75	0.16	1.09	0.12	0.43	0.10	0.54	0.23	0.14	0.09	0.10
Nonessential amino acids												
Alanine	1.52	7.17	2.82	19.25	0.37	1.33	1.44	7.98	0.28	0.38	0.12	0.18
Aspartic acid	2.71	12.08	4.51	29.85	0.55	1.99	1.24	6.87	0.06	0.07	0.02	0.04
½ Cystine	0.02	0.09	0.02	0.13	—	—	—	—	0.06	0.04	0.03	0.02
Glutamic acid	2.44	11.38	3.16	21.81	0.62	2.23	1.64	9.09	0.26	0.32	0.09	0.10
Glycine	2.22	10.30	1.92	13.20	1.06	3.82	1.01	5.60	0.34	0.19	0.31	0.12
Proline	0.25	1.17	0.25	1.74	0.19	0.68	0.24	1.33	0.31	0.28	0.16	0.12
Serine	2.97	13.63	3.03	21.12	1.80	6.48	1.47	8.14	0.42	0.33	0.26	0.19
Tyrosine	0.10	0.47	0.07	0.45	—	—	0.04	0.24	0.14	0.11	0.08	0.05

[1] Pooled specimens of livers or plasma from 2 to 5 animals were used in each experiment.
[2] C = complete diet; TD = threonine-devoid diet.
[3] Rats received cortisone acetate daily, 0.2 mg in one experiment and 0.4 mg in two experiments.

hypophysectomized animals receiving growth hormone. These changes could not be ascribed to hormonally controlled changes in the specific activity of the intracellular amino acid pool (14). Our previous studies (10–13) demonstrated that normal rats force-fed a threonine-devoid diet incorporated more radioactive amino acids into hepatic proteins in vivo and in vitro than did control animals force-fed a complete diet. It is now apparent that hypophysectomized rats, with a low level of hepatic protein synthesis, still respond to the feeding of a threonine-free diet with enhanced hepatic protein synthesis, in comparison with control animals. Here this increase occurs even though the pool of total hepatic free amino acids, particularly that of the precursor radioactive amino acid, leucine, is elevated at time of killing, 1 hour after administering the radioactive amino acid. Korner (14) has suggested that there is a defect in the hepatic microsomes of hypophysectomized rats responsible for the decreased protein synthesis. In our earlier studies (10) the hepatic ribosomes of rats force-fed the threonine-devoid diet have been shown to be chiefly responsible for the increased protein synthesis. Therefore, one may speculate that whatever action hypophysectomy has on hepatic microsomes this can most probably be overcome by force-feeding a threonine-free diet to the animals.

Other pathologic changes such as the increase in hepatic lipid and glycogen of hypophysectomized rats force-fed the threonine-devoid diet are of interest. It is known that hypophysectomy in the rat not only interferes with protein synthesis but also causes altered metabolism of carbohydrate and lipid (7). For example, it is known that after pituitary removal the ability of the liver to store glycogen is decreased and after moderate fasting the hepatic glycogen stores fall rapidly (7). This was encountered in experiment 423 where the hepatic glycogen was very low in the hypophysectomized control rats, not supplemented with cortisone acetate, force-fed the complete diet. Even under these conditions, however, the experimental animals force-fed the threonine-devoid diet still responded with a marked elevation in hepatic glycogen (table 2). Thus, even though the animals in these experiments were in an altered metabolic state due to hypophysectomy, they were still able to respond to the nutritional imbalance produced by force-feeding a threonine-devoid diet, with pathologic changes similar to those found in intact animals in earlier studies.

In our studies on the hepatic free amino acid levels in hypophysectomized control and experimental rats we observed several interesting points. The hepatic free amino acid levels in hypophysectomized rats fed the complete diet (table 4) are lower than in intact rats fed the complete diet (3). This decrease was more marked in the hypophysectomized animals which did not receive cortisone. This decrease was consistent with earlier findings of decreased size of the hepatic free amino acid pool in hypophysectomized animals (16). Experimental hypophysectomized rats force-fed the threonine-devoid diet showed an increase in many of the hepatic free amino acids, but not of plasma free amino acids, in comparison with control animals. An elevation, particularly in the hepatic nonessential free amino acids, has been described earlier in rats force-fed single essential amino acid-devoid diets (3).

The function of the anterior pituitary gland and its endocrine target organs in various forms of malnutrition has been reviewed frequently (17–19). With the development of the immunoassay technique for measuring human growth hormone, recent clinical studies (20, 21) have reported that the fasting level of human growth hormone in plasma is raised in kwashiorkor. This rise was considered to be related to the severity of protein depletion and it returned to normal only when protein was introduced into the diet. For this reason, it became of special interest to determine whether the pathologic change in our experimental kwashiorkor-like model in which young rats were force-fed a threonine-devoid diet could be related to increased secretion of growth hormone. Our acute studies in this report indicate that the pathologic changes can be induced in hypophysectomized rats as well as in intact normal rats (1, 11–13) and rule out the possible influence of increased secre-

tion of growth hormone in our experimental model. Whether these considerations based on experimental studies with young rats are in any way applicable to the human disease, kwashiorkor, is at present only highly speculative. If they are, however, it would suggest that the observed effect on the secretory activity of the pituitary gland in kwashiorkor is probably secondary rather than primary in this nutritional disorder.

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Germanium, Tin and Arsenic in Rats: Effects on growth, survival, pathological lesions and life span [1]

H. A. SCHROEDER,[2] M. KANISAWA, D. V. FROST AND M. MITCHENER
Department of Physiology, Dartmouth Medical School, Hanover, New Hampshire, and Brattleboro Memorial Hospital, Brattleboro, Vermont

ABSTRACT To evaluate innate effects of the trace elements germanium, tin and arsenic, 455 rats of the Long-Evans strain were fed a diet containing small amounts of these elements in an environment relatively free of trace contaminants. Groups of 100 or more, divided as to sex, were given 5 ppm germanate, arsenite or stannous ions in drinking water from weaning until natural death and compared with an equal number of controls. These levels were tolerable for growth. Innate toxicity in terms of life span and longevity occurred in females given tin and both sexes given germanium; increased incidences of fatty degeneration of the liver were observed for both these elements. Animals fed tin also showed an increased incidence of vacuolar changes in their renal tubules. Large amounts of arsenic accumulated in tissues, especially aorta and red blood cells, with no signs of toxicity. Tin accumulated to some extent and germanium less so. No element was tumorigenic or carcinogenic, there being somewhat fewer tumors in rats fed germanium than in the controls.

Life-term studies on mice and rats exposed to several abnormal trace metals have been reported (1–4). A more recent study involved germanium, tin and arsenic in mice (5). To confirm and extend these observations, rats were maintained on identical regimens. One purpose of these studies is to discover, if possible, recondite toxic effects in terms of growth, survival and extreme life span; another is to discover which elements, given orally, accumulate in several organs with age. In mice, tin was found to accumulate in heart muscle, as it does in man (6), and in spleen. Germanium accumulated in spleen, and arsenic given as arsenite to a small extent in liver, heart and lung. Mice fed germanium and arsenic had shorter survival times than their controls. The present report considers these and other variables in rats.

METHODS

The methods and materials used, the low metal diet, the drinking water and the special environmental conditions of the laboratory have been reported in detail and have not been altered (1). Random-bred rats of the Long-Evans strain were purchased; [3] their offspring were born and weaned in our laboratory. Groups of 50 or more of each sex, 4 in a cage, were given at weaning time sodium germanate, stannous chloride or sodium arsenite in drinking water at levels of 5 μg/ml of the element. The water also contained 1.0 μg/ml chromium (III) as the acetate, as well as soluble salts of zinc, copper, manganese, cobalt and molybdenum (1).

Animals were weighed at weekly intervals when weanlings, then at monthly intervals. They were disturbed for measurements of blood pressure, requiring anesthesia; for sampling of blood for analyses of fasting serum glucose levels and cholesterol, and for cleaning their cages at weighing time. At natural death, they were weighed, dissected and gross pathological changes described. Heart, lung, kidney, liver, spleen and tumors were fixed in Bouin's solution, sectioned, stained with hematoxylin and eosin and examined under light microscopy. Portions of these tissues were frozen in polyethylene bottles and at a suitable interval, ashed and analyzed for the elements given. A low tem-

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[1] Supported by Public Health Service Research Grant no. HE-05076 from the National Heart Institutes, Contract DA 2595 from the U.S. Army, Cooper Laboratories, Inc. and the CIBA Pharmaceutical Company.
[2] Address for requesting reprints: 9 Belmont Avenue, Brattleboro, Vermont 05301.
[3] Blue Spruce Farms, Altamont, New York.

perature asher [4] was used for arsenic (7) and germanium (8), as these elements are volatile; in the case of tin (6), tissues were ashed at 450° in muffle furnaces. Analytical methods used and their sensitivities have been reported in the study on mice (5).

Serum glucose was measured by the glucose oxidase method of Washko and Rice (9), using premixed reagents and a Berkeley Medical Instrument spectrophotometer. [5] Duplicate and replicate analyses agreed within 4%. Blood samples for fasting levels were obtained from 12 rats of each sex and each group, after they had been deprived of food for 18 hours.

Urine was tested semiquantitatively by sensitized paper [6] for protein and glucose. Blood pressure was measured on warmed, anesthetized animals by the method of Friedman and Freed (10).

During the summer of 1965, several studies involving general anesthesia and blood letting were made on female control animals, resulting in excessive mortality between the ages of 24 to 27 months. This mortality was confined to 5 cages. To provide adequate controls, the data on survival of the remaining 8 cages (32 animals) were combined with those of 11 cages (44 animals) in the control series previously reported (4). Erroneous comparisons would have resulted if the whole series of female controls were used. Survival of the 32 female rats used was similar to that of the 44 animals added. Growth rates were measured on the original series of 52 animals.

The lesions found in microscopic sections were evaluated by methods reported previously (11). Lesions were graded zero to 3+. Two changes in the liver were recorded: fatty degeneration, and cellular necrosis of peripheral, medial and central portions of the lobule. One lesion in the kidney was recorded: vacuolar changes in the renal tubules. The presence of pyelonephritis and other disorders was noted but not evaluated in this study, as no differences were found between groups. Blood vessels were examined for changes in their walls in heart, kidney, liver, lung and spleen.

The diet of seed rye flour (60%), dry skim milk (30%), corn oil (9%) and sodium chloride (1%), to which vitamins and ferrous sulfate were added (1), contained the following amounts of the trace elements under study (in μg/g wet weight): germanium 0.32, arsenic 0.46, and tin 0.28.

RESULTS

Growth rates. None of the 3 elements affected growth rates of rats significantly, except at isolated intervals (table 1). At 18 months of age, however, males fed tin and germanium weighed less, and females fed germanium weighed more than did their controls. Otherwise, mean weights of the 3 groups were remarkably similar. For the first few months the male rats in this series, given 5 ppm chromium in water, grew somewhat more rapidly than did those of the series reported previously (2), but by month 6 their weights were similar and so remained. Female rats of this series were larger at all ages including 1 year.

Heart weight. The mean body weights at death, the mean weight of the hearts and their mean ratios are shown in table 2. Hearts of male rats fed arsenic were smaller than those of their controls. Differences among other groups did not appear.

Survival. Median, 75% and 90% life spans are given in table 3 and the survival curves in figures 1 and 2. Differences of 2 months or more occurred in 3 groups at three intervals. Rats of both sexes fed arsenic and males fed tin were unaffected as to survival, according to these data, whereas both sexes given germanium and females given tin lived somewhat less long at these intervals. The difference of 134 days in the median life span of males fed germanium was significant.

Longevity. Defined as the mean age of the last surviving 10% of animals in each group, longevity is shown in table 3. Significantly lessened longevity was found in female rats given tin, and it was less by more than 2 months in females fed germanium. In the table also are the ages of the oldest survivors. A male fed arsenic lived a year longer than any of the male

[4] Tracerlab, 500-A, Richmond, California.

[5] Berkeley Medical Instruments. New England X-Ray and Electronic Equipment, Brookline, Massachusetts 02146.

[6] Combistex, Ames Company, Elkhart, Indiana.

TABLE 1

Mean weights [1] of rats given arsenic, germanium and tin

Age	Control	Arsenic	Germanium	Tin
days	*g*	*g*	*g*	*g*
Males				
30	72 ± 4.2	65 ± 4.1	78 ± 5.8	87 ± 6.3 [2]
60	190 ± 6.0	186 ± 8.8	193 ± 11.2	181 ± 8.2
90	270 ± 8.9	241 ± 6.7 [3]	272 ± 11.3	261 ± 7.7
120	313 ± 9.3	307 ± 4.9	305 ± 8.7	297 ± 7.7
150	342 ± 8.9	335 ± 5.2	339 ± 8.5	336 ± 8.6
180	365 ± 8.7	380 ± 7.2	363 ± 8.6	355 ± 11.3
360	444 ± 14.9	434 ± 6.5	464 ± 14.7	447 ± 10.4
540	507 ± 16.4	479 ± 11.0	455 ± 8.7 [4]	463 ± 10.5 [3]
Females				
30	65 ± 2.1	73 ± 4.6	64 ± 1.9	87 ± 4.9 [5]
60	154 ± 6.0	148 ± 7.4	132 ± 5.4	149 ± 6.7
90	197 ± 5.4	190 ± 4.0	183 ± 4.6	189 ± 5.5
120	225 ± 5.3	206 ± 5.5	213 ± 4.8	213 ± 4.6
150	239 ± 4.1	235 ± 4.9	235 ± 4.5	232 ± 3.6
180	251 ± 4.9	242 ± 5.0	243 ± 4.5	241 ± 3.8
360	268 ± 5.9	263 ± 6.5	286 ± 6.3 [4]	279 ± 7.0
540	267 ± 9.8	274 ± 5.4	301 ± 9.2 [4]	283 ± 5.2

[1] g ± SEM. There were 52–56 rats in each group.
[2] Differs from control, $P < 0.05$.
[3] $P < 0.025$.
[4] $P < 0.01$.
[5] $P < 0.005$.

TABLE 2

Heart and body weights of rats given arsenic, germanium and tin

	No. rats	Heart wt	Body wt [1]	Ratio × 1000
		mg	*g*	
Males				
Control	49	1498.1	333.9	4.69 ± 0.206
Arsenic	51	1349.1	322.1	4.02 ± 0.188 [2]
Germanium	49	1407.8	316.8	4.65 ± 0.186
Tin	44	1442.6	297.3	5.07 ± 0.243
Females				
Control	41	948.5	234.0	4.45 ± 0.216
Arsenic	44	1064.9	230.7	4.93 ± 0.231
Germanium	44	987.2	229.3	4.51 ± 0.211
Tin	51	914.8	197.8	4.65 ± 0.197

[1] At death.
[2] Differs from controls, $P < 0.025$.

TABLE 3

Mean life spans of rats given arsenic, germanium and tin

	No. rats	50% Dead	75% Dead	90% Dead	Longevity [1]	Oldest
		days	*days*	*days*	*days*	*days*
Males						
Control	56	872	974	1057	1160 ± 27.8	1232
Arsenic	53	825	937	1093	1220 ± 96.0 (1120 ± 18.4) [2]	1596
Germanium	51	738 [3]	902	1017	1177 ± 58.8	1319
Tin	56	876	957	1097	1134 ± 22.8	1222
Females						
Control	76	912	1050	1157	1304 ± 36.0	1347
Arsenic	55	912	1056	1175	1249 ± 24.9	1315
Germanium	52	833	932	1057	1231 ± 25.6	1308
Tin	56	830	989	1094	1160 ± 27.5 [3]	1267

[1] See text. Mean ± SEM.
[2] Excluding last survivor, which lived 376 days longer than next to last survivor.
[3] Differs from controls, $P < 0.005$.

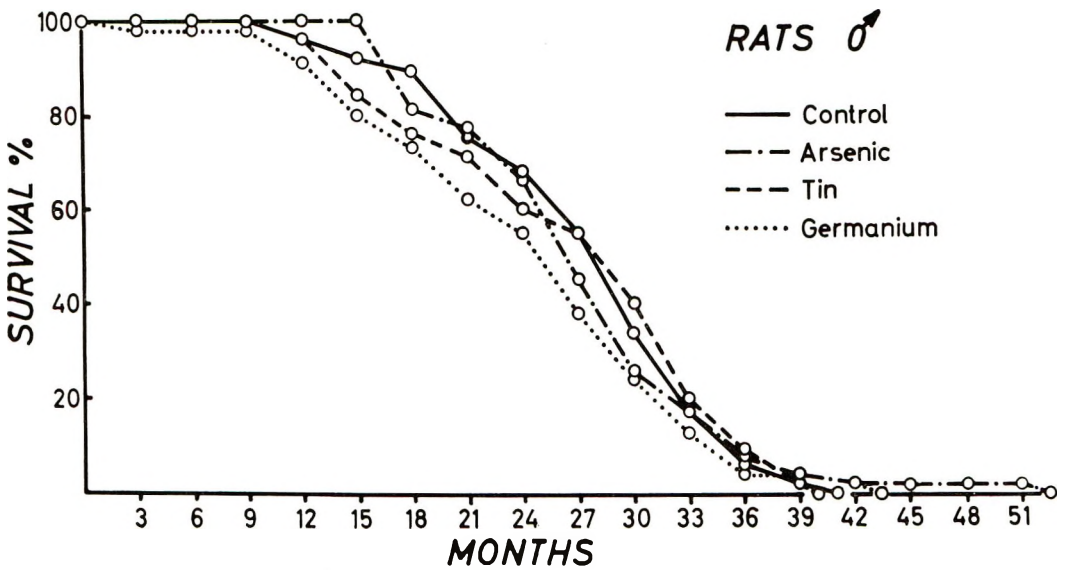

Fig. 1 Survival curves of male rats fed arsenite, germanate and stannous ions (5 ppm) in drinking water from the time of weaning. Although there were no significant differences from the controls at any 3-month interval, animals fed germanium survived somewhat less well from 9 to 27 months of age. Note the long survival of one animal fed arsenic.

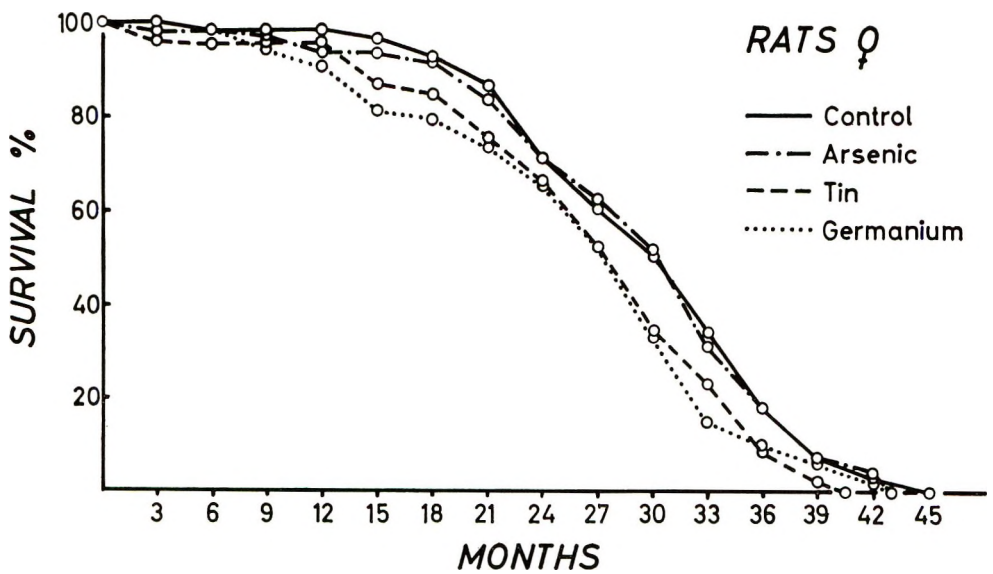

Fig. 2 Survival curves of female rats fed arsenite, germanate and stannous ions (5 ppm) in drinking water from the time of weaning. Although no significant differences from the controls were found for the rats fed tin, their survival was slightly less from 15 to 33 months of age. Animals fed germanium differed from the controls at 33 months of age by chi-square analysis ($P < 0.05$) and, in general, survived less well than their controls.

controls, more than 52 months, which was 141 days longer than our other oldest animal, one fed chromium (12).

Blood and urinary findings. Serum cholesterol levels on these rats have been reported (13); they were similar in all female groups. Males given arsenic had higher mean values ($P < 0.05$) and males given germanium had lower mean values ($P < 0.001$) than their controls.

Mean fasting serum glucose levels in male rats (± SEM) and the significance of differences in the values of the three groups from the controls were (in mg/100 ml): controls, 106.5±3.6, arsenic 78.5±4.4 ($P < 0.001$); germanium, 108.0±4.7; and tin, 101.3±4.2. In female rats they were: controls, 79.6±8.2; arsenic, 77.8±5.8; germanium, 99.7±3.6 ($P < 0.01$); and tin, 120.0±4.8. ($P < 0.001$). These measurements were made at approximately 2 years of age for the controls, 26 months for the arsenic group, 19 months for the germanium group and 18 months for the tin group.

Glycosuria was found in 15 of 66 control rats, 9 of 20 in the arsenic group, 3 of 20 in the germanium group and 6 of 22 in the tin group. It was of slight degree in all cases but one control, in which it was marked. Differences between groups were not statistically significant.

Proteinuria was common to almost all rats. Severe grades (3+ and 4+) were found in 27.3% of 66 control animals, 20.0% of those in the arsenic group, 45.0% of those in the germanium group and 36.4% of those in the tin group. For these two grades differences between groups were not significant. Grades 2 and 4+, however, occurred in 90% of the rats fed germanium and 63.7% of the controls ($P < 0.05$).

Blood pressure. Ten mature male and 10 female animals from each group were randomly selected for measurements of blood pressure at approximately 2 years of age. No abnormalities were found in three consecutive measurements at monthly intervals.

Tumors. We have reported that mice fed germanium had fewer tumors in all sites and those fed arsenic had fewer tumors of the lung than did their controls, whereas the feeding of tin was associated with no such effect (11). In rats, a difference appeared only in the germanium group. Tumor incidences in the 4 groups were as follows: controls 37.8%, arsenic 27.8%, germanium 22.2% ($P < 0.05$) and tin 29.9%. Clusters of abnormal liver cells appearing "pretumorous" (11) were more frequent in the arsenic group (20%) than in the others (4–10%). Of the 18 malignant tumors found, there were 9 in the controls, 3 in the arsenic group, 2 in the germanium group ($P < 0.05$) and 4 in the tin group. These data will be reported elsewhere.

Other pathological changes. The degrees of fatty degeneration of the liver found in the various groups are shown in table 4. Included are livers from previously reported animals (4) fed chromium and cadmium. There was a high incidence of fatty change in the livers of the germanium- and tin-fed rats compared with the controls, and a low incidence in the chromium group. Hepatic cellular necrosis occurred in all groups (12.1 to 22.2%) (table 5). Vacuolar changes in the proximal convoluted tubules of the kidney were significantly increased in animals fed tin (36.2%, $P < 0.025$) compared with controls (18.2%) (table 4). Vascular lesions were singularly rare or absent in the various groups.

Accumulation of elements in tissues. Mean values of the elements in tissues are shown in tables 6 through 8. Compared with controls, these elements accumulated in one or more organs.

Nine analyses on 23 stillborn or suckling rats showed no detectable tin in their whole carcasses. Three contained 0.68 μg/g. Certain tumors accumulated tin in concentrations of 0.91 to 7.77 μg/g, 5 containing more than 2.73 μg/g, whereas 4 other tumors had no detectable tin. Hearts and spleens showed more tin than did other normal tissues (table 6); which was also the case with mice (5). Accumulations were not large and, although the differences from the controls were not statistically significant, the prevalences were, in kidney, liver and lung.

Arsenic accumulated with age in all tissues analyzed to a remarkable extent. Although few young rats were available

TABLE 4

Fatty degeneration of the liver and vacuolar changes in the proximal convoluted tubule of the kidney in rats given trace elements [1]

	No. rats	Severity of lesion			Total no.	With lesions
		+	++	+++		
						%
Liver						
Control	88	6	22	5	33	37.5
Arsenic	83	12	26	4	42	50.6
Germanium	86	15	32	7	54 [3]	62.8
Tin	80	17	30	7	54 [4]	67.5
Chromium [2]	28	1	4	0	5 [5]	17.9
Cadmium [2]	24	2	4	2	8	33.3
Kidney						
Control	88	9	7	0	16	18.2
Arsenic	77	14	6	3	23	29.8
Germanium	84	14	7	1	22	26.2
Tin	81	15	10	1	26 [5]	32.1
Chromium [2]	27	2	1	0	3	11.1
Cadmium [2]	35	4	0	0	4	11.4

[1] Fatty changes in the liver were classified as peripheral, central and diffuse. There appeared to be no significant differences in the areas of degeneration among the various groups, except in rats fed chromium where peripheral changes were absent.
[2] Included from previous studies for comparison.
[3] Differs from controls by chi-square analysis, $P < 0.005$.
[4] $P < 0.001$.
[5] $P < 0.05$.

TABLE 5

Hepatic cellular degeneration and necrosis, and moderate and severe fatty degeneration, in rats given trace elements

	No. rats	Degeneration and necrosis	Fatty changes moderate and severe [1]	Total	
					%
Control	88	21	27	48	54.5
Arsenic	83	19	30	49	59.1
Germanium	86	12	39	51	59.3
Tin	80	14	37 [3]	52	65.0
Chromium [2]	28	12	4	16	57.2
Cadmium [2]	24	9	6	15	62.5

[1] Included from table 4. Differs from controls by chi-square analysis, $P < 0.05$.
[2] Included from previous studies (4) for comparison.
[3] Number differs by chi-square analysis, $P < 0.05$.

TABLE 6

Tin in tissues of rats, wet weight

	Controls [1]			Fed tin [2]		
	No. rats	No. present	Mean	No. rats	No. present	Mean
			μg/g			μg/g
Kidney	21	7 [3]	0.31	32	32	0.17
Liver	21	16 [4]	0.11	32	32	0.35
Heart	21	18	0.59	32	32	0.93
Lung	18	7 [3]	0.14	32	32	0.54
Spleen	21	21	0.96	36	36	1.88
Stillborn, whole	26	3	0.08	—	—	—
Tumors	18	11	1.77	2	2	1.12

[1] Control rats were 523 to 822 days old.
[2] Tin-fed rats were 602 to 953 days old.
Tissues were pooled in groups of 3 to 11 samples.
Differences between the means of the control and rats fed tin were not statistically significant.
[3] Differs from group fed tin by chi-square analysis, $P < 0.001$.
[4] $P < 0.01$.

for analysis because of the low mortality of this group, 10 aged less than 2 years had (in μg/g): kidney 9.20, liver 5.83, heart 11.90, lung 10.89, and spleen 14.18. Older rats had several times these values (table 7). Red blood cells of aged rats took up as much as 282 μg/g arsenic. This element also accumulated in the aorta. The oldest animal fed arsenic had lower concentrations in five tissues than the means of the others. Small amounts were found in most tissues of control animals and differences were highly significant. No arsenic was detected in kidneys, or in 16 lungs. This large accumulation of arsenic in rats can be compared with the relatively small amounts found in mice treated identically (5).

Germanium accumulated in the kidney (table 8) and was present in more livers and lungs than in controls; however, relatively small amounts were found.

TABLE 7

Arsenic in rat tissues, wet weight [1]

	Control [2]		Fed arsenic		Range [3]
	No. rats	Mean	No. rats	Mean	
		μg/g		μg/g	μg/g
Kidney	27	0.0	59	27.63	11–49
Liver	38	0.21	94	46.92	23–189
Heart	11	0.53	96	34.53	10–87
Lung	11	0.25	96	46.19	22–123
Spleen	11	0.31	83	39.79	14–99
Brain	3	0.38	—	—	—
Tumor	3	0.25	6	30.78	3–75
Aorta, ♂ (dry wt) [4]	—	—	10	315.60	—
Aorta, ♀ (dry wt) [5]	—	—	10	106.98	—
Washed RBC					
Young [6]	—	—	12	73.90	—
Old [5]	—	—	24	282.46	249–316
Whole blood	—	—	1	44.79	—

[1] Tissues were pooled in lots of 41 to 10.
[2] Control rats were 583 to 988 days old.
[3] Rats more than 2 years old.
[4] Less than 2 years old.
[5] More than 3 years old.
[6] Less than 1 year old.
Note: Oldest rat aged 1596 days had (μg/g): muscle 5.71, skin 2.23, hair 22.49, teeth 1.69, femur 2.28, kidneys 24.74, heart 18.06, lung 60.11, spleen 11.49, liver 20.75. Differences in the means of control and rats fed arsenic were highly significant ($P < 0.001$).

TABLE 8

Germanium in the tissues of rats, wet weight [1]

	Controls [2]			Fed germanium [3]		
	No. rats	No. present	Mean	No. rats	No. present	Mean
			μg/g			μg/g
Kidney	25	25	0.18	24	24	0.62 [4]
Liver	25	10 [5]	0.14	25	25	0.27
Heart	12	6	0.16	32	22	0.17
Lung	18	12 [6]	0.11	34	34	0.28
Spleen	9	9	0.58	18	18	0.50
Tumor	2	2	1.05	—	—	—
Whole, newborn	1	1	0.18	—	—	—
Fat	—	—	—	1	1	0.53

[1] Tissues were pooled in groups of 4 to 10.
[2] Control rats were 560 to 915 days old.
[3] Germanium-fed were 540 to 892 days old.
[4] Means of pooled samples differ from controls, $P < 0.025$.
[5] Differs from group fed germanium by chi-square analysis, $P < 0.0001$.
[6] $P < 0.005$.

DISCUSSION

In these and previous studies we have attempted to evaluate several trace elements present in human food and in the environment for effects on growth and longevity and for recondite toxicity in terms of chronic disease. Two common disorders have appeared in our laboratory, hypertension caused by excess cadmium (14) and diabetes mellitus resulting from chromium deficiency (12). This study uncovers no suggestion that specific disorders may result from arsenic, germanium or tin. None of these elements was tumorigenic or carcinogenic, nor was any associated with overt disease. No arsenic keratoses appeared, and this element was not toxic in terms of growth and life span.

In attempting to evaluate less obvious changes in the various criteria used in this study, we can arbitrarily classify values different from the controls as favorable or adverse. Thus, limited growth, lessened survival and longevity, elevated serum cholesterol and glucose levels, excess proteinuria, excess number of tumors, fatty changes in the liver and renal lesions can be considered as adverse, whereas their counterparts can be considered as favorable. In this light, female rats fed arsenic showed no significant differences from the controls; males had elevated serum cholesterol levels only. Glucose levels, however, were lower than the controls. These very few changes occurred in the presence of a remarkable accumulation of arsenic in the tissues, demonstrating that arsenite, given at this level, is not toxic to rats.

In animals fed tin, males showed one adverse effect, lower body weight; females exhibited decreased survival and longevity and elevated glucose levels, and both had increased incidences of fatty changes in liver and renal tubular vacuolization. No criterion was altered in a favorable direction. These changes were found in the presence of only small amounts of tin in tissues.

In rats given germanium, changes considered adverse were greater. Excess proteinuria, fatty livers and decreased survival were found in both sexes; males had lower weights and females decreased longevity and moderate hyperglycemia. Concerning presumed favorable effects, body weight in females was increased, serum cholesterol in males was decreased and there were fewer tumors. These changes occurred in the presence of slight accumulations of germanium in tissues. These data suggest therefore that both germanium and tin are slightly toxic to rats at the levels fed. Germanium showed similar toxicity to mice (5), although we did not find fatty degeneration of the liver increased in mice fed these elements.[7]

The approximate intakes of these elements were (in μg/100 g body weight/day): arsenic 37.76, germanium 36.92, and tin 36.68, while the controls ingested 2.76, 1.92 and 1.68, respectively. These intakes are relatively much larger than those of human beings, who take in food and water about 900 μg arsenic (7), 1500 μg germanium (8) and 4.0 mg tin (6) or 1.3, 2.1 and 5.7 μg/100 g body weight, respectively. Extreme but "normal" human intakes of arsenic and tin have been calculated as 4.8 mg and 40 mg, respectively (6.9 μg and 57 μg/100 g body weight), which places the intake of tin by rats comparable to that of certain human beings.

We have analyzed a few human tissues for arsenic. It was not detected in the kidney, liver, heart and pancreas of a 9-month-old male, but was found (μg/g, wet weight) in lung, 0.69; in muscle, 0.21; and in spleen, 1.43. In the tissues of a 69-year-old female, arsenic was not detected in kidney, heart and spleen, but occurred in liver (0.66 μg/g) and lung (0.59 μg/g). These concentrations are similar to those found in control rats.

We have not analyzed human tissues for germanium. Concentrations of tin found in rats fed this element were slightly larger in some organs than those reported in human beings (6), but were generally similar. Therefore, the human experience with respect to tin was reasonably well duplicated in rats. At these tissue concentrations, tin exhibited recondite toxicity in female rats in terms of life spans and survival, fatty degeneration of the liver and vacuolar degeneration of the renal tubules. This effect was not observed in mice (5).

[7] Schroeder, H. A., M. Kanisawa, D. V. Frost and M. Mitchener, unpublished observations.

The levels of these elements given to rats were tolerable in terms of growth. The level of arsenic was tolerable in terms of survival despite large accumulations of this element. Byron et al. (15) found no effect on survival of rats given arsenate or arsenite at much higher levels than we used. The level of germanium, however, was associated with decreased survival. Such an effect was curious since little germanium accumulated in tissues. Apparently germanium as germanate is readily excreted via the urine (8), but despite this characteristic, it apparently can exert recondite toxicity on liver, kidney, and perhaps on growing tumor cells.

ACKNOWLEDGMENT

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Changes in Cholesterol Concentrations in Rough and Smooth Endoplasmic Reticulum and Polysomal Profiles in Rats Fed Cholesterol [1,2]

ANDRZEJ PROŃCZUK AND LOUIS CHARLES FILLIOS
Massachusetts Institute of Technology, Cambridge, Massachusetts, and Boston University School of Medicine and University Hospital, Boston, Massachusetts

ABSTRACT A significant increase in the concentration of cholesterol (expressed in terms of protein) was found in both rough and smooth endoplasmic reticulum (ER) from rats fed cholesterol, with the smooth ER consistently showing the greater degree of increase. 2-^{14}C-mevalonate was then used to study the kinetics of newly synthesized cholesterol in vivo in both these fractions. The specific activity of newly synthesized cholesterol expressed in terms of total vesicle protein was consistently higher in the rough ER than seen in the smooth ER within the experimental period (up to 160 minutes) in control rats, but rats fed cholesterol show an opposite relationship. Also, cholesterol feeding resulted in a marked but expected depression in cholesterol synthesis. Sucrose gradient separations of ribosomes and polysomes showed that cholesterol feeding results in a relative increase in the monosomes (and possibly disomes and trisomes) at the expense of the larger polysomes. Furthermore, the free ribosomal protein (per liver) is significantly increased in rats fed cholesterol and accounts in part for the previously observed depression in hepatic protein synthesis in vivo and in vitro. The possible involvement of membranes in the intracellular transport of cholesterol is discussed.

It has been widely documented that various experimental diets containing cholesterol can elevate the circulating lipoproteins as well as cause a marked accumulation of hepatic lipids, including cholesterol, in relatively short periods of time. Such regimens can also produce significant depressions in certain aspects of hepatic protein synthesis in rats (1–4). To determine the nature of this phenomenon, other factors that may be related to this decreased capacity for total protein synthesis were examined. Since the endoplasmic reticulum (ER) is the principal site of synthesis of total protein (5) as well as the site of many of the steps in the synthesis of cholesterol (6, 7), certain changes associated with this membranous component were studied in rats fed standardized diets containing cholesterol.

MATERIALS AND METHODS

Female albino rats (7 weeks of age) of the Sprague-Dawley strain were selected for these experiments. The animals were individually caged in a temperature- and humidity-controlled room and fed a commercial laboratory ration [3] prior to receiving a semisynthetic diet. Three semisynthetic diets were used: a control diet (O-O), a cholesterol-supplemented diet (C-O), or a cholesterol-cholic acid diet (C-C). Cholesterol was added at 1.5% and cholic acid at 0.5% of the diet (at the expense of the carbohydrate level). This level of cholic acid does not interfere with food intake or body weight response (1, 2). The protein level (casein) was 20%, while the fat level (corn oil) was 10%; salt mixtures, choline chloride, inositol, trace nutrients, and vitamins were included in concentrations previously described.[4,5]

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[2] Part of these data were included in a preliminary report at the Annual Meeting of the Federation of American Societies for Experimental Biology, Chicago, Illinois, 1967.

[3] Purina Laboratory Rat Chow, Ralston Purina Company, St. Louis.

[4] All dietary ingredients including the cholesterol and cholic acid were purchased from Nutritional Biochemicals, Inc., Cleveland, with the exception of the corn oil which was obtained from Corn Products Company, New York.

[5] The levels of salt mixture (Hegsted salt mixture 1941 J. Biol. Chem., *138:* 459), choline, inositol, and vitamin mixture have been described (Fillios, L. C., and O. Yokono 1968 J. Nutr., *95:* 349).

After 2 weeks on an experimental diet, each rat was decapitated after an overnight fast (18 to 20 hours). This procedure was used for all rats in this study including those injected with labeled material intraperitoneally at 10-, 20-, 40,- 80-, or 160-minute intervals prior to killing. There was no significant difference in body weight response or food intake among the dietary groups.

Two minutes after an injection of 2-^{14}C-mevalonate (5 μCi/100 g of body weight),[6] an injection of unlabeled mevalonate [7] was given to dilute the labeled pool and allow a study of any possible "chasing" of newly synthesized cholesterol from one fraction to another.

After each rat was killed the liver was immediately perfused in situ with ice-cold sterile 0.9% NaCl. A portion of the liver was then taken and minced and homogenized in 5 volumes of ice-cold 0.88 M sucrose with a Tri-R tissue homogenizer. The homogenate was centrifuged for 30 minutes at 0 to 2° at 12,000 × *g* in a BD-2 International ultracentrifuge. The postmitochondrial supernatant was saved for the isolation of smooth and rough endoplasmic reticulum as well as free ribosomes according to a modification of the method of Hallinan and Munro (8). Freshly isolated pellets were precipitated with 0.2 N perchloric acid (PCA). This procedure was modified in the case of smooth-surfaced vesicle fractions. Before precipitation of the freshly isolated fraction with PCA, the residual isooctane of the pellet was removed by repeated extractions of this precipitate with methanol–chloroform (2:1). This precaution was found necessary to assure complete precipitation following PCA treatment. The precipitate was washed with 0.2 N PCA. The pellet protein was then dissolved overnight in 0.3 N KOH; one portion was taken for protein determination by the method of Lowry et al. (9); another portion was reprecipitated in 0.2 N PCA and the supernatant discarded. The final dried pellet was plated and dried on a stainless steel planchet. Radioactivity was determined using a Nuclear-Chicago thin window gas-flow counter.

The lipids of the isolated rough and smooth endoplasmic reticulum pellet were extracted with methanol–chloroform (2:1). The residual protein was determined as described above. The lipid extracts were washed twice with 0.2 volume of 0.05% $CaCl_2$ to remove any nonlipid contamination (10). The portion of lipid phase was then collected and evaporated overnight at 20°. The dried lipid materials containing cholesterol were reextracted with a 1:1 acetone–alcohol solution according to the Shapiro and Kritchevsky method (11). After filtration, the acetone–alcohol extracts were collected and made up to the previous volume. Two portions were then taken for further purification of the cholesterol by digitonide precipitation, according to the method of Sperry and Webb (12). Finally, the cholesterol precipitate was dissolved with a methanol–ethanol (1:5) solution and a sample saved for cholesterol determination (13); another portion was plated on and counted by means of a Nuclear-Chicago window gas-flow counter.

The polysomes were prepared after removal of interfering ferritin (14). Rats from two dietary groups (C-O or C-C versus O-O) were fasted 18 hours prior to killing. Polysomes obtained by Drysdale and Munro's (14) procedure were layered on a 4.6 ml linear sucrose gradient (10 to 40%) after the samples were equilibrated in terms of optical density at 260 mμ and centrifuged at 0 to 2° for 10 minutes at 38,000 rpm in a Spinco model L-2 ultracentrifuge, with a SW-50 rotor. After centrifugation, the bottom of the tube was punctured with a needle; the absorbancy at 260 mμ of the collected material was determined using a Gilford model 2000 recording spectrophotometer with a flow-through cell system. The C-ribosome profile method (15), widely used, does not permit complete determination of the monosomal peak (14) and for this reason the Drysdale and Munro method was found preferable for characterizing the monoso-

[6] 2-^{14}C-mevalonic acid as the lactone was purchased from Calbiochem, Los Angeles (spec. activity: 5.03 μCi/μmole). After its conversion to the potassium salt by the method of Shapiro and Kritchevsky (11), a dose of approximately 2 μmoles of labeled mevalonate was given to each rat in 0.9% NaCl.

[7] Mevalonic acid (dibenzylethylenediamine sodium salt A) was obtained from Calbiochem. After treatment with KOH the dibenzylethylenediamine was then extracted with petroleum ether and neutralized with HCl. Approximately 50 μmoles of the neutralized unlabeled mevalonate in 0.9% NaCl were injected into each rat to dilute the labeled pool.

TABLE 1

Relation of cholesterol to protein

Dietary group	No. of animals [1]	Body wt gain	Total liver wt	Rough vesicles			Smooth vesicles		
				Protein per liver [2]	Cholesterol per liver [3]	Cholesterol / Protein [4]	Protein per liver [2]	Cholesterol per liver [3]	Cholesterol / Protein [4]
		g	*g*	*mg*	*mg*		*mg*	*mg*	
O-O	16	46	5.80	94.6 [5]	4.21	4.45	36.3 [5]	1.63	4.49
C-O	5	46	7.38 [6]	124.8 [7]	7.09	5.68	43.2	5.45	12.59
C-C	16	44	8.04	117.1	8.83	7.54	39.9	5.39	13.68

[1] Initial body weights were approximately 144 g. Animals were fed the experimental diets for 2 weeks (see text).
[2] Protein = total protein of isolated particles.
[3] Cholesterol = total cholesterol.
[4] Ratio × 100.
[5] Mean values.
[6] Underlined values are significantly different from the O-O (control) value; $P < 0.02$ based on Student's *t* test.
[7] Broken line indicates only a suggestive difference; $P < 0.05$.

mal peak. Although comparisons were made between those two methods,[8] the data presented were based on the preferred method of Drysdale and Munro.

RESULTS

Cholesterol concentration in relation to total protein of smooth and rough endoplasmic reticulum. It has been widely observed that the hepatic cholesterol concentration can be greatly increased following cholesterol feeding. In the present study, the feeding of a high cholesterol diet also produced a significant increase in cholesterol concentration for both rough and smooth ER which generally agrees with an early report for total microsomal cholesterol (16). The absolute amount of whole liver cholesterol for rough ER obtained from rats fed cholesterol is almost twice as high as seen in normal rats, whereas the smooth portion of the ER shows an even greater cholesterol accumulation (table 1). Although the total liver cholesterol concentration can increase 5- to 10-fold (C-O diet) or 10- to 15-fold (C-C diet) in 2 weeks (1, 2), the cholesterol concentration for the microsomal fraction shows only a mild increase (16). In the present study, the cholesterol accumulation, which can be expressed in terms of either total cholesterol per liver or cholesterol to vesicle protein, shows that the smooth vesicles consistently have a greater increase than do the rough vesicles. For example, the cholesterol-to-protein ratio (× 100) for the smooth vesicles went from 4.49 to 13.68 whereas the value for rough went from 4.21 to 8.83 after 2 weeks of cholesterol-cholic acid feeding. A similar trend was observed in those rats fed cholesterol alone (4.49 to 12.59; 4.21 to 7.09).

The total vesicle protein per liver is only slightly increased (2). But the total protein of the free ribosomal fraction was found to be significantly increased ($P < 0.02$) (see legend to fig. 2); the significance of this latter increase is discussed in relation to the changes noted in the polysomal profiles. There were no statistical differences in body weight response

[8] Pronczuk, A. W., and L. C. Fillios 1967 Effect of dietary cholesterol on the hepatic endoplasmic reticulum. Federation Proc., 26: 409 (abstract).

or food intake for all experimental groups in this study.

^{14}C incorporation into the cholesterol of rough and smooth vesicles. Studies of ^{14}C incorporation into the newly synthesized cholesterol show marked changes following cholesterol feeding. In the control (O-O) group, the ^{14}C incorporation into cholesterol (expressed as specific activity per 100 mg of protein) appeared to be higher in the rough vesicles than in the smooth vesicles. After the initial 10-minute period, the activity of the rough ER fraction (until the 80-minute interval) remains significantly higher than the smooth ER but the curves appear to approach each other at 160 minutes (see fig. 1).

The high cholesterol diet group (C-C) shows a significantly lower incorporation of ^{14}C from mevalonate into newly synthesized cholesterol. If a 10- to 15-fold dilution of the cholesterol pool is taken into account, the ^{14}C activity of the fractions from the cholesterol-fed group will still represent only 20 to 30% of the activity of the control group; this degree of depression is consistent with the data of others (17, 18) when mevalonate was used as a precursor.

The initial rate of incorporation of ^{14}C into newly synthesized cholesterol is not apparently slower for either smooth or rough ER among those rats fed cholesterol. However, the total activity of the smooth fraction is higher through the entire ex-

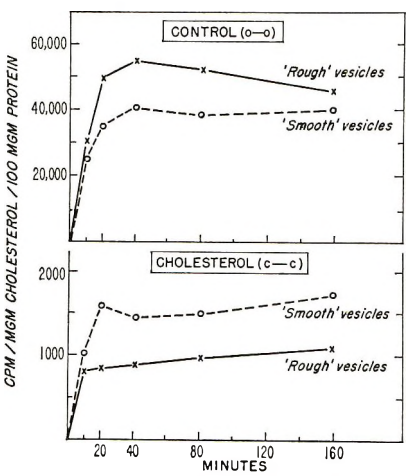

Fig. 1 ^{14}C incorporation into the cholesterol in vivo. Each point represents the mean of 4 to 8 liver values. (O-O) indicates those values which were derived from rats fed a control purified diet; (C-C) are values from rats fed a diet containing cholesterol and cholic acid. Each rat was fasted overnight before receiving 5 μCi of 2-^{14}C-mevalonate/100 g of body weight. Two minutes after receiving labeled mevalonate, each rat received a larger molecular dose of "cold" mevalonate to dilute the mevalonate pool. Kinetics for rats fed a diet supplemented with cholesterol alone (C-O) were not completed; but at 40 minutes, for example, the values for "smooth" and "rough" vesicles were 4630 and 2820, respectively.

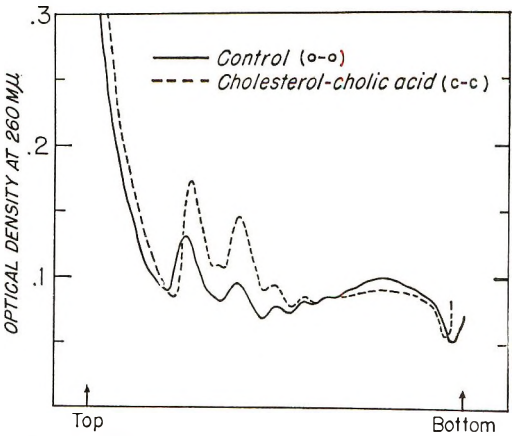

Fig. 2 Typical polysomal patterns from livers of rats fed control (O-O) or cholesterol-cholic acid diet (C-C). The postmitochondrial fraction was layered over a continuous sucrose gradient (10 to 40%) after the suspension was equilibrated in terms of optical density. The total protein of the free ribosomes was 20.73 mg/liver for control tissue whereas the rats fed cholesterol had a mean value of 25.70 mg/liver ($P < 0.02$).

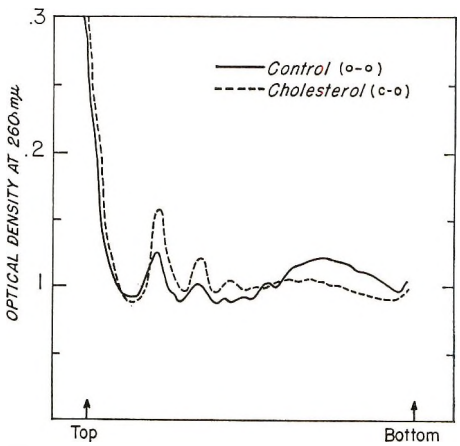

Fig. 3 Polysomal patterns from livers of rats fed the control (O-O) or the cholesterol diet (C-O).

perimental period. Hence, it was advisable to express these data as specific activity per 100 mg of protein (an index of total activity) since this would give a more meaningful picture of the changes taking place rather than expressing the data as specific activity alone (specific activities can be calculated from the cholesterol-to-protein ratios from table 1).

Polysomal profile analyses. A sucrose gradient profile of polysomal aggregates associated with the ER was examined, and it was found that the dietary treatment (cholesterol feeding) favored a shift of larger aggregates toward smaller aggregates, including monosomes. In figures 2 and 3 the employment of an improved method (14) which allows for a better characterization of the monosomal peak illustrates this observation. Admittedly, the differences are small in some cases but nevertheless this change in profile is a consistent observation in this laboratory in livers from rats fed diets supplemented with cholesterol-cholic or cholesterol. This method or the method of Wettstein et al. was found to show the same trend.[8]

It should be emphasized also that the quantitative differences, especially in the polysomal region, may be small since the polysome region is spread out. The overall profile changes are consistent, and the relative shift is always in favor of smaller units, especially monosomes. This change in the pattern has been seen in every comparison made and is not due to any effects related to anorexia, or the like, since control rats were treated identically and food intake patterns were similar. The effects of either (C-O or C-C) feeding on the polysomal profiles were similar.

DISCUSSION

Amino acid incorporation into the liver total proteins in vitro was found to be decreased in rats fed diets containing cholesterol or cholesterol and cholic acid (1–3), and this depression was reflected by a decrease in DNA-dependent RNA polymerase activity (4). The present report has attempted to determine whether these changes can be related to alterations of biochemical events associated directly with the endoplasmic reticulum. Even after a relatively short period of cholesterol feeding, the rat liver accumulates significant amounts of lipid, including cholesterol, and this largely accounts for the well-documented increase in liver weight. It is also known that total liver protein increases moderately, but the protein concentration in terms of DNA remains close to normal (2). Particular attention to the composition of the smooth and rough ER shows most of the microsomal protein associated with the rough ER. Actually, it was calculated by others (8, 19) that the rough ER accounts for 65% of the protein (and about 62% of phospholipids) of the total microsomal fraction; and this generally agrees with our values. Of considerable importance, however, is the more striking increase in the total free ribosomal protein. This increase in free ribosomes is supported by the polysomal profile analyses. (Livers from rats fed cholesterol showed a relative increase in monosomes but a decrease in the larger polysomes.) Therefore, the lowered capacity for protein synthesis of the endoplasmic reticulum following cholesterol feeding is related to these changes. Cholesterol feeding results not in a reduction of total ER protein but, as pointed out above, there is actually a modest increase as related to the whole liver. Therefore, the increases in the cholesterol-to-protein ratio of the endoplasmic reticulum are not due to any apparent decrease in total protein. The modest increase in cholesterol relative to protein for the rough ER is overshadowed by the relatively greater increase for the smooth ER. The significance of this difference in cholesterol accumulation between these two fractions is important in understanding the intracellular regulation of cholesterol metabolism.

It is customary to express the cholesterol concentration of various membranous components in terms either of protein or phospholipid (19). Under the present dietary conditions, it was felt prudent to use the cholesterol-to-protein ratio as the index of choice. Regardless, the total cholesterol of the liver endoplasmic reticulum is increased by our dietary treatments, and this increase is greater for the smooth vesicles. Whether these cholesterol increases represent an increase in membranous cholesterol or storage cholesterol

cannot be determined at the present time. Other data from this laboratory, however, have shown that the increase also represents a relatively greater percentage of esterified cholesterol for the smooth vesicles. Normally smooth vesicles have about 90% of the cholesterol in the free form; following cholesterol feeding, the percentage of free cholesterol decreases to about 75% but the percentage of free cholesterol in the rough vesicles remains greater than 90% (Fillios et al.).[9] This suggests that rough vesicles maintain a relatively smaller percentage of esterified cholesterol at all times even after the relatively high degree of hepatic cholesterolosis imposed by the present dietary treatments.

Dietary cholesterol is known to inhibit endogenous cholesterol synthesis in the liver microsomes, and the site of inhibition appears to take place before the synthesis of mevalonate (18, 20, 21). In the present study cholesterol synthesis was examined in rough and smooth vesicles isolated from rats fed cholesterol. As expected, both the cholesterol and cholesterol-cholic acid dietary groups demonstrate a very marked depression of cholesterol synthesis after measuring the activity of the cholesterol digitonide isolated from the membranes of rats injected with labeled mevalonate. An explanation for the very large depression in cholesterol synthesis, despite the use of labeled mevalonate in the present study, may be attributed in part to the dilution of the total cholesterol pool after 14 days of dietary treatment. It has already been shown, however, that prolonged cholesterol feeding brings about secondary feedback inhibitions between mevalonate and farnesyl pyrophosphate as well as between farnesyl and squalene; the degree of depression in cholesterol synthesis observed here is consistent with the experience of others (20).

Our data show that in control rat livers the specific activity or total activity is higher for the rough vesicles than the smooth vesicles. This implies that under normal conditions most of the newly synthesized free cholesterol of the microsomal fractions preferentially appears in the rough vesicles. The data also suggest that in time a crossover to smooth vesicles may take place, and experiments suggest such a possible relationship. In a strain of genetically obese rats such a crossover phenomenon is evident at about 180 minutes after labeled mevalonate was injected in the rats fed the control diets (Fillios et al.).[9] Following cholesterol feeding, cholesterol synthesis is depressed; and the total activity of newly synthesized cholesterol is actually higher in the smooth vesicles throughout the experimental period. This suggests that under these conditions the newly synthesized cholesterol preferentially accumulates within the smooth vesicles. This observation implies that there may be a threshold level for cholesterol in rough vesicles.

Under certain conditions, an hypertrophy of endoplasmic reticulum has been related to the production of hyperlipemia (22–24). In response to a specific lipid accumulation, it can be proposed that the endoplasmic reticulum (possibly in concert with other intracellular components) is intimately involved in the process of lipid transport out of the cell (3). This working hypothesis can involve the lipoprotein membrane of the smooth endoplasmic reticulum which through a process of vesiculation may carry, in smaller new vesicles, excess cholesterol esters and neutral fat to the Golgi apparatus where according to Jones et al. (24) the lipoproteins are packaged prior to their final extrusion into the circulation. The involvement of other membranes, like the plasma membrane, in such a mechanism has also been considered.[9] More likely the packets of osmophilic bodies observed by Jones et al. (24) and Hamilton et al. (25) may discharge their lipoprotein loads at the periphery of the cell. Two considerations can be conceived: a) cholesterol may preferentially accumulate in the rough and be transported via lipoprotein to the smooth ER, or b) the rough ER after losing adhering polysomes can become smooth, with the assembly of lipoprotein taking place within the cisternae of these smooth vesicles (probably using all or part of the original lipoprotein membrane itself). In cases where excess cholesterol accumulates in the liver pool, e.g., a high cholesterol diet, endogenous cholesterol

[9] Fillios, L. C., A. W. Pronczuk, O. Yokono and K. Kobayakawa, unpublished data.

synthesis is depressed but the accumulation of intracisternal cholesterol esters and other lipids may lead to an hypertrophy of smooth ER. Thus, lipoprotein may be stimulated by utilizing in part some of the original lipoprotein membrane of the smooth ER; other membranes such as the smooth elements of the Golgi apparatus (24, 25) as well as the plasma membrane, which is relatively rich in cholesterol (26), may be sequentially involved. The present data are compatible with the approach which includes the intimate involvement of membranes and provides further evidence on the relationship of the control of hypercholesteremia with the protein synthesizing apparatus of the liver cell.

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Cellular Antibody Synthesis in Vitamin B_6-deficient Rats [1]

MAHENDRA KUMAR AND A. E. AXELROD

Department of Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania

ABSTRACT Cellular antibody synthesis was determined by the Jerne agar-plaque technique in normal and vitamin B_6-deficient rats immunized with sheep erythrocytes. A severe reduction in the number of individual antibody-forming cells was observed in spleens from immunized deficient animals. This decreased cellular immune response was independent of the inanition associated with the deficiency and was restored to normal by the administration of pyridoxine shortly before immunization. Accumulation of antigen by rat spleen did not appear to be deranged in vitamin B_6 deficiency.

The significant role of vitamin B_6 in the development of numerous immune processes has been reviewed (1, 2). In our laboratories, it has been demonstrated in the rat that the level of circulating antibodies following the antigenic stimuli of human erythrocytes (3), diphtheria toxoid (4) and influenza virus (5) is reduced markedly in vitamin B_6 deficiency. However, these studies, concerned solely with the measurement of serum antibodies, fail to consider the effects of a vitamin deficiency upon the individual, discrete processes involved in antibody metabolism. The serum antibody level probably reflects an equilibrium state determined, on the one hand, by the rates of antibody synthesis and release from the site of formation and, on the other hand, by the rates of its destruction and excretion. Thus, a decreased antibody titer in the serum cannot be taken as unequivocal proof for the actual impairment of antibody synthesis (6, 7). Possible effects of a vitamin deficiency upon the metabolism of antigen before it reaches the target tissues such as spleen, and upon capacity of the cells of the antibody-forming apparatus to accumulate antigen, also merit consideration when evaluating the specific role of a vitamin. Evaluation of some of these factors by indirect means has indicated that the process of antibody synthesis is probably the site which is affected adversely in deficiencies of vitamin B_6 and pantothenic acid (7, 8).

In view of the complexity of factors involved in establishment of serum antibody level, it is apparent that a more direct approach to the problem of vitamin B_6 action is required. Such an approach would be afforded by an investigation of antibody synthesis at the cellular level. Progress in this area has been facilitated by the recent development of a method suitable for the study of antibody synthesis by individual cells (9, 10). Using this procedure, we have demonstrated in the present study a dramatic reduction in the number of antibody-forming cells in the spleens of vitamin B_6-deficient rats immunized with sheep erythrocytes. Further studies indicated that vitamin B_6 deficiency did not affect the ability of rat spleen to accumulate antigen and that a state of inanition had no adverse effect upon the cellular immune response of rats fed a complete diet.

MATERIALS AND METHODS

Animals and diets. Male, weanling albino rats of the Holtzman strain were used. The animals were housed individually in wide-meshed, screen-bottom, suspended cages. Composition of the purified basal diet fed to both the control and the vitamin B_6-deficient groups has been described previously (4). In addition, each rat received a vitamin pill which, for control animals, supplied adequate quantities of the B-vita-

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mins (4); vitamin B_6-deficient rats received the same pill devoid of pyridoxine. All animals were fed the basal diet ad libitum with the exception of the pair-fed inanition controls which received the complete B-vitamin pill and the amount of basal diet consumed the previous day by their vitamin B_6-deficient partners. Animals were weighed weekly. Various procedures were initiated after the rats had been fed the experimental diets for 8 to 9 weeks.

Antigen. Sheep red blood cells served as antigen in the present studies. Unwashed sheep erythrocytes were obtained from a commercial source [2] and washed with phosphate-buffered physiological saline solution.[3] A stock suspension of these packed red blood cells was prepared in phosphate-buffered saline solution at a concentration of 20 volumes percent and stored in glass vials at 4° for no longer than 1 week. Prior to immunization, this stock solution was diluted in phosphate-buffered saline to a concentration of 4×10^8 cells/0.5 ml. Cells were counted in a hemocytometer. Rats were immunized intravenously via the lateral tail vein by a single dose of 4×10^8 cells, except when indicated otherwise.

Measurement of antibody-forming cells of the spleen. The number of splenic cells capable of synthesizing antibody was determined by the method of Jerne et al. (9, 10) with the modification that the agar plates were prepared in Eagle's medium (11) buffered with Tris,[4] pH 7.2, and incubated in air rather than in a CO_2 incubator. The essential features of the method can be outlined as follows. At varying times after immunization, rats were exsanguinated by cardiac puncture under light ether anesthesia. The spleens were removed and placed in Eagle's medium contained in petri dishes resting on ice. Splenic cell suspensions were prepared by teasing the spleens with steel grids and filtering through cheese cloth. Cell concentration was determined after staining with crystal violet. Splenic cells were then incorporated with a dense population of sheep red blood cells in agar. Duplicate plates were prepared from each splenic suspension. The detection of individual antibody-forming cells is based upon the hemolytic property of the antibody produced. Magnification of the small amount of cellular antibody is achieved by incubating the splenic cells in agar, and thereby restricting their movement to a local area. Consequently, each antibody-producing cell releases hemolytic antibody which sensitizes the erythrocytes in its vicinity. The formation of clear plaques around each antibody-forming cell occurs following the addition of complement which causes lysis of the sensitized erythrocytes. Plaques are counted under low magnification and the number of individual antibody-forming cells thereby determined. Variations between the duplicate counts of like splenic suspensions were less than 5%.

Antigen metabolism. Rate of disappearance of labeled heterologous erythrocytes from the bloodstream and their corresponding accumulation and disappearance in tissues, e.g., liver and spleen, have been used as a measure of the activity of the reticuloendothelial (RE) system (12). We have conducted similar studies to evaluate the effect of vitamin B_6 deficiency on the functional activity of the splenic RE system in metabolizing the antigen, sheep erythrocytes, used in our present investigations. Sheep erythrocytes were labeled with radioactive chromium (^{51}Cr) on the day of their receipt from the suppliers as follows: red blood cells (50 ml) were washed three times in phosphate-buffered saline and a 20% suspension prepared in this buffer as described previously. Fifty milliliters of this 20% suspension were transferred to a siliconized glass vial containing 4 ml ACD solution.[5] Three millicuries of $Na_2{}^{51}CrO_4$ [6] dissolved in 10.9 ml sterile bicarbonate solution were added with slow shaking and the suspension incubated at 37° for 1 hour. Reaction was stopped by addition of 0.5 ml of a solution containing 84 mg of ascorbic acid at pH 7.0. Cells were washed in phosphate-buffered saline until washings were free of

[2] Erythrocytes were obtained in Alsever solution from Grand Island Biological Company, Grand Island, New York.

[3] Prepared by mixing 286 ml of 0.15 M Na_2HPO_4, 90 ml of 0.15 M KH_2PO_4 and 376 ml of 0.15 M NaCl and adjusted to pH 7.2.

[4] Tris (hydroxymethyl) aminomethane, 0.25 M solution was used to adjust pH of the medium to 7.2.

[5] Purchased from Abbott Laboratories, Chicago, Illinois. Each milliliter contained: (in mg) glucose, 12; sodium citrate, 25; and citric acid, 8.

[6] Purchased from Abbott Laboratories as Rachromate-51, specific activity 464 mCi/mg Cr.

radioactivity. Usually 3 to 4 washings were sufficient. This is essentially the procedure of Silver (13) as modified by Koros.[7] Tagged erythrocytes (4×10^8 cells, containing 2.76×10^5 cpm) were injected intravenously via the lateral tail vein into each rat. At varying times thereafter, rats were anesthetized with ether and a sample of blood withdrawn by cardiac puncture. Spleens were obtained after exsanguination. The total spleen of each rat or 0.5 to 1.0 ml of blood was added to 1.0 ml of 0.5 M NaOH and incubated for 16 to 18 hours at 45°. Radioactivity of the resulting solution was determined in a well-type automatic scintillation counter.[8] Appropriate corrections for background and decay were applied.

Measurement of serum antibody level. Rats were bled by cardiac puncture at varying times after immunization as indicated in the text. Sera were decomplemented at 56° for 30 minutes and stored at −20° until used. Serial double dilutions of sera were prepared in 0.066 M phosphate buffer, pH 7.4, containing 0.00015 M $CaCl_2$, 0.0005 M $MgCl_2$ and made isotonic with NaCl. Serum hemolysin titrations were conducted in the presence of sheep erythrocytes and guinea pig complement by a standard method (14). The last tube showing 100% hemolysis was taken as the end point. This method measures both 19S and 7S immunoglobulins.

RESULTS

Effects of vitamin B_6 deficiency and caloric intake on the development of antibody-forming cells. There was a dramatic reduction in the number of antibody-forming cells in the spleens of immunized deficient rats as compared with that in the immunized control rats (table 1). The decrease in number of antibody-forming cells did not appear to be the result of the state of inanition associated with the deficiency since no deleterious effects were observed in the immunized pair-fed inanition control rats. Actually, the inanition controls exhibited a higher response than control rats fed ad libitum. Although no explanation for this increased cellular response can be offered at present, we have noted in previous studies at our laboratories that serum antibody levels of immunized inanition controls were frequently higher than those of the corresponding immunized controls fed ad libitum. Also, pyridoxine therapy begun 3 days prior to immunization and continued for 4 days until killing could produce a marked increase to normal level in the number of antibody-forming cells in the spleens of immunized vitamin B_6-deficient rats (table 1). Irreversible degeneration of competent cells resulting from vitamin B_6 deficiency was, therefore, not evident. The absence of any adverse relationship between caloric intake and cellular immune response was further indicated by the observation that the increased production of antibody-forming

[7] Koros, A. M. C. 1965 A study of primary immune response employing the technique of plaque formation in agar by single antibody-producing cells. Ph.D. Thesis. University of Pittsburgh, Pennsylvania, p. 53.

[8] Packard Auto-gamma Spectrometer model 5313, Packard Instrument Company, Inc., Downers Grove, Illinois.

TABLE 1

Antibody-forming cells (AFC) in the spleens of immunized rats [1]

Group		AFC/10^6 splenic cells		Initial body wt [2]	Final body wt [2]
		Avg	Range		
				g	g
Nonimmunized control [3]	(6) [4]	0.04	0–0.12	56 ± 0.9	242 ± 9.2
Ad libitum-fed control	(8)	232	133–390	56 ± 0.9	241 ± 9.3
Pair-fed control	(5)	1012	462–1644	56 ± 0.9	186 ± 10.0
Vitamin B_6 deficient	(10)	4	1–12	56 ± 1.0	116 ± 10.5
Vitamin B_6 deficient, treated [5]	(6)	200	143–311	56 ± 1.0	108 ± 8.1

[1] Sheep erythrocytes, 4×10^8, per rat were injected intravenously 4 days before removal of spleens.

[2] Body weight is expresssed as average ± SE. Final weights were recorded at the time of killing, 8 to 9 weeks after determination of initial weights.

[3] Similar background counts were observed in the other groups.

[4] Figures in parentheses indicate the number of rats.

[5] Each vitamin B_6-deficient rat received a daily intraperitoneal injection of 5 mg of pyridoxine, beginning 3 days before immunization and continued until the end of the experiment. During this 7-day period of pyridoxine administration, each rat received daily an amount of basal diet equal to that consumed per day immediately before the period of pyridoxine therapy.

cells following pyridoxine therapy occurred during a period of severe restriction of food intake.

Studies on antigen metabolism. The accumulation of antigen in the RE apparatus of spleen may precede actual antibody formation and can be considered an important step in the sequence of events leading to antibody formation by splenic lymphoid cells. Accordingly, we must consider the possibility that the adverse effect of vitamin B_6 deficiency upon antibody formation may be related to a disturbance in this function of the RE system. To assess this possibility, we have determined the accumulation and rate of disappearance of radioactivity in blood and spleen following the administration of the same dosage of ^{51}Cr-tagged sheep erythrocytes to control and vitamin B_6-deficient rats. To facilitate comparison, the number of tagged sheep erythrocytes administered in these experiments was identical to that used in the previous investigation on the development of antibody-forming cells. Our procedures permitted determination of the antigen-accumulating capacity of the entire spleen in both control and vitamin B_6-deficient rats.

The radioactivity pattern of disappearance in the blood was the same in both control and deficient rats (fig. 1). The higher radioactivity per milliliter of blood in the deficient rats could be attributed to

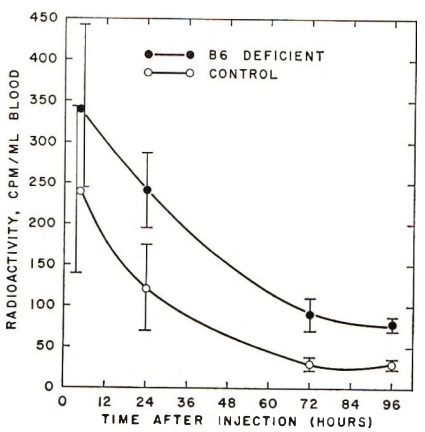

Fig. 1 Radioactivity recovered in blood at various times after administration of 4×10^8 ^{51}Cr-tagged erythrocytes containing 2.76×10^5 cpm. Values are given as average ± SE. Each point represents 4 to 8 rats.

TABLE 2

Radioactivity recovered from spleens after administration of ^{51}Cr-tagged sheep erythrocytes [1]

Time after erythrocyte injection	No. of rats	Individual splenic wt		Radioactivity per 100 mg spleen		Radioactivity per total spleen	
		Control	Deficient	Control	Deficient	Control	Deficient
hr		*mg*	*mg*	*cpm*	*cpm*	*cpm*	*cpm*
4	4	672 ± 35.6 [2]	330 ± 43.4 [2]	1100 ± 190 [2]	2300 ± 160 [2]	7400 ± 1200 [2]	7500 ± 660 [2]
24	4	679 ± 25.1	243 ± 49.5	800 ± 120	1900 ± 650	5500 ± 1000	4200 ± 880
72	5	774 ± 58.2	273 ± 15.8	600 ± 90	1800 ± 380	4600 ± 390	5000 ± 800
96	4	625 ± 68.5	240 ± 21.2	500 ± 100	2300 ± 250	2900 ± 630	5100 ± 430

[1] Each rat received intravenously 4×10^8 cells containing 2.76×10^5 cpm.
[2] Values are expressed as averages ± SE.

the lowered blood volume of these smaller animals.

The ability of spleen from vitamin B_6-deficient rats to accumulate and retain radioactivity of tagged sheep erythrocytes was not impaired despite the decreased splenic size in this deficiency (table 2). It seems reasonable to conclude that the number of sheep erythrocytes accumulated by the RE system of the spleen was not diminished in vitamin B_6-deficient rats.

Since it has not yet been established that the radioactive chromium is indeed attached to the antigenic site of the erythrocytes, it appeared necessary to investigate the relationship between dosage of antigen and formation of antibody-forming cells. It could be reasoned that any adverse effect of the deficiency on the metabolic function of the RE system might be mitigated by increasing the dosage of antigen beyond the level found to be optimum for the production of antibody-forming cells in the controls. The data of this study are presented in figure 2. In the control rats, the number of antibody-forming cells in the spleen increased with an increase in dosage of sheep erythrocytes. The optimum dosage was 4×10^8 erythrocytes/rat. On the other hand, we observed an extremely poor response to those doses when given to vitamin B_6-deficient rats. Increasing the antigenic dosage of erythrocytes in the deficient rats to a level 10 times that of 4×10^8 produced no perceptible change in the number of antibody-forming cells.

Hence, evidence from both lines of investigation described in this section indicates that the decreased formation of antibody-forming cells in vitamin B_6 deficiency is not due to a diminished rate of accumulation of antigen by the spleen.

Studies on the rate of formation of antibody-forming cells. The appearance of antibody-forming cells was observed in control rats at varying times during a 7-day period following immunization. The number of antibody-forming cells increased exponentially to a maximum value at 4 days after immunization (fig. 3). Thereafter, a sharp decline ensued. A similar time-response pattern in rats has been noted by other investigators (15). On the basis of this information, determinations of antibody-forming cells reported thus far in this study were performed 4 days after immunization. To ascertain whether the formation of these cells is delayed in vitamin B_6 deficiency, a similar time study was performed in vitamin B_6-deficient rats. In contrast to controls, the number of antibody-forming cells did not increase as rapidly and reached a plateau 4 days after immunization (fig. 3). It is apparent, there-

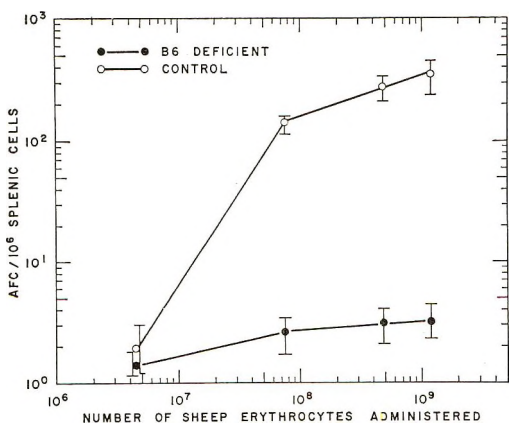

Fig. 2 Effect of dosage of sheep erythrocytes upon the formation of antibody-forming cells (AFC) determined 4 days after administration of erythrocytes. AFC are given as average ± SE. Each point represents 4 to 6 rats.

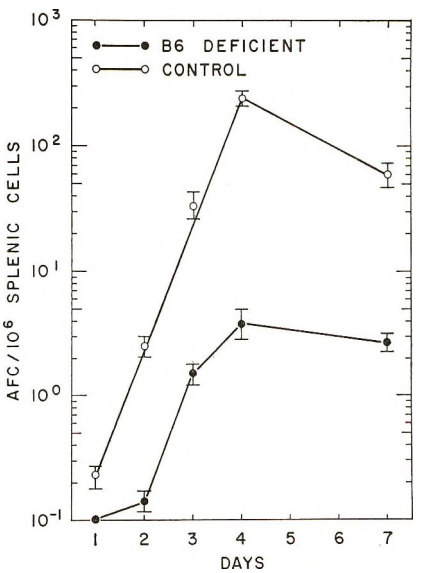

Fig. 3 Kinetics of response after administration of 4×10^8 sheep erythrocytes. Antibody-forming cells (AFC) are given as average ± SE. Each point represents 4 to 6 rats.

fore, that the decreased formation of antibody-forming cells observed in vitamin B_6-deficient rats 4 days after immunization is not simply a manifestation of a delayed response but, rather, is the result of a profound disturbance in the basic mechanism involved in the formation of these cells.

Serum antibody determination. Antibodies, as immunoglobulins, appear in the bloodstream subsequent to stimulation of lymphoid tissues with antigen. Determination of serum antibody content, therefore, provides an additional parameter of the systemic immune response. In the present study, serum antibodies (hemolysins) were determined in the control and vitamin B_6-deficient rats 4 to 7 days after administration of erythrocytes. Results of this study presented in table 3 demonstrate that the serum antibody content was markedly depressed in the deficient rats.

TABLE 3

Antibody-titer values of sera

Days after immunization [1]	Control group		Deficient group
	Avg	Range	Avg
4	3.99 [2]	2.00–4.99	< 1.00
5	8.24	6.99–8.99	< 1.00
7	7.50	5.99–8.99	< 1.00

[1] Rats were immunized by intravenous injection of 4×10^8 sheep erythrocytes per rat.

[2] Values are expressed as $\log_2$ of the reciprocal of last dilution shownig 100% hemolysis. Each average value was obtained from 4 rats.

DISCUSSION

The data in this paper indicate that the deleterious effects of vitamin B_6 deficiency on the immune process are evidenced at the cellular level. A decreased production of antibody-forming cells could account for the lowered level of circulating antibodies observed in this deficiency. Any attempt to explain the mode of action of vitamin B_6 in the dynamic expression of the immune response must encompass the following considerations with the recognition that the exact mechanism of antibody formation is not yet known. The process of antibody synthesis is initiated by an antigen stimulus or its biologically "processed" derivative. A relationship between cellular antibody synthesis and cytodifferentiation is evidenced by the following sequential changes exhibited by the stimulated cells: a) triggering of the progenitor cells to differentiate, b) proliferation, and c) initiation of antibody synthesis by the progeny of the cells so formed. The role of cellular proliferation in the process of antibody formation has been documented by the studies of Koros et al. [9] and Urso and Makinodan (16) demonstrating the high turnover of DNA in antibody-forming cells. Studies of Trakatellis and Axelrod (17) using labeled precursors of nucleic acids demonstrated that the synthesis of both DNA and RNA was diminished in the spleens of vitamin B_6-deficient rats. These adverse effects of vitamin B_6 deficiency were linked to the role of pyridoxal phosphate in the formation from serine of one-carbon units required for the synthesis of nucleic acids. The decreased biosynthesis of ribosomal and messenger RNA fractions in vitamin B_6 deficiency was revealed in subsequent investigations from this laboratory through measurements of incorporation of radioactivity from labeled orotic acid into these RNA fractions (18). On the basis of these observations, we suggested that the deterrent effects of vitamin B_6 deficiency on the immune response could be related to the inhibition of cell proliferation resulting from an inadequate supply of DNA as well as to a decreased production of messenger RNA essential for the synthesis of immunoglobulins (1, 18). Results of the present experiments demonstrating the inability of vitamin B_6-deficient rats to fabricate antibody-forming cells following an antigenic stimulus offer strong support for this concept.

ACKNOWLEDGMENT

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Influence of Sodium Chloride and Certain Other Food Components on Fluoride Absorption in the Rat [1,2]

YNGVE ERICSSON [3]

Department of Cariology and Chemical Laboratory, School of Dentistry, Karolinska Institutet, Stockholm, Sweden

ABSTRACT The effect of sodium chloride, wheat flour and some other food components on the intestinal absorption and skeletal storage of ^{18}F-labeled fluoride (F) was investigated in the rat, with a view to the possible use of staple foods instead of drinking water for the caries-preventive administration of F. The main parts of the experiments were carried out as 4-hour tests following tube ingestion of 8 ml of water or food slurry containing 4 ppm F as ^{18}F-labeled NaF. Femoral uptake of F was greater after ingestion of flour or egg yolk than after water. Addition of 350 mM NaCl reduced femoral F uptake from water and flour slurry but not from egg yolk. Egg white did not differ significantly from water. Individual variations were, as always, greater regarding intestinal absorption than femoral accumulation of F. The greater femoral uptake of F given with egg yolk (with or without NaCl), despite the large unabsorbed rest, was not explained by experiments with any of the egg yolk components, glutamic acid, aspartic acid, lecithin, lysine, cholesterol, orthophosphate or fat. Lysine reduced femoral uptake of F whereas fat (olive oil) had no effect. Rats given a calcium phosphate-rich diet prior to the ingestion of F retained more in the intestine and less in the skeleton provided F was ingested with water only, but not if the ingested volume was made viscous with carboxymethyl cellulose (CMC). The CMC increased the 4-hour femoral fixation of F about as much as flour, pure starch and egg yolk, and CMC gave an unchanged femoral F uptake after 1 hour despite delayed intestinal absorption. Skeletal fixation of parenterally administered F was not measurably influenced by the simultaneous ingestion of egg yolk, olive oil or cholesterol. With only 1 hour survival time after ingestion, sodium chloride produced a notably strong reduction of skeletal F uptake from both water and flour.

Alternatives to fluoridation of drinking water are of particular interest for several reasons. Drinking water fluoridation is practicable only in water works of a certain technical standard and hence great populations, particularly in the developing countries with their rapidly increasing caries rate, cannot benefit from this measure. The fact that all the distributed water has to be fluoridated while generally less than 1% is used for human ingestion makes drinking water fluoridation uneconomical in principle, even if the cost of the added salt is very low compared with the value of the dental benefits. The control of drinking water fluoridation requires daily analyses and other safety measures. Finally, psychological difficulties are often created by ignorance and fear.

At the same time the interest in systemic fluoride supply has been considerably extended by the increasing number of reports on preventive and therapeutic effects of fluoride in osteoporotic bone disorders, e.g., Purves (1), Rich and Ensinck (2), Cohen and Gardner (3), Cass et al. (4), Bernstein et al. (5), Aeschlimann et al. (6) and Bilginturan and Özsoylu (7).

Commonly used foods have often been suggested as water-replacing vehicles for automatic supply of optimal caries-preventive fluoride quantities. Salt, milk, flour, bread or sugar are the alternative food vehicles for fluoride that have been discussed mostly and to some extent used and scientifically tested.

Salt is already utilized as a fluoride vehicle in Switzerland and carries considerable promise for a great part of the world. The results of a limited number of physiological and clinical studies also indicate its effectiveness: increased urinary excretion of fluoride following the ingestion

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[2] Presented in part at the annual meeting of the International Association for Dental Research, San Francisco, 1968.

[3] Present address: Karolinska Institutet, Box 3207, S- 103 64 Stockholm, Sweden.

of fluoridated kitchen salt (8), increased uptake in enamel surfaces (9), and notable caries reduction in children despite a low fluoride dosage with the salt (10). Cereals also appear promising as fluoride vehicles (11), possibly in combination with salt (8, 12). Such application, however, requires a mapping of the consumption and its variation in different countries and areas; further, a thorough knowledge of the intestinal absorption and the local effect on the teeth of fluoride ions in the vehicles in question, and finally, adequate proof of a clinical caries-preventive effect of fluorides ingested with these vehicles. Our knowledge in these fields is at present very limited.

The present investigation aims to study the effect of salt and several other food components on the absorption and utilization of fluoride in the rat.

MATERIAL AND METHODS

Male albino rats of standardized stock and feeding,[4] weighing about 200 g, were used for the experiments. The rats were kept fasting but with access to water from the afternoon preceding the morning of the experiment. Every rat was given, by stomach tube, 4 ml of the liquid or semiliquid test food containing 4 ppm F as ^{18}F-labeled NaF, immediately followed by 4 ml of the same test food containing the same concentration of unlabeled NaF; the latter quantity had the purpose of rinsing down the active solution from the stomach tube.

After 4 hours the rats were killed by overstretching the spine under ether anesthesia and the abdominal part of the digestive tract and one femur were dissected out and weighed. These organs were assumed to reflect unabsorbed fluoride and skeletal uptake of absorbed fluoride, respectively. Femoral uptake was regarded as a suitable parameter of physiological fluoride utilization, particularly owing to its moderate variation and its parallelism with the uptake in dental tissues during tooth mineralization (13). The 4-hour interval was chosen because previous investigations have shown that femoral uptake of fluoride from an ingested dose has reached its maximum after 4 hours (13, 14).

After weighing the specimens, intestines with their contents were homogenized in a Polytron homogenizer, and weighed aliquots were taken for ^{18}F-analysis in a well-type scintillation detector. The femurs were dissolved in a standard volume of half-concentrated nitric acid and similarly analyzed for ^{18}F. Samples of the ingested substances were diluted to the same geometry in counting tubes and similarly analyzed.

The ^{18}F-activity remaining in the whole digestive tract was calculated as percent of the total dose. Femoral activities were calculated as percent of the dose given per gram animal (= g-dose) that was found per gram femur; this compensates for individual variations in body and femur weights of the rats.

Series 1. Tests with water, flour, egg yolk and egg white — with and without a high salt concentration

The following food components, all containing 4 ppm of added F, were tested in series 1: 1) distilled water; 2) distilled water plus 350 mM NaCl; 3) distilled water plus 12% wheat flour; 4) distilled water plus 12% wheat flour plus 350 mM NaCl; 5) egg yolk (containing about 50% water) plus ⅛ weight distilled water; 6) egg yolk (containing about 50% water) plus ⅛ weight distilled water plus 350 mM NaCl in total water; 7) egg white (containing about 88% water) plus ¼ weight distilled water; and 8) egg white (containing about 88% water) plus ¼ weight distilled water plus 350 mM NaCl in total water.

The ratio 4 ppm F/350 mM NaCl is the same as the ratio 440 mg NaF/kg NaCl, which has been calculated as optimal for the fluoridation of kitchen salt under Swiss conditions (8).

The chosen flour concentration was found to be the highest concentration that could be pressed through the feeding tube after dissolving in water by heating to near boiling. Egg yolk was chosen as a suitable type food from the animal kingdom, egg white as a suitable protein food.

Results. The results of series 1 are given below together with those of series 2.

[4] Anticimex Co., Stockholm. Rat diet: (in percent) protein, 20.8; non-protein nitrogen, 2.8; fat, 3.7; N-free extract, 53.8; fibers, 5.8; ash, 7.4; water, 8.5; and fluoride about 28 ppm.

Series 2. Tests with some amino acids, lecithin and cholesterol

Following the observation in series 1 of a notably high femoral uptake of ^{18}F-labeled fluoride when given with egg yolk, a second series was run with 4 ppm F solutions containing the following components of the egg yolk: 1) 4% glutamic acid; 2) 4% aspartic acid; 3) 4% lecithin; 4) 4% lysine; and 5) 1.4% cholesterol.

Some of these components, which have a low solubility in water, were given as well-homogenized suspensions.

Results. The results of series 1 and 2 are given in figures 1 and 2. The variation in the numbers of rats given the different food ingredients depends in part on the fact that the experiments had to be carried out in several sets owing to the short half-life of ^{18}F, in part on the different importance attached to these food ingredients.

As in our previous ^{18}F experiments, unabsorbed intestinal remains vary much more between individuals than the femoral uptake. (Ten samples of the same intestinal homogenate gave a standard error of ^{18}F activity of only 0.85%.) Figure 2, however, indicates a number of significant differences between groups.

Sodium chloride significantly reduced the femoral uptake of fluoride given with water and flour, whereas no such effect was found with NaCl plus egg yolk and only an insignificant reduction with NaCl plus egg white. Only in the case of flour was there an indication that reduced ab-

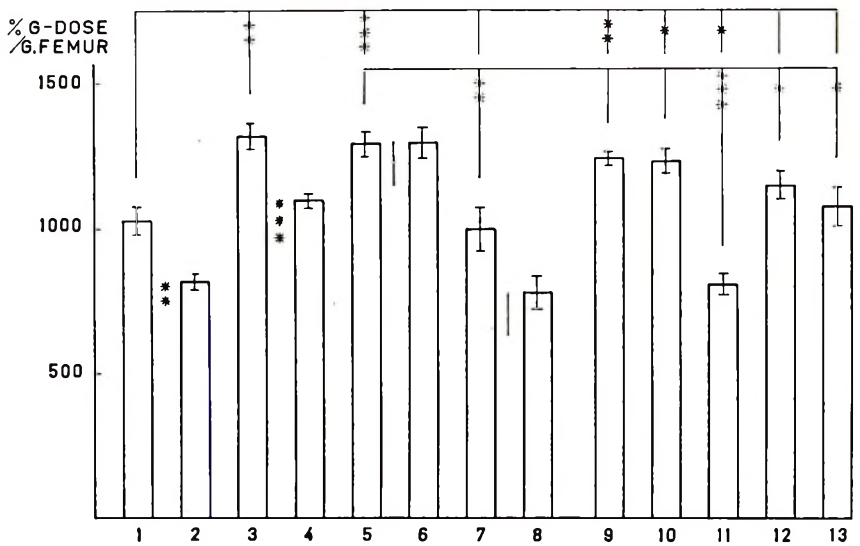

Fig. 1 Femoral uptake of labeled fluoride after four hours.

Group	No. of rats	Food components	Group	No. of rats	Food component
1	23	Water	9	8	Glutamic acid
2	12	Water + NaCl	10	8	Aspartic acid
3	12	Flour	11	4	Lysine
4	12	Flour + NaCl	12	8	Lecithin
5	12	Egg yolk	13	4	Cholesterol
6	8	Egg yolk + NaCl			
7	8	Egg white			
8	4	Egg white + NaCl			

Columns indicate means ± SE. One to three stars indicate statistically significant differences at the 5, 1 and 0.1% P level, respectively; solid line, a statistically nonsignificant difference. Stars and lines between columns indicate significance of difference between groups represented by these columns. Stars and lines in systems above columns indicate significance of differences between group 1 (water) and other groups, and between group 5 (egg yolk) and other groups, respectively.

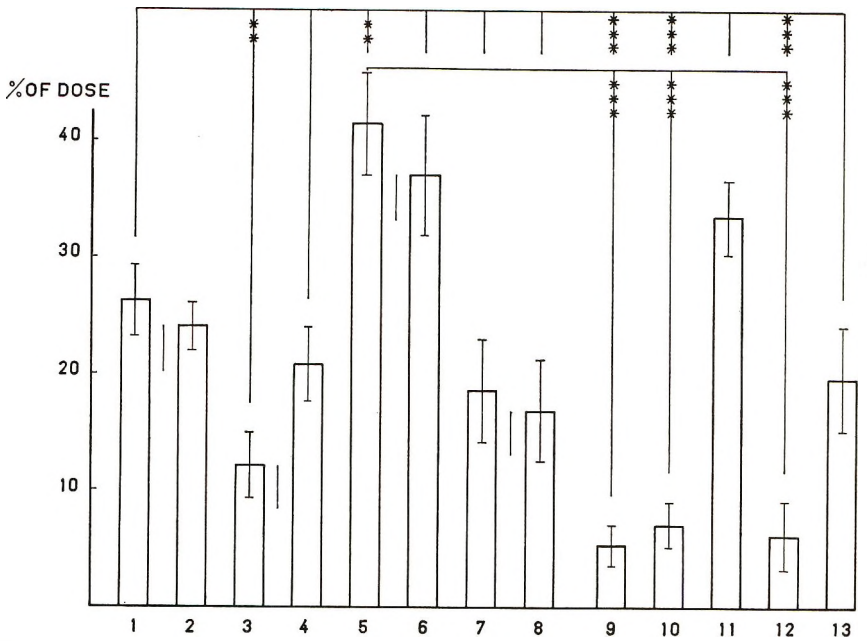

Fig. 2 Digestive tract residue of labeled fluoride after 4 hours. Group, column, and difference denotations as in figure 1.

sorption after 4 hours might be the mechanism of reduced fluoride utilization in the presence of salt.

There was a better utilization of fluoride from both flour and egg yolk than from water. Different mechanisms possibly may have mediated this effect, since flour gave a low intestinal rest which was increased by NaCl, whereas egg yolk gave a very high unabsorbed rest which was not significantly influenced by NaCl.

Egg white and egg white plus NaCl did not differ significantly from water and water plus NaCl, respectively.

Of the egg yolk components tested in series 2, lysine resulted in a low femoral uptake and a high unabsorbed rest of ^{18}F-labeled fluoride. Glutamic acid, aspartic acid and lecithin led to remarkably complete absorption but still not quite as great a femoral uptake as egg yolk.

Cholesterol, finally, showed intermediate figures which hardly indicated any specific action on fluoride absorption and storage.

Series 3. Tests with fat and orthophosphate

Fat, which comprises over 30% of the egg yolk and about 1% of wheat flour, has previously been reported to enhance fluoride absorption (15, 16).

The phosphate content of the tested foods might be thought to influence the fluoride absorption (17) and possibly its skeletal fixation.[5]

Tests were carried out according to the standard technique. The results obtained with addition of either 0.12% or 28% olive oil emulsified with 0.08% Tween 80, or 10 mM orthophosphate buffer, pH 6, were entirely negative and the detailed procedure and results are therefore omitted here.

Series 4. Tests at an interval following the ingestion of a high or low calcium diet

Since the intestinal fluoride residue showed a very great interindividual variation in this as well as in many previous investigations, and since notable variations were observed in the fecal contents of the large intestines of the fasting rats, it was thought that differences in residual calcium of the digestive tract might cause at least some of the variation in fluoride ab-

[5] Alcock, N. W. 1963 Some effects of fluoride on mineral metabolism in the rat. Federation Proc., 22: 2349 (abstract).

sorption. This was tested with groups of rats kept on the diets indicated in table 1 for 2 days immediately before the preexperimental fasting time.

Groups of 4 rats from each of these dietary categories were given: 1) 4 ml water containing 4 ppm F as ^{18}F-labeled NaF plus 4 ml of the same solution unlabeled; 2) the same solutions as in 1) with the addition of 1.6% carboxymethyl cellulose (CMC); and 3) the same solutions as in 2) with the addition of 350 mM NaCl.

The addition of 1.6% CMC gave nearly the maximal viscosity that could be handled in the tube-feeding procedure. This viscosity was intended to delay contact between the ingested solution and the intestinal residue. The addition of NaCl had the same study purpose as in the previous experiments.

Intestinal residue and femoral uptake were analyzed after 4 hours as previously described. The homogenized digestive tracts were also analyzed for phosphorus and calcium plus magnesium.

Results. The results are given in tables 2 and 3. The intestinal contents of total phosphate were similar, and of calcium not significantly different.

It should, however, not be overlooked that a difference in calcium or phosphate residues, or both, may have occurred in the

TABLE 1

Diet	Food	Round figures for Ca and P contents	
		Ca	P
		mg/100 g	*mg/100 g*
Ca-rich diet	Cheese	870	610
	(equal parts)		
	Soybeans	225	590
Ca-poor diet	Fat pork	6	110
	(equal parts)		
	Maize	18	270

TABLE 2

Total intestinal contents of phosphorus and calcium [1]

	Calcium		*t*	*P*	Phosphorus	
	Range	Mean			Range	Mean
High Ca-P rats	3.4–47.5	19.9	1.87	0.05–0.1	19.5–40.0	30.2
Low Ca-P rats	2.8–24.9	12.0			24.0–39.5	29.3

[1] Millimolar concentrations of Ca and P in hydrochloric acid solution of ashes (1 ml 0.1 N HCl/gram weight of intestines).

TABLE 3

Effect of different calcium and phosphate contents of previous ingestion, combined with differences in viscosity and/or NaCl content of test ingestion

Group	No. of rats	Preingestion	Ingestion	Femur	Digestive tract
				% g-dose/g	*% dose*
1	4	Low Ca+P	Water	905 ± 27.6 [1]	6.1 ± 0.4 [1]
2	4	High Ca+P	Water	786 ± 30.6	14.2 ± 1.8
3	4	Low Ca+P	1.6% CMC	902 ± 43.1	14.3 ± 2.8
4	4	High Ca+P	1.6% CMC	996 ± 41.6	7.2 ± 0.3
5	4	Low Ca+P	1.6% CMC+NaCl	841 ± 20.5	7.2 ± 1.2
6	4	High Ca+P	1.6% CMC+NaCl	854 ± 31.9	10.4 ± 1.8

[1] Mean ± SE.
Significance of differences (P values): femur: 1–2, < 0.05; 4–6, < 0.05; digestive tract: 1–2, < 0.01; 3–4, 0.05; 3–5, 0.05; other differences insignificant.

proximal part of the intestine despite the lack of significant group differences as regards the calcium and phosphate content of the total intestinal tract.

It appears that the "Ca-P-rich" rats had a much greater intestinal retention and significantly less femoral uptake of the fluoride given in plain water solution. With the addition of CMC, however, the Ca-P-rich rats had a significantly lower intestinal fluoride retention and an insignificantly greater femoral uptake. With the further addition of NaCl the differences were reduced and there was an insignificant tendency toward a reduced femoral uptake when compared with the rats given CMC but not NaCl.

The apparent contradiction of these results might be resolved by the hypothesis that at least two main factors were active in the skeletal utilization of fluoride: one intestinal retention factor, which may have dominated the tests with low viscosity ingestion, and one factor inherent in circulation, metabolism or bone fixation of fluoride, which might have dominated the tests where contact between ingested fluoride and intestinal residue was prevented or delayed by a high viscosity. In the latter case NaCl apparently had a reducing effect on skeletal utilization of fluoride just as in experimental series 1.

Series 5. Tests with parenteral fluoride administration and peroral administration of various food components

If factors other than the absorption factors were active the former factors should be more clearly noticeable if the femoral fixation of intraperitoneally injected fluoride was measured following the ingestion of foods which could supply such factors. The following experiment was designed to test this hypothesis.

Groups of rats were given 8 ml of one of the following substances through stomach tube: 1) distilled water; 2) egg yolk diluted with ⅛ volume of distilled water; 3) distilled water with 28% olive oil emulsified with 0.1% Tween 80; and 4) 1.75% cholesterol emulsified with 0.1% Tween 80.

Fifteen, thirty or forty-five minutes after the ingestion 1 ml of saline containing 1 ppm F as ^{18}F-labeled NaF was injected intraperitoneally.

Only the femoral ^{18}F activity was determined in this series, 4 hours after the injection.

Results. The results appear in table 4. The differences found were insignificant with the exception of one of the olive oil-water differences ($P \sim 0.02$).

Series 6. Comparative tests on absorption and bone fixation differences 1 hour after fluoride ingestion with certain food types

If absorption differences were the main reason for the variations in skeletal fixation of fluoride ingested with different food types, then these differences might be greater 1 hour after the ingestion than after the 4-hour period chosen as standard for these experiments.

To test this possibility an experimental series was carried out where unabsorbed intestinal residue and femoral uptake of labeled fluoride were determined 1 hour after the ingestion of fluoride with some test foods.

As test substances the following foods were selected, which in the 4-hour experiments had given notable variations in bone

TABLE 4

Effect of food components on femoral uptake of intraperitoneally injected fluoride

Ingested food	Femoral uptake of ^{18}F-labeled fluoride after 4 hours					
	No. of rats	F injected 15 min after feeding	No. of rats	F injected 30 min after feeding	No. of rats	F injected 45 min after feeding
		% g-dose/g bone		*% g-dose/g bone*		*% g-dose/g bone*
Water	5	1.246 ± 25 [1]	6	1.100 ± 52 [1]	5	1.128 ± 17 [1]
Olive oil	5	1.161 ± 51	6	1.224 ± 21 [2]	5	1.192 ± 17
Egg yolk	5	1.091 ± 27	7	1.183 ± 32	5	1.113 ± 30
Cholesterol	5	1.260 ± 27	—	—	5	1.125 ± 28

[1] Mean ± SE.
[2] Only significant difference, between water and olive oil in 30-minute test: $P \sim 0.02$.

uptake and which represented probably the most promising vehicles for mass prophylaxis with fluoride: 1) distilled water; 2) distilled water plus 350 mM NaCl; 3) distilled water plus 12% wheat flour; and 4) distilled water plus 12% wheat flour plus 350 mM NaCl.

Results. Figure 3 shows the various results. The differences between salt and nonsalt groups were striking and would have been greater still in the water and water plus NaCl comparison, had not one of the rats fed water shown the extreme values, 572 and 40.7, for femoral uptake and intestinal content, respectively.

Between the corresponding water and flour groups the differences were smaller and indeed significant only as regards the water-flour comparison for the digestive tract contents.

Series 7. Tests on the effect of viscosity on intestinal distribution and femoral uptake of fluoride

In several experiments with both NaF and Na_2PO_3F it was established that pure starch and carboxymethyl cellulose had an enhancing effect similar to wheat flour on the femoral uptake of the label of these fluorides. This effect appeared to be due to the viscosity. It suggested that a higher viscosity gave a prolonged retention of the ingested fluoride in the stomach where fluoride absorption is known to occur. To test this hypothesis an experimental series was carried out according to the standard technique, with the following variations: 1) labeled NaF in distilled water (20 rats), and 2) labeled NaF in 2% CMC solution (20 rats).

Ten rats of each group were sacrificed after 1 hour, the other ten after 4 hours. The gastric and intestinal remains were determined separately, as well as the femoral uptake.

Results. The results are shown in figure 4. It is seen that there were greater intestinal remains in the CMC group after 1 hour *both* in the stomach and the intestine whereas these differences were largely leveled out after 4 hours. In this series there was no difference in femoral uptake after 1 hour but the typical difference was observed after 4 hours.

If these results are compared with those obtained with flour (figs. 1–3) there is agreement concerning the equal femoral uptake after 1 hour and higher uptake after 4 hours, and also agreement concerning the digestive tract remains after 1 hour but not after 4 hours.

DISCUSSION

The extremely complex process of absorption and the limitation of the main part of these investigations to 4 hour tests may be the main reasons why the relationship of fluoride absorption to femoral fixation

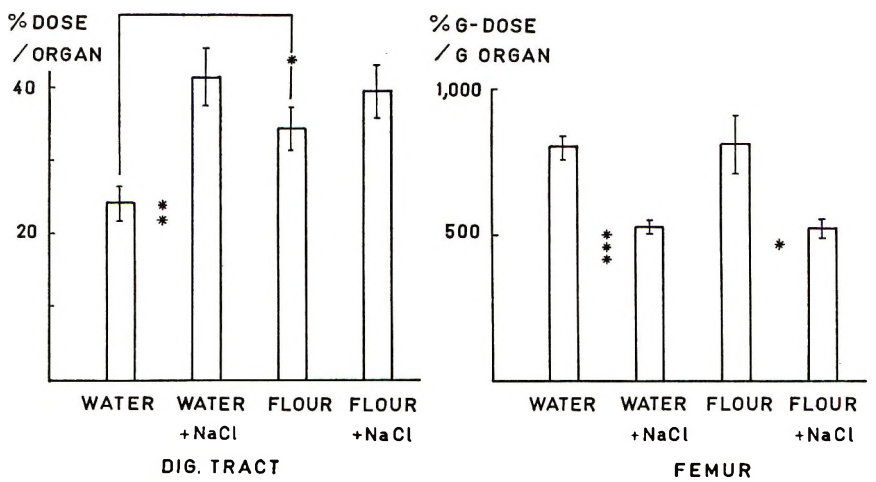

Fig. 3 Femoral uptake and digestive tract residue of labeled fluoride after 1 hour. Column and difference denotations as in figure 1.

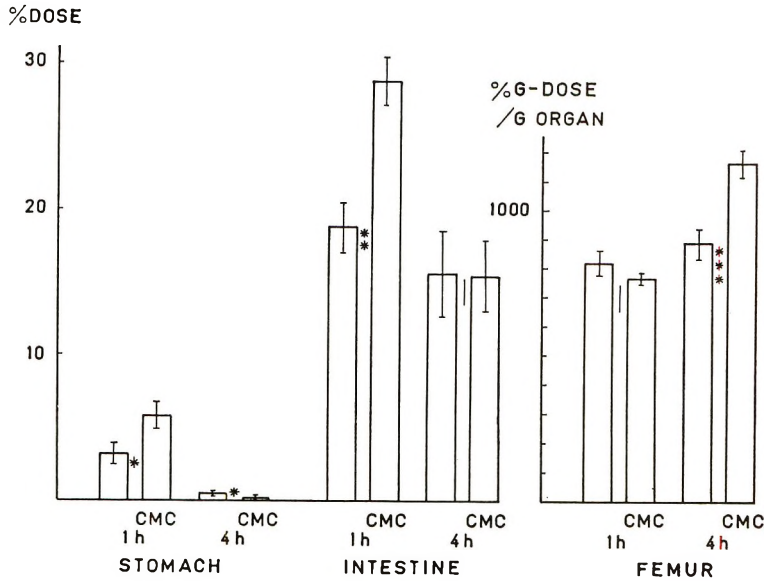

Fig. 4 Femoral uptake and gastric and intestinal residues of labeled fluoride 1 and 4 hours after ingestion with water and CMC solution, respectively. Column and difference denotations as in figure 1.

appears rather weak in the majority of the experiments; this was found also to be the case when scanning the values for the individual rats. A high chloride concentration will probably exert its depressing effect on skeletal utilization of fluoride via an early postingestive influence on absorption; this is supported by the results of the 1-hour tests. There may be a competition between halogens for transport through the gastric or intestinal wall, or other specific effects of chloride, and an osmotic movement of fluid toward a high chloride concentration in the gastric or intestinal lumen may also reduce fluoride absorption, particularly immediately following the ingestion, before the chloride has been diluted or absorbed.

The increased skeletal utilization of fluoride ingested with flour is probably due to the physical consistency since both CMC and pure starch exerted a similar effect. This may also be the mechanism of egg yolk since none of its tested components was found to increase the skeletal fluoride uptake despite more complete intestinal absorption. An increased viscosity does not give any greater skeletal uptake of fluoride during the first hour after ingestion in contrast to the opposite effect of chloride, and the only conclusion on the mode of action of the viscosity that can be ventured is that it appears to give an unchanged femoral uptake during the first hour despite less total absorption and greater remains in the digestive tract.

The 1-hour effect of a 350 mM NaCl concentration on skeletal fluoride uptake seems to be a reduction not exceeding 20%. The lower chloride concentrations more commonly occurring in foods should have a correspondingly lower effect, and in addition chloride is often supplied with foods which according to our results give a correspondingly higher degree of utilization of the fluoride. There are also investigations with mixed solid diets or fish diets which indicate small differences in the utilization of food-borne and water-borne fluoride (18, 19). It is therefore probable that fluoride supplied with salt will give about the same systemic effect as fluoride in water in all normal cases. As regards the local cariostatic effect in the oral cavity it has previously been shown that chloride has an enhancing action on the fluoride uptake of enamel surfaces (9).

It is notable that lysine gave a rather low absorption and utilization of fluoride while the reverse occurred with aspartic acid,

glutamic acid and lecithin. The caries-preventive effect of lysine that was extensively investigated some years ago — review: Brislin and Cox (20) — thus does not seem to be mediated by any systemic effect on fluoride metabolism.

Experiments to test the possible effect of magnesium on the absorption and storage of fluoride were considered but not carried out, because the data of previous investigators [6] indicated that even the magnesium content of our flour mixtures would be much too low to exert any measurable influence on the fluoride utilization (21).

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Absorption and Utilization of Glucose by Meal-fed and Nibbling Rats [1]

GILBERT A. LEVEILLE AND KRISHNA CHAKRABARTY
Division of Nutritional Biochemistry, Department of Animal Science, University of Illinois, Urbana, Illinois

ABSTRACT The absorption and utilization of glucose by meal-fed (animals having access to food for a single, daily, 2-hour period) and nibbling (fed ad libitum) rats was studied. Meal-fed rats absorbed glucose more rapidly than nibbling animals and this enhanced absorptive rate could be explained by an increase in the weight of the small intestine. The weight of the intestine relative to body weight and the glucose absorption coefficient were both increased by about 40% in the meal-fed as compared with the nibbling animal. As a consequence of the enhanced glucose absorption rate the amount of glucose entering the blood stream, per unit time, would be approximately 40% greater in the meal-fed animal. Yet, blood glucose levels increased more slowly in meal-fed than in nibbling rats following the oral administration of glucose. The rate of utilization of absorbed glucose was significantly greater in meal-fed as compared with nibbling rats. Also, the rate of glycogen storage in muscle (abdominal, gastrocnemius and diaphragm) following food ingestion was significantly greater for meal-fed than for nibbling animals. The physiological significance of these findings and the possible regulatory mechanisms involved are discussed.

The laboratory rat normally consumes its food in numerous small meals throughout the day (1). Although some variation exists with respect to the relative amounts of food ingested during the light and dark periods of the day, the frequency of feeding is sufficient to ensure an essentially constant availability of nutrients for absorption from the intestine. Meal-feeding, the limitation of access to food to a single 2-hour period daily, therefore, would present an abnormal situation to the laboratory rat. To cope with this imposed stress the animal must develop the ability to store energy during the meal periods for use during the ensuing period of fast between meals.

Tepperman and Tepperman (2) reported that glucose absorption was increased in the meal-fed rat, implying that the amount of glucose presented to tissues per unit time is increased. This increased glucose presumably would be stored in the form of glycogen and lipid. In accord with this is the demonstration that fatty acid synthesis is markedly enhanced in adipose tissue (3–5) and to some extent in liver (2) of the meal-fed rat. Also, glycogen apparently accounts for a significant proportion of the energy stored during the meal period (6,7). Accompanying the increase in lipid synthesis is an increase in the activity of enzymes related to fatty acid synthesis in liver and adipose tissue of meal-fed rats (3,8,9). However, the uptake of substrate, namely glucose, by the tissues must also be enhanced, although this has not been demonstrated.

The experiments were undertaken to evaluate glucose absorption and utilization in the meal-fed rat and to compare the observed values with those obtained in animals fed ad libitum. The results show that glucose absorption and utilization are greatly enhanced in meal-fed animals as is glycogen storage in muscle.

EXPERIMENTAL

Male rats of the Sprague-Dawley strain weighing 250–350 g were used for all studies. The animals were divided into 2 groups: the meal-fed group was allowed access to food from 8 AM to 10 AM only, while the other group was fed ad libitum (nibblers). The animals were maintained on these feeding schedules for at least 3 weeks before use, since this period has been shown to be of sufficient duration to induce the lipogenic response to meal-feeding (6).

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The rats were housed individually in wire cages having raised floors and in a temperature-regulated room (21°). Water was available at all times. The animals were fed a commercial diet before initiation of these investigations [2]; during the experimental period a semipurified diet was fed. The composition of the experimental diet has been described (10).

In the experiments food was removed from the nibbling animals at 10 AM on the day preceding the experiment. The experiments were initiated at 8 AM on the following day, thus both meal-fed and nibbling animals were without food for 22 hours before use. The treatment on the day of experiment is indicated in the Results section.

For the oral glucose tolerance tests the rats were force-fed 5 ml of a 40% glucose solution and blood was obtained from the tail for glucose determination. For glycogen analysis the animals were decapitated and exsanguinated, the muscles were rapidly removed, weighed and digested in 30% KOH. Glycogen was determined as described earlier (7).

The absorption of glucose was determined by the Cori procedure (11). Two hours following oral glucose administration, the animals were killed. The digestive tract was tied anterior to the stomach and at the origin of the colon. The contents of the stomach and the small intestine were collected and the glucose content was determined. The weight of the small intestine was also obtained.

The glucose content of the circulating "glucose pool" was estimated from the blood glucose concentration and an assumed glucose space of 30% of body weight (12). Blood glucose was determined by means of glucose oxidase [3] and serum free fatty acids by the titrimetric method of Dole and Meinertz (13) using nile blue A as the indicator.

RESULTS AND DISCUSSION

The change in blood glucose levels following the oral administration of glucose was studied in meal-fed and nibbling rats deprived of food for 22 hours. The results of this experiment, shown in figure 1, indicate that blood glucose increased more rapidly in the nibbling rats than in meal-

[2] Rockland Rat/Mouse diet (complete), Teklad Inc., Monmouth, Illinois.

[3] Glucostat, a prepared enzymatic glucose reagent, Worthington Biochemical Corp., Freehold, New Jersey.

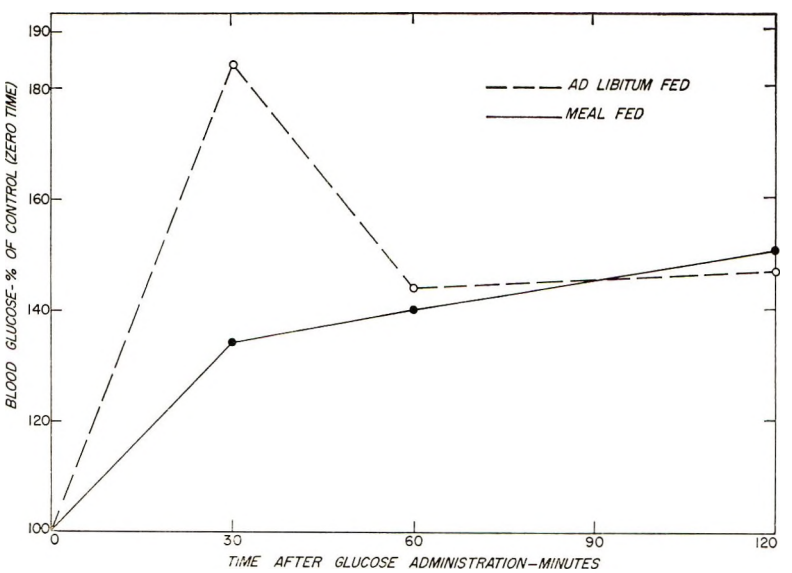

Fig. 1 Relative change in whole blood glucose concentration of fasting meal-fed and nibbling rats given glucose orally. Meal-fed and nibbling rats maintained without food for 22 hours were given 5 ml of a 40% glucose solution orally and blood was taken for glucose determination at the times indicated. Each point is the mean value for 4 rats. Final body weights ± SE were 336±8 and 392±13 g for the meal-fed and nibbling rats, respectively.

fed animals. However, 60 or 120 minutes after glucose administration blood glucose levels were similar, relative to the fasting blood glucose values, for the meal-fed and nibbling rats. The pattern of change for each group was quite different. In the meal-fed rats blood glucose levels increased gradually over the 2-hour period studied, whereas the values for the nibbling animals increased abruptly during the first 30 minutes to over 180% of control values, decreased to about 140% of control in the next 30 minutes, and remained at this level during the subsequent hour. Blood glucose levels are the net result of glucose absorption and tissue glucose uptake. If, as reported by Tepperman and Tepperman (2), glucose absorption is more rapid in meal-fed than in nibbling rats, the results shown in figure 1 would suggest that the uptake of glucose by tissues is also more rapid in the meal-fed rat. These data further imply that the mechanism(s) responsible for initiating the uptake of glucose by peripheral tissues responds more rapidly in the meal-fed rat.

In an attempt to overcome any differences in absorption rate between the meal-fed and nibbling animals an experiment was conducted in which glucose was administered intraperitoneally. The changes in serum glucose and free fatty acid levels were followed over a 2-hour period after glucose administration as shown in figures 2 and 3. Serum glucose levels rose very markedly and rapidly in both groups of animals, but more so in the nibbling than in the meal-fed rats (fig. 2). Fifteen minutes after glucose administration the serum glucose levels of the meal-fed and nibbling rats were 425 and 325% of the zero time control values. The levels dropped rapidly to 200 and 125% of control for the nibbling and meal-fed animals, respectively, in the next 15 minutes. One hour after glucose administration the serum glucose values of the meal-fed animals had essentially returned to control levels, whereas the nibbling rats still had serum glucose values equivalent to 150% of control. Even 2 hours after glucose administration the serum glucose level of the nibbling rats had not returned to the control value. These data are in accord with the results of the experiment summarized in figure 1, and imply that glu-

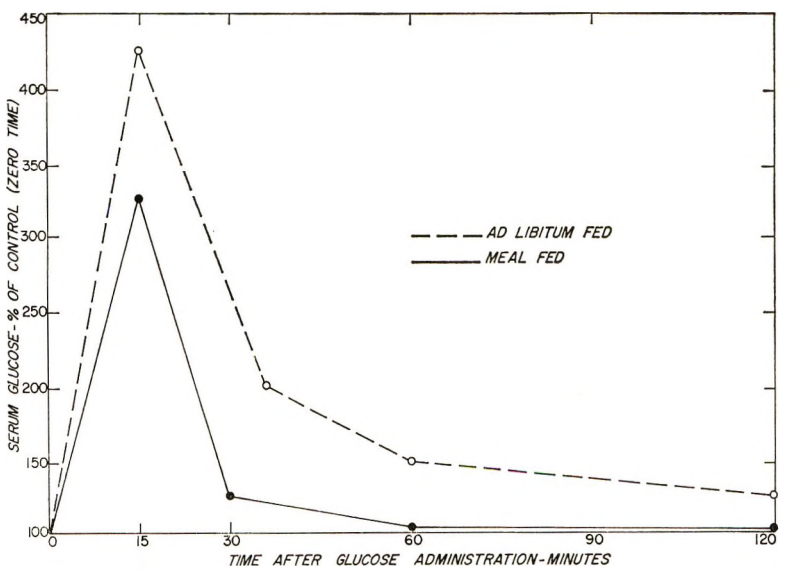

Fig. 2 Relative change in serum glucose concentration of fasting meal-fed and nibbling rats given glucose intraperitoneally. Meal-fed and nibbling rats maintained without food for 22 hours were given 800 mg of glucose in 2 ml of normal saline and animals from each group were killed at the times indicated. Each point represents the mean value for 5 rats. Final body weights ± SE were 296 ± 4 and 383 ± 5 g for the meal-fed and nibbling rats, respectively.

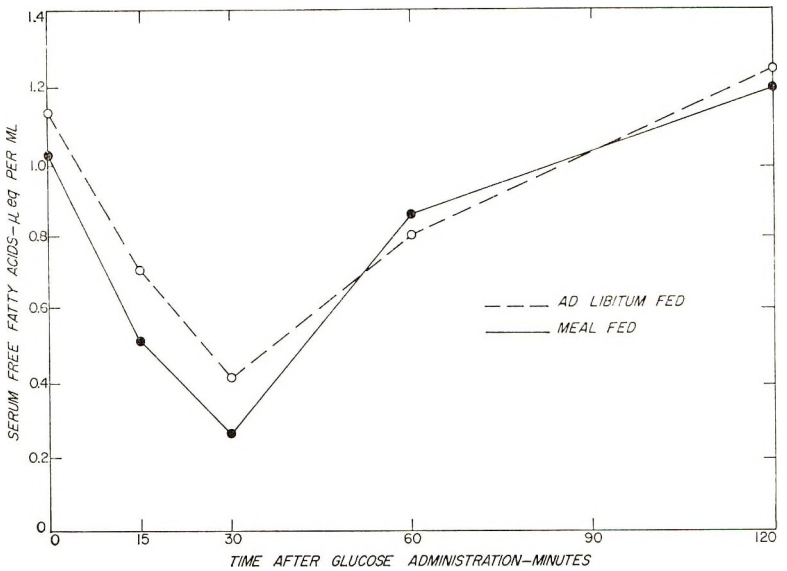

Fig. 3 Serum free fatty acid concentrations of fasting meal-fed and nibbling rats given glucose intraperitoneally. Each point represents the mean value for 5 rats. Other experimental details are as indicated in figure 2.

TABLE 1

Glucose absorption and intestinal weight of meal-fed and nibbling rats [1]

	Meal-fed	Nibbling	P value [2]
Body weight, g	337 ± 10 [3]	404 ± 11	< 0.01
Intestinal weight, g/100 g body wt	2.80 ± 0.14	2.01 ± 0.07	< 0.01
Glucose absorption:			
mg/100 g body wt/hour	268 ± 9	195 ± 12	< 0.01
mg/g intestine/hour	96 ± 3	98 ± 3	ns

[1] Meal-fed and nibbling rats maintained without food for 22 hours were given 5 ml of a 40% glucose solution orally. Two hours later the animals were killed, and the intestinal weight and glucose remaining in the digestive tract was determined.
[2] Probability of differences between meal-fed and nibbling rats being significant; ns = not significant.
[3] Mean ± SE for 5 rats.

cose uptake by tissues is more rapid in meal-fed than in nibbling rats. Circulating free fatty acids decreased for 30 minutes after glucose administration (fig. 3) and rose steadily thereafter, returning to fasting levels after 2 hours. The pattern of change in circulating fatty acids was similar for the meal-fed and nibbling rats and is in accord with present concepts concerning the regulation of fatty acid release from adipose tissue (14).

The experiment summarized in table 1 was designed to study the influence of intestinal absorption and tissue uptake of glucose on blood glucose levels. Meal-fed and nibbling rats maintained without food for 22 hours were force-fed 5 ml of a 40% glucose solution. Blood was taken before feeding and 30, 60 and 120 minutes after feeding. The amount of glucose remaining in the intestine after 2 hours was determined and the absorption coefficient (mg glucose absorbed/100 g body weight/hour) was calculated. The intestinal weights and absorption coefficients are shown in table 1. The relative size of the small intestine was increased by about 40% as a consequence of meal-feeding. This finding was anticipated, since a similar hypertrophy of the digestive tract has been observed in the intermittently starved rat (15) and the meal-fed chicken

(16,17). The absorption coefficient was approximately 40% higher in meal-fed as compared with nibbling rats, a difference which was highly significant. The absorption coefficient observed for the nibbling rats is in excellent agreement with the value reported by Cori (11). When the absorption of glucose was expressed per gram of intestine, the rates were identical for the meal-fed and nibbling animals. These results indicate that the rate of absorption per unit of intestinal surface area is not altered by meal-feeding. The glucose absorption capacity is increased in the meal-fed rat as a result of the greater absorptive area resulting from hypertrophy of the intestine. Thus, the amount of glucose absorbed and presented to the tissues per unit time would be greater by about 40% in the meal-fed rat.

Despite a greater inflow of glucose from the intestine the rise in blood glucose is less rapid in the meal-fed rat than in its nibbling counterpart, as shown in table 2. These results are in general agreement with those shown in figure 1, except that blood glucose values were somewhat higher in the experiment shown in table 2. Other data presented in table 2 include: 1) changes in circulating glucose, estimated by assuming a circulating glucose space (extracellular space) equivalent to 30% of body weight (12); 2) the glucose absorbed; and 3) the percentage of absorbed glucose not accounted for as circulating glucose (presumed to be utilized or taken up by the tissues). Blood glucose values were consistently higher following glucose administration in the nibbling rats as compared with the meal-fed animals, although only the 30-minute difference attained statistical significance. The increase in circulating glucose, however, was significantly higher at all time periods in the nibbling rats, whereas the amount of glucose absorbed was higher in the meal-fed animals. The percentage of glucose utilized was significantly greater at all time periods for the meal-fed rats than for the nibbling animals. The greatest difference was observed 30 minutes after glucose administration, at which time the meal-eaters had utilized 93% of the absorbed glucose, while the nibblers had only utilized 82%.

These results show that the meal-fed rat not only has a greater capacity to absorb glucose from the intestinal tract but the peripheral tissues also have a greater capacity to assimilate glucose. The major tissues utilizing and presumably storing

TABLE 2

Blood glucose concentration, glucose absorption and utilization of absorbed glucose by meal-fed and nibbling rats [1]

Treatment	Time after glucose administration	Whole blood glucose	Change in circulating glucose [2]	Glucose absorbed [3]	Absorbed glucose utilized [4]
	min	*mg/100 ml*	*mg*	*mg*	*%*
Meal-fed	0	92 ± 7 [5]	—	—	—
	30	125 ± 5	33 ± 8	452 ± 13	93 ± 2
	60	141 ± 8	50 ± 6	903 ± 25	94 ± 1
	120	148 ± 2	55 ± 4	1355 ± 38	96 ± 0.4
Nibbling	0	92 ± 4	—	—	—
	30	151 ± 10	71 ± 9	393 ± 11	83 ± 3
	60	153 ± 8	73 ± 8	787 ± 22	91 ± 1
	120	147 ± 6	66 ± 2	1180 ± 33	94 ± 0.3
P value (meal-fed vs. nibbling) [6]	0	ns	—	—	—
	30	< 0.05	< 0.02	< 0.01	< 0.01
	60	ns	< 0.05	< 0.01	< 0.02
	120	ns	< 0.05	< 0.01	< 0.01

[1] Experimental details are given in table 1, footnote 1.
[2] Circulating glucose space was assumed to be 30% of body weight (12). Change is calculated relative to zero time blood glucose values.
[3] Based on rates of absorption determined over a 2-hour period, see table 1.
[4] $\frac{\text{Absorbed glucose} - \text{change in circulating glucose}}{\text{Absorbed glucose}} \times 100.$
[5] Mean for 5 rats ± SE.
[6] Probability of difference between meal-fed and nibbling rats being significant; ns = not significant.

glucose are adipose tissue and muscle. Numerous studies have shown that adipose tissue of meal-fed rats has an increased capacity to convert glucose carbon to fatty acids (3–5). Muscle, however, by virtue of its mass would also contribute significantly to the removal of glucose from the circulation. Previously we have shown that diaphragm muscle from meal-fed rats accumulates glycogen more rapidly upon refeeding than muscle of nibbling animals (7). This particular muscle, although convenient for study, is not necessarily representative of all muscles. To determine how other muscles of meal-fed rats respond to refeeding, with respect to glycogen storage, the experiment summarized in table 3 was conducted. Meal-fed and nibbling rats were maintained without food for 22 hours. Some of the animals were killed at this time; the remaining rats were allowed access to food for 2 hours and were killed 6 hours after the start of feeding. The glycogen content of abdominal, gastrocnemius, diaphragm and heart muscle was determined. As shown in table 3, the initial tissue glycogen values were similar for both groups, but after refeeding the values were significantly higher for all muscles of the meal-fed compared with those of the nibbling rats. The rates of glycogen accretion were much higher for the meal-fed rats than for the nibblers. Diaphragm muscle showed the greatest accumulation of glycogen and heart muscle the least. The low rate of glycogen accumulation in heart muscle presumably reflects the fact that energy storage is not one of the major functions of this muscle. These data demonstrate that the mass of skeletal muscle, represented by the abdominal and gastrocnemius muscles, in the meal-fed animal has a much greater capacity to accumulate glycogen than does muscle of the nibbling rat. This is also true of diaphragm muscle, although, as these data show, this muscle is not truly representative of other muscles because of its greater capacity to take up glucose and to synthesize glycogen.

This experiment might be criticized because of the difference in food consumption between the nibblers and meal-eaters. In this study, during the 2-hour meal, the meal-fed rats consumed an average of 13 g of food per rat as compared with only 8 g for the nibblers. The ingestion of 8 g of diet represents an intake of 5.3 g of glucose, and the expected absorption of glucose during the 6-hour period would be 3.37 g (using the absorption coefficient of 195 mg/100 g body wt/hour, table 1). Consequently, since less than 65% of

TABLE 3

Change in glycogen content of various muscles from meal-fed and nibbling rats upon refeeding [1]

	Muscle tissue			
	Abdominal	Gastrocnemius	Diaphragm	Heart
	glycogen content, mg/g tissue, wet wt			
Meal-fed:				
Fasted (4)	2.7 ± 0.4 [2]	3.0 ± 0.4	1.1 ± 0.2	1.2 ± 0.1
Refed (5)	14.0 ± 3.3	14.0 ± 0.8	20.4 ± 3.1	2.6 ± 0.1
Nibbling:				
Fasted (4)	2.1 ± 0.3	2.5 ± 0.3	0.7 ± 0.2	1.7 ± 0.2
Refed (5)	5.4 ± 0.4	4.4 ± 0.6	4.9 ± 0.9	1.8 ± 0.2
P value (meal-fed vs. nibbling) [3]:				
Fasted	ns	ns	ns	ns
Refed	< 0.05	< 0.01	< 0.01	< 0.02
	rate of change — mg glycogen/g tissue, wet wt/hour			
Meal-fed	1.9	1.8	3.2	0.2
Nibbling	0.6	0.3	0.7	0.02

[1] Meal-fed and nibbling rats weighing 283 ± 8 and 288 ± 12 g (± SE), respectively, were maintained without food for 22 hours at which time the fasted animals were killed, refed animals were allowed access to food for 2 hours and were killed 6 hours after the start of feeding.

[2] Values are means ± SE for the number of rats shown in parentheses.

[3] Probability of differences between meal-fed and nibbling animals being significant; ns = not significant.

the ingested glucose would have been absorbed in the nibbling animal, glycogen storage should not have been limited by the unavailability of glucose for absorption.

The two major tissues responsible for the increased glucose utilization in meal-fed rats, muscle and adipose tissue, are both markedly insulin sensitive (18,19). It is particularly attractive to consider that insulin may be largely responsible for the enhanced glucose uptake by these tissues. Such an effect could be mediated by an increased release of insulin induced by meal ingestion or perhaps an increased sensitivity of the tissues to insulin. Partial support for the latter proposal is found in the report of Braun et al. (20), which indicates that adipose tissue of meal-fed rats is more sensitive to insulin than is tissue of nibbling animals. These authors have also reported that diaphragm muscle of the meal-fed animal is less sensitive to insulin than is muscle of nibbling rats. Although it is difficult to assess the role of insulin from these observations they do suggest that the hormone plays a role in the adaptation induced in the rat by meal-eating. Studies are presently in progress to assess the possible role of insulin in the meal-fed rat.

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Influence of Periodicity of Eating on the Activity of Various Enzymes in Adipose Tissue, Liver and Muscle of the Rat [1]

KRISHNA CHAKRABARTY AND GILBERT A. LEVEILLE
Division of Nutritional Biochemistry, Department of Animal Science, University of Illinois, Urbana, Illinois

ABSTRACT The activities of various enzymes involved in the conversion of glucose to lipid were compared in tissues of meal-fed (access to food limited to a single daily 2-hour period) and nibbling (fed ad libitum) rats. Activities of hexokinase, pyruvate kinase, α-glycerophosphate dehydrogenase, acetyl CoA carboxylase and pyruvate carboxylase were significantly elevated in adipose tissue of meal-fed, as compared with nibbling rats. Muscle hexokinase activity, liver pyruvate carboxylase, and liver phosphoenolpyruvate carboxykinase activities were also enhanced by meal-feeding. The activities of liver glucokinase, hexokinase, pyruvate kinase, α-glycerophosphate dehydrogenase and acetyl CoA carboxylase, of muscle pyruvate kinase and α-glycerophosphate dehydrogenase, and of adipose tissue phosphoenolpyruvate carboxykinase were similar in meal-fed and nibbling animals. The possible significance of these observations to the metabolic economy of the meal-fed rat is discussed.

Rats restricted to a single short daily meal period (meal-fed) show an increased metabolic efficiency in comparison with ad libitum-fed control animals (nibblers). To cope with the period of fast between meals, the meal-fed animal becomes more efficient in converting carbohydrate to storage materials, mainly lipid and glycogen. The meal-fed rat shows an increased rate of glucose utilization and a higher rate of lipid and glycogen synthesis from glucose (1–5). The activity of several enzymes related to fatty acid synthesis (glucose 6-phosphate dehydrogenase, NADP-malic dehydrogenase and citrate cleavage enzyme) are increased in adipose tissue and liver of meal-fed rats (6–8). The changes observed are most marked in adipose tissue. This is not surprising, since adipose tissue is the major site of lipogenesis in rats (9) and in meal-fed animals accounts for over 95% of the total fatty acids synthesized (3). Meal-fed rats also have a greater capacity to accumulate glycogen in diaphragm muscle and adipose tissue than nibbling animals (4). To further delineate the enzymatic changes taking place in the meal-fed animal, we have studied the activity of enzymes involved in glucose utilization, especially those related to the conversion of glucose to fatty acids and glyceride glycerol.

It is probable that several enzymatic adaptations occur in the meal-fed rat which permit an enhanced rate of lipogenesis. In this report we have compared some of the key enzymes involved in 1) the phosphorylation of glucose, 2) the conversion of glucose to fatty acid and glycerol, and 3) the synthesis of NADPH essential for reductive lipogenesis, in various tissues of meal-fed and nibbling rats. Our purpose was to gain a clearer insight into the factors responsible for the elevated rates of lipogenesis and glucose utilization observed in the meal-fed animal. Some significant enzymatic changes are shown to result from meal-feeding, especially in adipose tissue of the rat.

MATERIAL AND METHODS

Animals. Male rats of the Sprague-Dawley strain were used for all experiments. The animals were housed individually in metal cages having raised wire floors and in a temperature-regulated room (22°). The animals weighed 250–280 g initially and were randomly divided into 2 groups. One group was fed ad libitum (nibblers) and the other had access to food from 8 AM to 10 AM only (meal-eaters).

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Water was available at all times. The composition of the diet fed during the experimental period has been described previously (10), and supplied approximately 19, 12 and 70% of the calories as casein, corn oil, and glucose, respectively. The animals were maintained on these feeding schedules for at least 3 weeks, a period shown to be sufficient to induce the lipogenic and other meal-feeding responses (11). For enzyme determinations, the rats were usually killed at 8 AM on the day of the experiment. The rats weighed 275-350 g at the time of the enzyme studies. The body weight changes and food consumption in the meal-eaters and nibblers were similar to those reported previously (12).

Materials. Sodium bicarbonate-^{14}C,[2] malonic acid-2-^{14}C,[2] and Omnifluor [2] were purchased as was inosine 5-diphosphate [3] and other biochemicals. [4] Acetyl CoA was prepared by a modification of the method of Stadtman (13), wherein concentrated acetic anhydride was used rather than a 0.1 M solution. The method of Trams and Brady (14) was used for the preparation of 2-^{14}C-malonyl CoA.

Enzyme assays. Phosphoenolpyruvate (PEP) carboxykinase (EC. 4.1.1.32) was assayed by a modification of the method of Chang and Lane (15) as described by Ballard et al. (16). A blank tube without PEP was included in each assay. Approximately 1 g tissue (liver or adipose tissue) was homogenized in 5 ml of 0.25 M sucrose in a glass-Teflon homogenizer. The homogenate was centrifuged in a refrigerated centrifuge (0°), and the resulting 20,000 $\times$ *g* supernatant was used for assaying the enzymes. It should be noted that for adipose tissue, the "supernatant" used is really the clear intermediate layer below the fat cake.

Pyruvate carboxylase (EC. 6.4.1.1) was assayed by the method of Ballard and Hanson (17). Each assay value was corrected for blank activity by using a tube from which acetyl CoA was omitted. The homogenizing medium contained 0.02 M tris buffer, pH 7.4, 0.2 M sucrose, 1 mM EDTA (ethylenediaminetetraacetic acid) and 1 mM reduced glutathione. The preparation of the enzyme extract was similar to that indicated above.

Acetyl CoA carboxylase (EC. 6.4.1.2) was assayed by the method of Chang et al. (18). Citrate was omitted from the blank tube. The tissue (liver or adipose tissue) was homogenized in a medium containing 0.15 M KCl, 0.05 M tris (Cl^-), pH 7.2, and 0.1 mM EDTA. The homogenate was centrifuged as described above and the supernatant was recentrifuged at 105,000 $\times$ *g*. This supernatant was used directly for assay. The enzymatic product was tentatively identified as malonyl CoA by demonstrating that it was isochromatographic with 2-^{14}C-malonyl CoA prepared chemically (14). The solvent system was 0.1 M K-acetate, pH 4.5: alcohol (1:1). Further identification was obtained by digesting the product in 3 N NaOH at 50° for 1 hour, acidifying and extracting with ethyl acetate. The radioactive compound obtained after digestion was found to have the same R_F as malonic acid-2-^{14}C in a solvent system containing ether : acetic acid : H_2O (13:3:1).

Liver glucokinase (EC. 2.7.1.2) and hexokinase (EC. 2.7.1.1) were measured by the method of Walker and Perry (19). The final concentration of glucose used was 100 mM and 0.5 mM for glucokinase and hexokinase, respectively. Adipose tissue and diaphragm muscle hexokinase activities were assayed by the same method, except that the final concentration of glucose was 10 mM. Since crude extracts were used, it was assumed that 2 molecules of NADP were reduced per molecule of glucose phosphorylated.

Pyruvate kinase (EC. 2.7.1.40) was assayed by the method of Bücher and Pfleiderer (20). α-Glycerophosphate dehydrogenase (EC. 1.1.1.8) was assayed by a modification of the method of Beisenherz et al. (21), wherein the final concentration of dihydroxyacetone phosphate was 5×10^{-4} M instead of 1×10^{-4} M. This concentration of substrate yielded maximum activity.

For the assay of glucokinase, hexokinase, pyruvate kinase and α-glycerophosphate dehydrogenase, the tissues were homogenized in ice-cold medium containing 0.15 M KCl, 0.004 M $MgSO_4$, 0.004 M EDTA, and 0.004 M N-acetyl cysteine, pH 7.0. A Sorvall omnimixer was used to

[2] Purchased from New England Nuclear Corp., Boston, Massachusetts.
[3] Obtained from Calbiochem, Los Angeles.
[4] Sigma Biochemical Corporation, St. Louis.

homogenize the muscle and adipose tissue, whereas for liver a glass-Teflon homogenizer was used. The 105,000 × *g* supernatant, prepared as indicated above, was used for these assays.

Protein determinations were carried out according to Lowry et al. (22) on the supernatants used for the assay of enzyme activity. Ten milliliters of toluene scintillant (4 g Omnifluor, 230 ml ethanol and toluene to 1 liter) were used for radioactivity determinations, which were carried out in a Packard Tri-Carb liquid scintillation counter. The data were analyzed statistically by the analysis of variance or by *t* test comparisons as indicated in the tables.

RESULTS

The enzymes which phosphorylate glucose are responsible for initiating its metabolism. Consequently, the activity of these enzymes, glucokinase and hexokinase in liver and hexokinase in adipose tissue was studied, and the results are presented in table 1. Adipose tissue hexokinase activity was severalfold higher in tissue of meal-fed as compared with nibbling animals. Liver hexokinase and glucokinase activities were not significantly altered by meal-feeding. In this particular experiment, the analysis of variance does indicate a significant difference between the hepatic glucokinase activities of meal-fed and nibbling rats. This difference, however, was not observed consistently, and when observed was small. The data for glucokinase activity suggest an increased activity following meal-ingestion in the meal-eating rat. This has been noted repeatedly; however, the difference is not great enough to attain statistical significance. Although not shown in table 1, the data were analyzed for possible time trends and no significant time effect was noted for these enzymes.

In a second experiment, the activity of glucose-phosphorylating enzymes in liver and muscle was studied. The activities of pyruvate kinase, a key glycolytic enzyme and of α-glycerophosphate dehydrogenase, the enzyme responsible for supplying α-glycerophosphate for the synthesis of triglycerides, were also assayed in liver, muscle and adipose tissue of meal-eating and nibbling rats. The results of this study are shown in table 2. The data for liver hexokinase and glucokinase are comparable to those in table 1, although there was a small significant difference between the hepatic hexokinase activities of the meal-eaters and the nibblers. Muscle hexokinase activity was significantly higher in diaphragm muscle of the meal-fed rat. The difference, although amounting to only about 20%, has been observed consistently. Pyruvate kinase and α-glycerophosphate dehydrogenase activities were significantly higher in adipose tissue of the meal-fed rat. The activity of these two enzymes in liver or muscle however, was not influenced by meal-feeding.

TABLE 1

Activity of glucose phosphorylating enzymes in liver and adipose tissue of fasted and fed meal-eating and nibbling rats

Treatment	Dietary status [1]	Liver hexokinase	Liver glucokinase	Adipose tissue hexokinase
		units/mg protein [2]	*units/mg protein* [2]	*units/mg protein* [2]
Meal-eaters	Fasted	2.60 ± 0.18(10) [3]	6.77 ± 0.85(5)	22.2 ± 2.2(10)
	Refed	3.15 ± 0.16(10)	8.30 ± 1.21(5)	23.0 ± 2.7(10)
Nibblers	Fasted	3.10 ± 0.26(8)	6.36 ± 1.25(3)	8.8 ± 0.7(8)
	Fed	2.98 ± 0.16(8)	5.66 ± 0.71(3)	6.2 ± 1.0(8)
	Refed	3.11 ± 0.10(9)	3.43 ± 0.60(4)	6.2 ± 0.7(9)
P value [4]	Meal-eaters vs nibblers	ns	< 0.05	< 0.01

[1] Fasted animals were maintained without food for 22 hours; refed animals were maintained without food for 22 hours, allowed access to food for 2 hours and were killed 6 hours after the start of feeding; fed animals had access to food until the time of killing.
[2] A unit is defined as 1 μmole glucose phosphorylated per minute.
[3] Mean for the number of rats indicated in parentheses ± SEM.
[4] P = probability of significance based on analysis of variance; ns = not significant.

TABLE 2

Activity of hexokinase, pyruvate kinase and α-glycerophosphate dehydrogenase in various tissues of meal-fed and nibbling rats [1]

Tissue	Treatment	Hexokinase	Glucokinase	Pyruvate kinase	α-Glycerophosphate dehydrogenase
		units/mg protein [2]	*units/mg protein* [2]	*units/mg protein* [2]	*units/mg protein* [2]
Adipose tissue	Meal-eaters	—	—	102 ± 5(5) [3]	1190 ± 167(5)
	Nibblers	—	—	73 ± 4(5)	704 ± 98(5)
	P value [4]	—	—	< 0.01	< 0.05
Muscle	Meal-eaters	22.6 ± 1.0(8)	—	762 ± 62(5)	382 ± 36(5)
	Nibblers	18.6 ± 1.1(8)	—	704 ± 53(5)	366 ± 18(5)
	P value	< 0.02	—	ns	ns
Liver	Meal-eaters	2.78 ± 0.14(5)	6.68 ± 0.93(5)	348 ± 63(8)	628 ± 59(5)
	Nibblers	2.33 ± 0.02(5)	6.35 ± 0.81(5)	338 ± 28(8)	726 ± 65(5)
	P value	< 0.02	ns	ns	ns

[1] Meal-eaters were fasted for 22 hours before the experiment, whereas the nibblers had access to food until the time of killing.
[2] A unit is defined as 1 μmole substrate converted to product per minute.
[3] Mean for the number of rats indicated in parentheses ± SEM.
[4] *P* = probability of significance determined by *t* test; ns = not significant.

TABLE 3

Activity of acetyl CoA carboxylase, pyruvate carboxylase and phosphoenolpyruvate carboxykinase in liver and adipose tissue of meal-fed and nibbling rats [1]

Tissue	Treatment	Acetyl CoA carboxylase	Pyruvate carboxylase	Phosphoenolpyruvate carboxykinase
		units/mg protein [2]	*units/mg protein* [2]	*units/mg protein* [2]
Liver	Meal-eaters	4.2 ± 0.04 [3]	18.2 ± 2.2	48.8 ± 6.1
	Nibblers	4.8 ± 0.05	5.3 ± 1.1	31.0 ± 3.3
	P value [4]	ns	< 0.01	< 0.05
Adipose tissue	Meal-eaters	3.0 ± 0.4	141.7 ± 17.6	10.1 ± 1.1
	Nibblers	0.35 ± 0.09	31.5 ± 6.5	10.4 ± 1.0
	P value	< 0.01	< 0.01	ns

[1] Meal-eaters were fasted for 22 hours before the experiment, whereas the nibblers had access to food until the time of killing.
[2] A unit is defined as 1 μmole substrate carboxylated per minute.
[3] Mean ± SEM for 6 rats.
[4] *P* = probability value as determined by *t* test; ns = not significant.

The activities of 3 carboxylase enzymes were assayed in liver and adipose tissue of meal-fed and nibbling rats and the results of this experiment are summarized in table 3. The activity of acetyl CoA carboxylase, which is responsible for initiating fatty acid synthesis, was similar in liver of meal-fed and nibbling animals, but in adipose tissue, the activity of this enzyme was increased about 10-fold as a result of meal-feeding. Pyruvate carboxylase has been implicated in the generation of reducing equivalents to support fatty acid synthesis (17). The data in table 3 show that the activity of this enzyme is 3- to 4-fold higher in liver, and 4- to 5-fold higher in adipose tissue of meal-fed as compared to nibbling animals. The PEP-carboxykinase is an enzyme essential for gluconeogenesis, but not lipogenesis. The activity of this enzyme was increased in liver, but not in adipose tissue of the meal-fed animals.

DISCUSSION

The data presented in this report add several additional enzymes to the growing list of those whose activity is enhanced in adipose tissue of the meal-fed rat. The adaptive enzymes studied to date can all be related in some way to lipogenesis and it is not surprising, therefore, that of the tissues studied adipose tissue shows the greatest adaptability (3,8,12,23,24). Hepatic fatty acid synthesis is higher in fasted meal-fed rats when compared with fasted nibbling animals, but not when compared with nibbling animals which have not been fasted (2,11). Since liver plays a very

minor role in the total fatty acid synthesis of the meal-fed animal (3,10), the above observation seems reasonable.

The findings of the present study agree with the conclusion that adipose tissue is the major adaptive tissue with regard to lipogenesis in the meal-fed rat. Thus, the observation that hexokinase, pyruvate kinase, α-glycerophosphate dehydrogenase and acetyl CoA carboxylase activities are elevated in adipose tissue, but not in liver, of the meal-fed rat supports the above concept. These enzymes play obvious roles in supporting high rates of lipogenesis: hexokinase, by initiating the utilization of glucose, provides a substrate for further metabolism; α-glycerophosphate is an essential substrate for fatty acid esterification; pyruvate kinase controls the formation of pyruvate and acetyl CoA; and acetyl CoA carboxylase is responsible for initiating fatty acid synthesis via the malonyl CoA pathway. When other enzymes, such as the pentose pathway dehydrogenases, NADP-malic dehydrogenase and citrate cleavage enzyme, the activity of which is also enhanced in adipose tissue of meal-fed rats, are considered, it becomes apparent that adipose tissue of the meal-fed rat is enzymatically capable of supporting rapid lipogenesis. Yet, the actual mechanism(s) involved in "triggering" these adaptive changes is not known. It appears likely that the increased pentose pathway activity is the result of hyperlipogenesis rather than the cause. This is implied by the observation that in the meal-fed animal lipogenesis is enhanced for some time before pentose pathway dehydrogenase activity is increased (11). With regard to the pentose pathway enzymes in particular, and in general concerning all the enzymes studied so far in adipose tissue of the meal-fed rat, it is our feeling that these changes are best explained by the concept that the increased use of an enzymatic pathway stimulates an increase in the activity of the enzyme(s) involved in that pathway, as suggested by Freedland and Harper (25) and by Fitch and Chaikoff (26). Thus, in adipose tissue of the meal-fed rat increased uptake of glucose during the meal period would necessitate a greater flow of substrate through the glycolytic sequence and conversion of the original glucose carbon to fatty acids. As a result of this increased substrate flow the enzymes controlling "key" or rate-limiting reactions would be enhanced. In liver the rate of glucose utilization is not markedly increased since glycogen formation proceeds at the same rate as observed in the nibbling animals (4) and fatty acid synthesis at a lower rate than in the nonfasted nibbler (11). Consequently, the adaptive enzymatic changes either do not appear or are much less marked than those noted in adipose tissue.

The activity of cytoplasmic pyruvate carboxylase was studied since this enzyme apparently makes an important (50%) contribution in supplying NADPH for fatty acid synthesis by the following "malate transhydrogenation cycle" (17):

1) Pyruvate + CO_2 + ATP $\xrightarrow{\text{pyruvate carboxylase}}$ oxaloacetate + ADP + P_i

2) Oxaloacetate + NADH + H^+ $\xrightarrow{\text{NAD-malate dehydrogenase}}$ NAD^+ + malate

3) Malate + $NADP^+$ $\rightleftharpoons$ $NADPH + H^+$ + pyruvate + CO_2

The activity of NADP-malic dehydrogenase has already been shown to be increased in adipose tissue of the meal-fed rat (8) and the activity of NAD-malic dehydrogenase is high enough not to be rate-limiting (8). Hence, the enhancement of pyruvate carboxylase activity in the adipose tissue of the meal-fed rat permits the tentative conclusion that the malate transhydrogenation cycle is functioning at an increased rate to support hyperlipogenesis in this tissue.

Pyruvate carboxylase is generally associated with gluconeogenesis. To determine whether this enzyme was functioning in the supply of reducing equivalents or in the conversion of pyruvate to PEP, the activity of another gluconeogenic enzyme, PEP-carboxykinase was assayed. In adipose tissue the activity of this enzyme was not increased by meal-feeding implying that PEP formation from pyruvate was not enhanced in this tissue. It has been demonstrated previously that the conversion of pyruvate-2-^{14}C to glyceride glycerol is not increased in the adipose tissue of the meal-fed rat (27). In liver, however, both pyruvate carboxylase and PEP-carboxykinase

activities were enhanced by meal-feeding. The possibility that meal-feeding may alter the gluconeogenic capacity of the liver is presently under investigation.

The finding that muscle hexokinase activity is increased in the meal-fed animal may in part account for its greater capacity to accumulate glycogen (4). Muscle, because of its mass, contributes significantly to overall glucose utilization, which has been shown to be enhanced in the intact meal-fed rat as compared with the nibbling animal (5).

We have previously suggested that insulin plays an important role in controlling metabolism in the meal-fed rat (5) and Braun et al. (28) have presented evidence supporting this concept. Muscle and adipose tissue contain predominantly type II hexokinase and insulin is essential for maintaining the activity of this enzyme (29). Consequently, the increased hexokinase activity in adipose tissue and muscle of meal-fed animals lends support to the concept that insulin plays a role in the overall adaptation to meal-eating. Furthermore, this hormone would, to a large degree, regulate the diurnal shifts in metabolism in the meal-fed animal. Because insulin is essential for the uptake of glucose by muscle and adipose tissue, an increased release of the hormone or a greater sensitivity of the tissues to it would enable the more rapid utilization of glucose which has been shown for the meal-fed rat (5). As suggested above, the increased cellular metabolism of glucose could be responsible for the observed increase in enzyme activity. During the period of fast between meals, insulin release would be inhibited, thus inhibiting the uptake of glucose by muscle, and more important by adipose tissue. This would result in an increased release of fatty acids by adipose tissue, an increased uptake of fatty acids by liver and muscle with consequent inhibition of glycolysis as proposed by Randle et al. (30) and by Weber et al. (31).

More recent evidence (32) suggests that acetyl CoA produced by the oxidation of fatty acids may reinforce the inhibitory effects of fatty acids on glycolysis and glucose phosphorylation. Whether such mechanisms function with any greater degree of efficiency in tissues of meal-fed animals remains to be determined.

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Vitamin B_6 in Blood, Urine, and Liver of Monkeys [1]

EVA M. BENSON, JEAN M. PETERS, MARGARET A. EDWARDS, M. R. MALINOW [2] AND CLARA A. STORVICK
Nutrition Research Laboratory, School of Home Economics, Oregon State University, Corvallis, Oregon

ABSTRACT Blood and urinary vitamin B_6 and urinary 4-pyridoxic acid values are reported for 3 rhesus monkeys before and after a 5-day period of pyridoxol·HCl supplementation. Blood and liver vitamin B_6 values are given for a group of free-ranging howler monkeys. Microbiological procedures employing *Saccharomyces carlsbergensis* ATCC 9080, *Streptococcus faecium* Ø 51 NCDO 1229 and *Lactobacillus casei* ATCC 7469 were used for the vitamin B_6 analyses and a microprocedure of the lactone method was used for 4-pyridoxic acid determinations. Large increments of vitamin B_6 in blood and urine and increased excretion of 4-pyridoxic acid were observed in the rhesus monkeys following supplementation. Of significance was the prolonged elevation of values several days after supplementation had ceased. Blood values for the howler monkeys were higher than those for the rhesus. Differential assay of liver hydrolysates from howler monkeys revealed that most of the vitamin was present in the pyridoxamine form. A lesser amount was in the pyridoxal form and the amount of pyridoxol was negligible.

Previous reports on vitamin B_6 values for monkey blood include those of Greenberg and Rinehart (1), Marsh et al. (2), and Marquez.[3] Marquez also determined the vitamin B_6 content of monkey urine. All these studies were done on the rhesus monkey and the method of determination was microbiological assay employing the yeast *Saccharomyces carlsbergensis*. No information on vitamin B_6 values for the howler monkey is available. Data of any kind for this species are limited since efforts to maintain it in captivity have been largely unsuccessful.

Vitamin B_6 values are reported here for 2 species of monkey, the rhesus *(Macaca mulatta)* and the howler *(Alouatta caraya)*. Blood, urine and liver samples were made available through collaboration with the Oregon Regional Primate Research Center. The effect of a period of vitamin B_6 supplementation was studied in rhesus monkeys. These animals were maintained in the controlled environment of the Primate Center and for this study blood and urinary vitamin B_6 and urinary 4-pyridoxic acid determinations were made before and after a period of pyridoxol·HCl supplementation. The howler monkeys were free-ranging in their native habitat of Argentina. Hence, for this species, vitamin B_6 determinations were made on blood and liver samples that had been obtained in the field and constituted part of a study to be reported elsewhere on the possible relationship between vitamin B_6 deficiency and the presence of coronary lesions (3).

For the studies reported here the same routine microbiological procedure of Atkin et al. (4) employing *S. carlsbergensis* ATCC 9080 was used. In addition, 2 other organisms, *Streptococcus faecium* Ø 51 NCDO 1229 and *Lactobacillus casei* ATCC 7469, provided a differential type assay based on that of Rabinowitz and Snell (5) and modified by Gregory (6). *S. carlsbergensis* responds to all 3 forms of the vitamin (pyridoxal, pyridoxol, and pyridoxamine). *S. faecium* responds to pyridoxal and pyridoxamine, and *L. casei* responds to pyridoxal. To our knowledge vitamin B_6 values for monkey blood, urine and liver based on the latter two vitamin

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[2] Chairman, Division of Cardiovascular Physiology, Oregon Regional Primate Research Center, Beaverton, Oregon.

[3] Marquez, L. R. 1955 Studies on the metabolism of vitamin B_6. Ph.D. Thesis, University of Wisconsin, Madison.

B_6-dependent assay organisms have not been reported.

The problems encountered in applying microbiological procedures to the determination of vitamin B_6 in blood have been discussed by Storvick and Peters (7) and by Haskell and Wallnöfer (8). Blood appears to contain growth-promoting, or inhibitory substances, or both, for the various organisms that invalidate use of the differential technique. Moreover, inhibition has been mentioned by Rabinowitz and Snell (5, 9) and by Short and Fairbairn (10) as a problem in *S. carlsbergensis* assays for the vitamin B_6 content of urine.

EXPERIMENTAL

Rhesus monkey study

Vitamin B_6 supplementation procedure. This experiment was designed to demonstrate the effect of an extended period of pyridoxol·HCl [4] supplementation on the vitamin B_6 content of blood and urine of 3 adult rhesus monkeys. These animals had been conditioned to restraining chairs and were fed a pelleted commercial diet [5] plus small amounts of apple and banana. The individual monkeys consumed approximately 200 g daily of the commercial diet, which by analysis with *S. carlsbergensis* contained approximately 7 μg of vitamin B_6/g of diet. Therefore, the estimated vitamin B_6 intake during the unsupplemented period was about 1.4 mg/day.

To establish base levels for the vitamin in blood and urine, control samples were obtained before the period of supplementation. For the 3 days preceding supplementation 24-hour collections of urine were made and peripheral blood samples were drawn 48 hours before supplementation.

During the period of supplementation the monkeys were fed, in addition to their regular diet, 20 mg of pyridoxol·HCl daily for 5 consecutive days. Blood was not taken and urine was not collected during this 5-day period.

Beginning 24 hours after the last supplement was given, daily urine collections were made for 3 consecutive days. Peripheral blood samples were drawn 48 hours after supplementation had ceased.

Preparation of the samples for analysis. Blood samples were oxalated and kept frozen until hydrolyzed before microbiological assay. The samples were protected from light as much as possible. Hydrolysis was done with 0.055 N HCl in the ratio of 1 ml of blood plus 20 ml of acid by autoclaving for 5 hours at 121°. After hydrolysis, the samples were adjusted to pH 7 with KOH and then back to pH 5.2 with HCl before adjustment of the volume and filtration through Whatman no. 50 filter paper. The filtrates were refrigerated overnight, autoclaved for 5 minutes at 121°, and refiltered to remove any precipitated protein. If not assayed immediately the samples were layered with benzene and refrigerated.

Urine collections were made under toluene in plastic bottles surrounded by ice and protected from light as much as possible. They were stored frozen until hydrolyzed before analysis. The urine samples were prepared for microbiological assay according to the method of Sauberlich.[6] A 10 ml aliquot of urine was autoclaved with 50 ml of 0.1 N HCl for 30 minutes at 121°. The sample was cooled, adjusted to pH 5.2 with KOH, and diluted to 100 ml before filtration through Whatman no. 1 filter paper. For the determination of urinary 4-pyridoxic acid the microprocedure of Woodring et al. (11) was used.

Howler monkey study

The monkeys were trapped on the islands opposite Bella Vista, Corrientes and transported to Buenos Aires. During captivity, water and food were provided ad libitum. Within 72 hours of their capture, blood and liver samples were obtained under thiopental sodium anesthesia.

Preparation of the samples for analysis. Blood samples were oxalated and frozen for air shipment to the Oregon Regional Primate Research Center. They remained frozen until hydrolyzed and assayed as described for the rhesus monkey samples.

[4] Hexa-betalin, pyridoxine·HCl, 100 mg/ml, Eli Lilly and Company, Indianapolis, Indiana.

[5] Purina Monkey Chow, Ralston Purina Company, St. Louis.

[6] Sauberlich, H. E., U.S. Army Medical Research and Nutrition Laboratory, Fitzsimons General Hospital, Denver, Colorado, personal communication.

Samples weighing 3 to 4 g from each of 6 livers were received frozen for determination of the vitamin B_6 content. Approximately 1 g of each sample was homogenized in a portion of the 100 ml of 0.055 N HCl used for hydrolysis. The homogenates were hydrolyzed for 5 hours at 121°. The sample preparation was continued in the same manner as for blood, except that it was unnecessary to heat and refilter the hydrolysate following refrigeration overnight since no additional protein precipitation took place upon heating.

RESULTS AND DISCUSSION

Rhesus monkeys. Response to a vitamin B_6 supplement. The limitations of the 3 organisms, utilizing the differential technique with regard to the assay of blood, have been pointed out (7, 8). Therefore, it must be recognized that vitamin B_6 values for blood, particularly for samples drawn before supplementation, are probably not valid due to the very small amount of vitamin B_6 present in relationship to interfering substances. All 3 assay organisms, however, were able to measure the response of the blood to vitamin B_6 supplementation, since large increments of the vitamin were found in blood following supplementation (table 1). The results showed that a high proportion of the vitamin was still in the form of pyridoxol. A significant feature was the time of sampling at which these high values were obtained, namely, 48 hours after supplementation had ceased. In studies reported by Marquez [3] on humans there was a rapid return to normal levels of vitamin B_6 in blood when a single 100 mg test dose of pyridoxol was given. On the other hand Marsh et al. (2) studied both humans and rhesus monkeys receiving pyridoxine supplements over extended periods of time. Elevation of blood vitamin B_6 continued for some time after supplementation had ceased. These investigators suggested that return to initial levels following supplementation depends to some extent upon the duration of the period of increased intake. The prolonged high levels of vitamin B_6 in blood found in our study may be due to the sustained 5-day period of supplementation as opposed to a single test dose. They may suggest that the laboratory diet, although adequate for prevention of clinical vitamin B_6 deficiency symptoms, did not provide for tissue saturation.

The literature indicates that the requirement for vitamin B_6 in all species studied including the monkey is still controversial. It varies with a number of factors, including the type of diet and the maturity of the animals, and must be reported in terms of the criteria of adequacy used. The requirement for rhesus monkeys indicated by Rinehart and Greenberg (12) of 50 μg/kg of body weight is frequently cited if optimum growth and freedom from obvious clinical lesions are the criteria employed. Based on the same parameters a somewhat higher requirement, namely 1 to 2 mg/day, has been suggested by Marquez [3] and by Emerson et al. (13). When biochemical parameters such as maximum enzyme levels are used as the

TABLE 1

Vitamin B_6 in blood of rhesus monkeys before and after supplementation with pyridoxol·HCl (calculated from pyridoxal·HCl standard curves)

Monkey no.	Weight	Day sample drawn [1]	*S. carlsbergensis*	*S. faecium*	*L. casei*
	kg		*μg/100 ml*	*μg/100 ml*	*μg/100 ml*
339	3.4	2	1.77	22.72	37.84
		10	392.21	153.24	148.56
1385	3.8	2	1.63	19.22	34.25
		10	198.98	156.52	157.68
1481	6.9	2	5.13	19.98	40.38
		10	109.20	76.91	104.48

[1] Day 2 represents 48 hours before vitamin B_6 supplementation was initiated. Day 10 represents 48 hours after vitamin B_6 supplementation was discontinued.

indexes of adequacy, however, the intake needed to achieve such levels may be even higher. For example, Marsh et al. (2) have shown that 4 mg/day are necessary to maintain maximum transaminase levels in the blood of rhesus monkeys. Whether or not maximum enzyme levels are necessarily optimal has not yet been established.

In the study reported here, the only criteria of adequacy were apparently normal growth and the absence of obvious clinical lesions. However, the blood values as measured by *S. carlsbergensis* for the period before supplementation approximate those found by Marquez [3] for monkeys on what she considered a less than optimum intake of 0.5 mg/day. It may be that the 1.4 mg/day provided by the laboratory diet at the Oregon Regional Primate Research Center was still only marginal for these monkeys. Certainly the continued elevation of blood levels after supplementation had ceased, together with the considerably delayed urinary excretion of the vitamin, indicates that the monkeys had the capacity to retain larger amounts of the vitamin than were provided by the regular laboratory diet. Whether this represents an adaptation by the organism to larger amounts of the vitamin or whether it can be related to the achievement of tissue saturation remains speculative.

Table 2 shows the urinary vitamin B_6 and 4-pyridoxic acid levels before and after supplementation. The assay values obtained by *S. carlsbergensis* on the control samples were somewhat lower than those obtained by *S. faecium*. This type of problem has been reported in assays on human urine by Rabinowitz and Snell (5, 9). They suggested that it was due to the presence of materials toxic to the yeast which suppressed its response to vitamin B_6. Nevertheless, *S. carlsbergensis* has been the organism of choice in all studies to determine vitamin B_6 require-

TABLE 2

Vitamin B_6 and 4-pyridoxic acid in urine of rhesus monkeys

Monkey no.	Day	Pyridoxal·HCl supplement	Urine volume	Vitamin B_6 (calculated from pyridoxal·HCl standard curves)			4-Pyridoxic acid
				S. carlsbergensis	*S. faecium*	*L. casei*	
		mg/day	*ml/24 hr*	*mg/24 hr*	*mg/24 hr*	*mg/24 hr*	*mg/24 hr*
339	1	0	480	0.024	0.036	0.017	0.358
	2	0	340	0.071	0.103	0.044	0.270
	3	0	100	0.023	0.030	0.015	0.330
				(0.039) [1]	(0.056)	(0.025)	(0.319)
	4–8	20 (no collections made)					
	9	0	90	4.01	2.56	1.62	8.60
	10	0	378	19.05	9.50	6.67	9.15
	11	0	210	13.60	3.23	1.99	8.50
				(12.22)	(5.10)	(3.43)	(8.75)
1385	1	0	400	0.069	0.106	0.044	0.220
	2	0	130	0.009	0.017	0.006	0.175
	3	0	374	0.017	0.025	0.007	0.210
				(0.032)	(0.049)	(0.019)	(0.202)
	4–8	20 (no collections made)					
	9	0	210	13.08	5.96	3.67	5.00
	10	0	268	16.42	7.10	4.86	6.10
	11	0	326	11.90	9.42	6.51	4.70
				(13.80)	(7.49)	(5.01)	(5.27)
1481	1	0	405	0.025	0.043	0.019	0.310
	2	0	216	0.068	0.132	0.067	0.330
	3	0	175	0.058	0.104	0.057	0.295
				(0.050)	(0.093)	(0.048)	(0.312)
	4–8	20 (no collections made)					
	9	0	755	19.25	9.51	6.44	7.85
	10	0	392	6.86	4.59	3.06	5.55
	11	0	273	8.97	5.07	3.58	7.55
				(11.69)	(6.39)	(4.36)	(6.98)

[1] Numbers in parentheses indicate averages for 3 days.

ments. In this study the urine values obtained by the yeast for the control samples approximate those reported by Marquez [3] for rhesus monkeys on a pyridoxol·HCl intake of 0.5 mg/day. With regard to urinary vitamin B_6 values following supplementation, all 3 organisms measured large increments of vitamin B_6. The *S. carlsbergensis* assays revealed that there was considerable excretion of the vitamin as pyridoxol. It seems likely that this pyridoxol represents excess intake and probably was not involved coenzymatically. However, there was also a marked increase in the excretion of 4-pyridoxic acid in the urine following supplementation, indicating considerable conversion of some of the extradietary pyridoxol to the metabolite. These results show that a prolonged elevation of urinary vitamin B_6 occurs with a sustained period of pyridoxol·HCl supplementation and corroborate the observations for blood. Somewhat analogous findings on the delayed elimination of pyridoxol supplements in humans and some interesting hypotheses to explain these phenomena have been made by Johansson et al. (14).

It is hoped that in future studies samples might be analyzed at close intervals both during and after supplementation, and that sampling might be continued long enough to allow the blood and urinary vitamin B_6 levels to return to those of the presupplementation state.

Howler monkeys. Vitamin B_6 content of blood and liver. The vitamin B_6 content of the blood of 19 free-ranging howler monkeys is shown in table 3. With one exception, the values obtained by the *S. carlsbergensis* assay of the blood ranged between 4 and 10 μg/100 ml. These values are higher than those found in the study on rhesus monkeys reported here, but are similar to values found for rhesus monkeys by Greenberg and Rinehart (1).

The livers of 6 of these monkeys were assayed for vitamin B_6 (table 4). Significant was the fact that the differential assay technique could be applied to liver hydrolysates. It is possible that the inhibitory or stimulatory substances, or both, that interfere with such assays for blood do not exist in liver. A more logical explanation, however, may be based on the observation that the vitamin B_6 content of liver is much higher than that of blood. This higher concentration requires considerable dilution before assay which may result in diluting out the interfering substances. The differential technique reveals that most of the vitamin in liver hydrolysates is present in the pyridoxamine form. A lesser amount is in the pyridoxal form and the amount of pyridoxol is negligible.

TABLE 3

Vitamin B_6 in blood of howler monkeys (calculated from pyridoxal·HCl standard curves)

Monkey no.	*S. carlsbergensis*	*S. faecium*	*L. casei*
	μg/100 ml	*μg/100 ml*	*μg/100 ml*
32	5.37	38.50	39.38
33	1.74	45.54	39.16
34	7.13	37.40	42.68
35	4.05	36.08	34.98
36	6.25	38.94	37.18
37	9.90	50.82	42.90
38	8.10	46.42	32.56
39	5.32	48.18	41.14
40	8.71	52.14	44.44
41	7.70	41.58	39.38
42	7.30	46.64	41.36
43	6.42	45.32	47.74
44	6.69	45.98	45.76
45	8.32	49.06	51.48
46	8.40	50.16	39.38
48	7.96	41.36	42.68
49	5.94	45.76	41.80
50	6.12	46.86	39.60
51	6.64	49.94	42.68

TABLE 4

Vitamin B_6 in liver of howler monkeys

Liver no.	Pyridoxal and pyridoxamine content mixture standard [1]		Pyridoxal content pyridoxal·HCl standard
	S. carlsbergensis	*S. faecium*	*L. casei*
	values expressed as μg/g of wet wt		
33	9.25	8.55	2.62
40	13.44	12.96	3.59
45	13.36	15.64	4.12
46	14.68	15.76	4.44
50	13.72	15.84	4.17
51	14.24	16.52	3.83

[1] The composition of this standard (30% pyridoxal·HCl and 70% pyridoxamine·2HCl) was determined by application of differential calculations to a preliminary assay and denotes approximate ratio of pyridoxal to pyridoxamine in the liver hydrolysates.

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Thiamine Instability in Experimental Wet Diets Containing Commercial Casein with Sulfur Dioxide

M. A. JOSLYN AND J. LEICHTER
Department of Nutritional Sciences, University of California, Berkeley, California

ABSTRACT The effect of casein on destruction of thiamine during storage in aqueous suspensions, both at 25°, and frozen at −15°, was investigated. This destruction was found to be caused by residual sulfur dioxide present in some lots of commercial casein, apparently fumigated with sulfur dioxide. Considerable variation in sulfur dioxide level in such casein was observed. Destruction of thiamine by sulfur dioxide in aqueous solution at the same pH and concentration was considerably faster, indicating an unknown protective effect of casein.

It is recognized that losses of thiamine content in experimental diets for laboratory animals occur during storage at room temperature. These losses have been ascribed primarily to the catalytic effect of salt mixtures (1–3) but even when their composition was changed losses were still encountered (1). Calloway [1] observed that losses of thiamine appeared to be directly proportional to casein content of mixed diets during freezing storage.

Thiamine is known to be subject to oxidation (4) or reduction and to cleavage by thiaminase (5, 6), by sodium acetate or barium nitrite (7) or by sulfite (8–10). Thiamine also has been reported to be decomposed by thermostable factors in ferns which have been identified as both nitrogenous (11) and flavonoid (12, 13). Thiamine also has been reported to be bound by adsorption to casein and other proteins (14) or by heat-stable nitrogenous constituents or flavonoids present in carp entrails (15). In the former case the adsorbed thiamine should still be largely biologically available. The chemistry of the reaction in the latter case still remains to be elucidated. A heat-stable thiamine-destroying factor was reported to occur in Oriental millet by Bhagvat and Devi (16) and this was confirmed by Weakley et al. (17). On the other hand, an alleged thiamine-destroying factor in soybean meal, reported by Indian colleagues, on basis of low recovery of added thiamine by their modifications of the thiochrome assay, was not confirmed. Weakley et al. (17) reported this to be due to the unreliability of the thiochrome assay procedure used. The nature of the thiamine-destroying factor in millet, a staple food grain in India, is still unknown.

We have observed that residual sulfite present in some commercially available caseins is largely responsible for the decomposition of thiamine at room temperature or during freezing storage in mixed diets. Although the chemistry of sulfite cleavage is well established, the rate-determining factors are still largely unknown. We are investigating the kinetics of the reactions occurring in the presence and absence of casein but are reporting our observations on rate and extent of loss in thiamine because of their importance to other investigators using premixed casein diets, particularly when these are pelletized in the presence of added water.

EXPERIMENTAL

Materials. Several commercial preparations of casein were analyzed for their residual sulfite and thiamine-decomposing action. Only the data on four samples of vitamin-free casein [2,3,4,5] are reported here,

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[1] Calloway, D. H., University of California, Department of Nutrition, Berkeley, unpublished data.

[2] Vitamin-Free Test Casein, Lot no. 661806, was obtained about February 1967 from General Biochemicals, Inc., Chagrin Falls, Ohio.

[3] Lot no. 671943 was obtained from above source about February 1968.

[4] Vitamin-Free Casein, Control no. 8217, was obtained from Nutritional Biochemicals Corporation, Cleveland.

[5] Casein, vitamin free, Lot no. 34065, was obtained from Calbiochem, Los Angeles.

because these would be used in experimental diets. The thiamine hydrochloride was a USP preparation [6] and the sodium metabisulfite a reagent grade chemical.[7]

Methods. Thiamine was determined by the thiochrome method of the Association of Vitamin Chemists (18) omitting the enzyme digestion and column purification steps. Sulfur dioxide determinations were made by both the gravimetric method of Monier-Williams (19) and the colorimetric method described by Nury et al. (20).

To determine the rate and extent of destruction of thiamine by the residual sulfur dioxide present in casein, 20% aqueous suspensions of casein [2] were prepared containing 200 μg thiamine hydrochloride/100 g suspension which had a pH of 5.5. These suspensions were stored both in a water bath at 25°, and frozen at −15°. To prevent microbial growth 100 ppm merthiolate [8] were added to the samples held at 25°. The casein was dispersed in distilled water by shaking in a glass-stoppered Erlenmeyer flask in a shaker for 10 minutes, then thiamine was added, and the suspension brought to weight with water.

Aliquots for residual thiamine and sulfur dioxide determinations were removed periodically during storage for 1 month. The casein suspensions to be frozen were divided into small portions to avoid repeated thawing and refreezing of the whole preparation for each determination.

To establish that the sulfur dioxide present in the casein was responsible for the thiamine destruction, the sulfur dioxide present was oxidized by addition of an equivalent amount of hydrogen peroxide to the casein suspension prior to the addition of the thiamine. The oxidation of the sulfite present was confirmed by analysis. Data were obtained also with a casein free from sulfite.[5]

To determine the effect of casein itself on the rate and extent of thiamine destruction by sulfur dioxide, losses of thiamine were determined under identical conditions but in the absence of casein.

RESULTS AND DISCUSSION

The sulfur dioxide content of several lots of commercially available vitamin-free caseins is shown in table 1. The sulfur dioxide content of casein preparations containing it was found to be surprisingly constant even when the samples were stored in partially filled containers at room temperature for as long as 1 year.

TABLE 1

Sulfur dioxide content of several commercial casein preparations

Name	Sulfur dioxide
	ppm
Vitamin-free test casein	
Lot no. 661806 [1]	400
Lot no. 671943 [2]	1750
Vitamin-free casein [3]	0
Casein, vitamin free [4]	0

[1] Vitamin-Free Test Casein, Lot no. 661806, was obtained about February 1967 from General Biochemicals, Inc., Chagrin Falls, Ohio.

[2] Lot no. 671943 was obtained from above source about February 1968.

[3] Vitamin-Free Casein, Control no. 8217, was obtained from Nutritional Biochemicals Corporation, Cleveland.

[4] Casein, vitamin free, Lot no. 34065, was obtained from Calbiochem, Los Angeles.

Vitamin-free casein obtained from only one source contained appreciable amounts of sulfur dioxide and this varied in different lots tested. Usually sulfur dioxide is not used in commercial production of casein (21, 22), at least not in the United States, but it is possible to precipitate casein from skim milk by sulfurous acid and at least one process for doing so has been patented (23). The casein obtained by precipitation with sulfurous acid would not be expected to vary as widely in sulfur dioxide content as that found for the General Biochemicals preparation.[9] Sulfur dioxide, however, is used also to fumigate casein to prevent insect infestation. Fumigation of casein usually is carried out by using 1 lb. of sulfur dioxide gas/100 cubic feet of storage space.[11] This would result

[6] Thiamine hydrochloride, USP, B grade, Lot no. 53755, was obtained from Calbiochem, Los Angeles.

[7] Sodium metabisulfite, reagent powder, Matheson Coleman and Bell, Norwood, Ohio.

[8] Merthiolate, solution (1:1000), Eli Lilly and Company, Indianapolis, Indiana.

[9] The Technical Services Department of General Biochemicals in a personal communication dated May 1, 1968 admitted that during the processing of casein a compound is added that will decompose to sulfur dioxide. The treated casein is then subjected to repeated washing with alcohol and vacuum-dried. They ascribe the presence of residual sulfite in such casein to reactions with calcium lactate but this has not been confirmed by us.

[10] (Fig. 1.) Sulfur dioxide, 80 ppm, equivalent to the sulfur dioxide content in the casein suspension used.

[11] The Borden Chemical Company 1961 General procedures in manufacture of casein, p. 9 (mimeo circular).

in a level of 400 to 2000 ppm depending on the proportion of space occupied by casein and the absorption of sulfur dioxide by the sacked casein. Since most of the casein marketed in the United States is now imported and standards for casein have not been established, it is likely that fumigation to destroy insects may be practiced as needed on lots of casein imported from abroad. Until 1947, the imports of casein amounted to less than 20% of domestic production but in 1947, imports amounted to over 50% and since 1952 the major supply of casein marketed was imported (21, 24). The present high support for non-fat dry milk solids, insuring much greater revenue from skim milk than from casein, is the primary cause of the present small production of casein in the United States (21, 24). This amounted in 1964 to less than 2 million pounds in comparison with imports of about 110 million pounds from New Zealand, Australia, Argentina, Canada, France and Poland.

The effect of casein on thiamine stability is shown in figure 1. Thiamine losses occurred in samples in which sulfur dioxide was present either in casein used or added as such. The sulfur dioxide-free casein suspensions, obtained by either oxidation of residual sulfite by hydrogen peroxide or by use of caseins free of sulfur dioxide, did not change in thiamine content. An aqueous solution of thiamine also remained unchanged under the conditions of the experiment. It may be assumed, therefore, that the losses of thiamine observed are exclusively due to the residual sulfur dioxide.

The destruction of thiamine by casein containing sulfur dioxide is slowest in frozen suspensions and considerably more rapid at 25°. In aqueous solutions, containing the same level of sulfur dioxide, the destruction is much more rapid. Plots of logarithm of residual concentration of thiamine against time were found to be linear and the destruction thus was first order with respect to thiamine. Farrer (25) also reported that the thermal destruction of thiamine was of first order but increased with increase in pH.

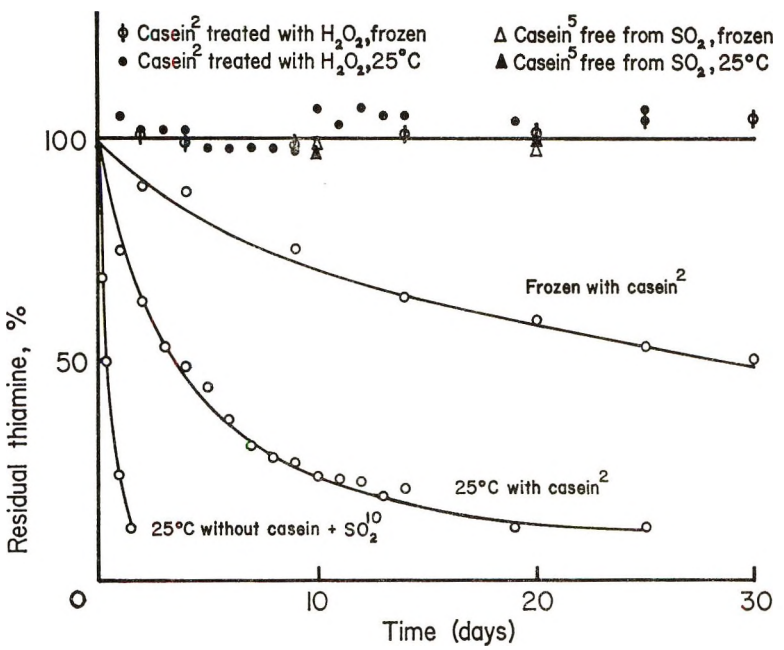

Fig. 1 Rate of change in residual thiamine by sulfur dioxide in presence and absence of casein at 25° and in freezing storage. (Casein was present at a level of 20% by weight, thiamine concentration was 200 μg/100 g solution, and the sulfur dioxide concentration was 8 mg/100 g solution.) See footnote 10 on preceding page.

The rate constants of thiamine destruction by sulfur dioxide under the different conditions used are shown in table 2. The rate constant of thiamine at 25° is about four times that in the frozen suspensions. The rate constant of thiamine loss, however, at an equivalent concentration of sulfur dioxide, in the absence of casein, is significantly greater (15 times). This protective effect of casein may be due to the partial adsorption of sulfur dioxide or thiamine by casein or a combination of both. The nature of the protective effect of casein is being investigated. We have found that when a casein suspension containing thiamine was centrifuged, there was six times as much thiamine present in the sediment as in the supernatant, on a wet weight basis. When the same suspensions were freeze-dried about 100, 80 and 20% of thiamine added was recovered after storage in a desiccator at room temperature for 1, 34 and 78 days, respectively. No change in thiamine content occurred during rapid freezing in solid carbon dioxide-cooled alcohol.

The change in sulfur dioxide content was undetectable both at 25° and in frozen casein suspensions. There was a slight decrease in sulfur dioxide content in aqueous solution, from 80 to 74 ppm over a 2-day period. An appreciable decrease in sulfur dioxide, however, occurred during freeze-drying and storage for 78 days. The sulfur dioxide level decreased from the initial 400 ppm to 300 ppm.

The above findings are in agreement with Calloway's [1] observations on destruction of thiamine in a cooked cornstarch-base diet containing vitamin mixture (equivalent to 0.25 mg thiamine hydrochloride/100 g diet solids) when casein was substituted for part of the cornstarch by adding it, with stirring, to the base, cooling at room temperature, freezing in cottage cheese cartons and storing at −20°. The dry solids content of the final diets was approximately 50%. Destruction appeared to be directly proportional to casein content of the diets (10, 40, and 70% in lieu of cornstarch).

Weanling male rats fed the 70% casein diet showed symptoms of thiamine deficiency after 2.5 to 3 weeks of feeding. These animals recovered uneventfully when given supplementary thiamine added to the thawed diet at the time of feeding. Weight loss also occurred after about 5 to 6 weeks of feeding with the 40% diets. Subsequent analysis of the 40% diets revealed only about 20 μg of thiamine/100 g dry solids; the 10% diets contained about 120 to 180 μg.

When the 10 and 40% diets were essentially dry and stored under refrigeration no loss in thiamine occurred.

TABLE 2

Specific reaction rate constants for thiamine destruction by sulfur dioxide under various conditions [1]

Experimental conditions	Rate constant,[2] k
Thiamine in water at pH 5.5, 25° and −15°	0
Casein [3] + H_2O_2 + thiamine at 25° and frozen	0
Casein [4] + thiamine at 25° and frozen	0
Casein [3] + thiamine, frozen	25×10^{-3} days^{-1}
Casein [3] + thiamine at 25°	104×10^{-3} days^{-1}
Thiamine in water at pH 5.5, 25° and sulfur dioxide (8 mg/ g solution)	1560×10^{-3} days^{-1}

[1] Casein was present at a level of 20% by weight, thiamine concentration was 200 μg/100 g solution, and the sulfur dioxide concentration was 8 mg/100 g solution.

[2] $k = \frac{2.303}{t} \log_{10} \frac{a}{a-x}$

where t = days
a = initial concentration of thiamine
x = amount of thiamine destroyed in time t
(a–x) = concentration remaining after time t.

[3] Vitamin-Free Test Casein, Lot no. 661806, was obtained about February 1967 from General Biochemicals, Inc., Chagrin Falls, Ohio.

[4] Casein, vitamin free, Lot no. 34065, was obtained from Calbiochem, Los Angeles.

In the presence of 1% glycerol as a stabilizing agent in a diet containing 18% casein and 4% USP salts no. 2, 88% of the thiamine was recovered after 1 month of storage in a refrigerator plus 24 hours in a rat feeder at 23°. This, however, was not the case in our freeze-dried casein-thiamine mixture. The source of casein used by Calloway [12] is not known.

In mixed guinea pig diets made with casein containing sulfite and pelletized, the thiamine content was found to be 0.87 mg/kg dry solids when analyzed after 4 weeks storage in a dry condition, in comparison with an initial calculated level of 16 mg/kg, indicating 94% destruction.

It is, therefore, of great importance to bring to the attention of experimental nutritionists the possible presence of sulfur dioxide in some commercially available caseins, and to caution those investigators using premixed wet diets. In addition to thiamine destruction, high levels of sulfur dioxide in the diets may be the source of other adverse effects on the laboratory animals. It is recommended that sulfite-free casein be used and the sulfur dioxide content of caseins be tested before incorporation into a wet mixed diet.

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[12] See footnote 1.

Caloric Intake, Weight Loss and Changes in Body Composition of Rats as Influenced by Feeding Frequency [1]

CLARENCE COHN AND DOROTHY JOSEPH
Division of Nutritional Sciences, Department of Medicine, Michael Reese Hospital and Medical Center, Chicago, Illinois

ABSTRACT The relationship between the effects of caloric intake levels and feeding frequency on body composition was studied during a weight reduction regimen for obese rats. After rats had been made obese by force-feeding excess calories, they were allowed to ingest their food ad libitum or were pair-force-fed against a control. The rats with free access to food ate sparingly until a body weight consistent with their age and sex was achieved; at this time, the food intake increased to approximate "normal" with consequent slow gain in body weight. The force-fed animals not only tended to lose less and regain more weight than their partner eating ad libitum but, in addition, the rats fed by tube ended the experiment containing more body fat and less protein and water. Starvation for 7 days before food availability did not affect final body weight or body composition. When the amount of food eaten was restricted in amount, there was no influence of feeding frequency on the rate of weight loss and on body composition. The results suggest that there is some "threshold" quantity of caloric intake that must be exceeded if changes in body composition, which result from differences in the periodicity of food intake, are to be observed.

Differences in the periodicity of consumption of the diet (feeding frequency and rate of ingestion of the diet) by an animal are accompanied by enzymatic adaptations to the magnitude of the load of nutrients requiring disposition per unit of time (1–3). Under certain circumstances, the results of integrated enzymatic responses and other responses (here called "physiologic" adaptations) also can be seen and measured. Thus, the body of the rat that has been forced to consume "normal" amounts of food divided and ingested twice daily contains more lipid and less protein and water than that of the animal eating the same amount of ration but in muliple small feedings (4). The activities of a number of enzymes, especially those concerned with lipogenesis, reflect the rate of ingestion of the diet (5, 6).

Changes in feeding frequency, however, are not followed invariably by alterations in gross overall "physiology." Although it has been reported that the body composition of rats or farm animals "trained" to eat their food in a limited time period daily may not differ from that of controls eating the same quantity of nutrients in frequent small meals (7–10), it was noted during a period of weight and fat loss that accelerated lipogenesis in vitro was exhibited by the fat tissue of rats that had formerly consumed their day's restricted food intake in a limited time period (11–13). Studies in man comparing the metabolic effects of feeding frequency likewise have yielded divergent results. Hence, early studies suggested marked success with a combination of decreased calorie intake eaten with increased periodicity, as an adjunct to a weight reduction program for humans (14). Subsequent reports however, indicated that with a decreased food intake, the rate of ingestion of the diet plays no role in determining either the rate of weight loss or the composition of the tissues lost (15, 16).

To reconcile the discrepant data, the experiments described below were performed. The results obtained, when correlated with previous findings, suggest that while modifications of intermediary metabolism occur rather regularly with differences in feeding frequency, "physiologic" changes (exemplified by alterations in body composition)

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may not be observed unless the magnitude of the daily caloric load exceeds some "threshold."

METHODS

Male rats of the Holtzman strain, received when 100–120 g in body weight, were used in the studies. During a preliminary period of "overfeeding" they were force-fed a moderate carbohydrate diet (table 1) in excess (100 to 120 kcal/rat/day) for a 2 to 3 month period, at the end of which they weighed between 430 and 530 g. This method of force-feeding has been shown to result in the production of "normal" obese rats (17). Distilled water was freely available to all rats during this period of rapid excessive weight gain, as well as in the experimental ones that followed. The tube-feeding was performed before 8:00 AM and after 4:15 PM.

TABLE 1

Composition of diet

Constituent	%
Cellu flour	4.8
Salt (Wesson)	4.0
Casein hydrolysate (enzymatic)	16.5
Lactalbumin hydrolysate (enzymatic)	2.0
Cornstarch	17.0
Sucrose	17.0
Dextrin	16.0
Corn oil (Mazola) [1]	16.0
Yeast extract powder	3.2
Liver powder	1.6
Methionine	0.3
Wheat germ oil	0.8
Cod liver oil	0.8
2-Methyl-1,4-naphthoquinone	0.008

[1] Corn Products Company, Argo, Illinois.

In subsequent experimental periods of measured food intake, rats were allowed to eat the moderate carbohydrate diet ad libitum in solid form or were force-fed the diet in liquid suspension. Each force-fed animal was pair-fed against a control with free access to food; the latter animals received distilled water by stomach tube twice daily to equalize the handling and stomach distension attendant on tube-feeding. All rats were caged individually and weighed daily.

Four experiments were conducted after the animals were considered to be suitably obese. The details of these studies follow. In all experiments, day 1 refers to the day when "overfeeding" was stopped and controlled feeding instituted.

Experiment 1. On day 1, 10 obese rats were given access to food immediately, 5 under ad libitum and 5 under pair-force-feeding conditions. For the first 7 days of the experiment, 6 additional rats were starved, after which 3 were offered food ad libitum and 3 individually pair-force-fed against their control. The study lasted 61 days; then the rats were killed and each animal was analyzed in toto for fat and water by the method of Mickelsen and Anderson (18).

Experiment 2. Of 12 animals made obese, 4 were killed on day 1 to obtain original values for body water, protein and fat. The two experimental groups of rats consisted of 4 animals with free access to food for a 36-day period and 4 others each of which was pair-force-fed against a control. When killed, the animals were analyzed for total body water, protein and fat (18).

Experiment 3. The methods and protocol were essentially those of experiment 2, except that the study was conducted for 64 days. Of 12 rats that had been force-fed to obesity, 4 animals were killed on day 1, 4 allowed to eat ad libitum and 4 pair-force-fed; total body analyses were performed on all animals.

Experiment 4. Of the 11 obese rats used, 4 animals were killed for body composition studies on day 1; the experimental animals were given a restricted food intake under one of two conditions: 1) three rats were force-fed their food twice daily and 2) four animals were pair-fed the same amount of food in 24 small aliquots 24 times per day on a "feeding wheel" (13) and were tube-fed water twice daily. Under these conditions, all rats received the identical amount of food for a 63-day period when they were killed for measurements of body constituents.

RESULTS

Experiment 1. Figure 1 shows that rats, force-fed to obesity and then offered food immediately ad libitum lost body weight as a result of a subnormal food intake. On achieving a body weight consistent with their age and sex (data derived

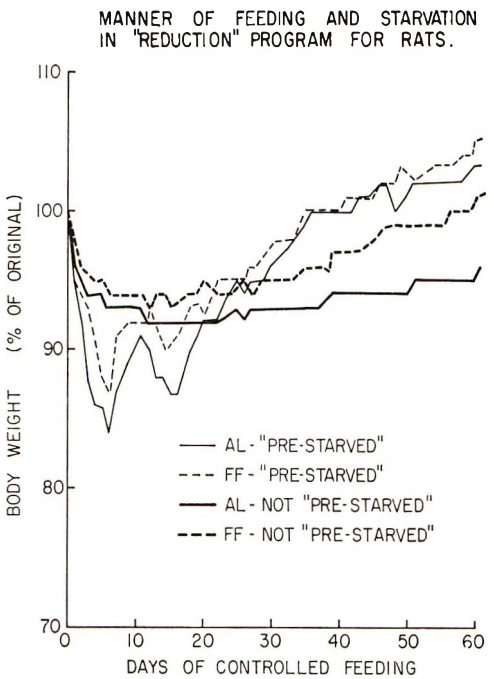

Fig. 1 Body weights, given as a percentage of the body weights on day 1, of obese rats eating ad libitum or pair-force-fed. (Obesity was produced by force-feeding an excess of calories for a 2-month period.) Two groups (not starved) were offered food on day 1; two other groups (pre-starved) were fasted for 7 days before food availability. In this and figures 2 and 3, AL = rats eating ad libitum, FF = force-fed animals.

from both published (17), and unpublished observations on male animals eating the same diet from the age of 2 months to that of 9 months) food intake increased and a weight gain ensued at a rate comparable to that of animals that had not been subject to the "overfeeding" regimen. The body weight curve of the pair-force-fed animals, as might be expected, behaved similarly except that these animals lost less and regained more. Under the conditions of the experiment, as evident in table 2, the rate of ingestion of the diet greatly influenced final body composition; the animals that had had free access to food contained more water and significantly less fat than their force-fed partners. Starvation before controlled feeding was associated with both a greater initial weight loss and a greater subsequent weight gain, but the starvation did not influence the differences in body composition attendant on the subsequent different feeding frequencies. The total food intake over the 61 days of the experiment was not influenced by food deprivation before its availability; the animals starved for 7 days ate an average of 645 g over a 54 day interval and those allowed to eat immediately consumed 654 g over 61 days.

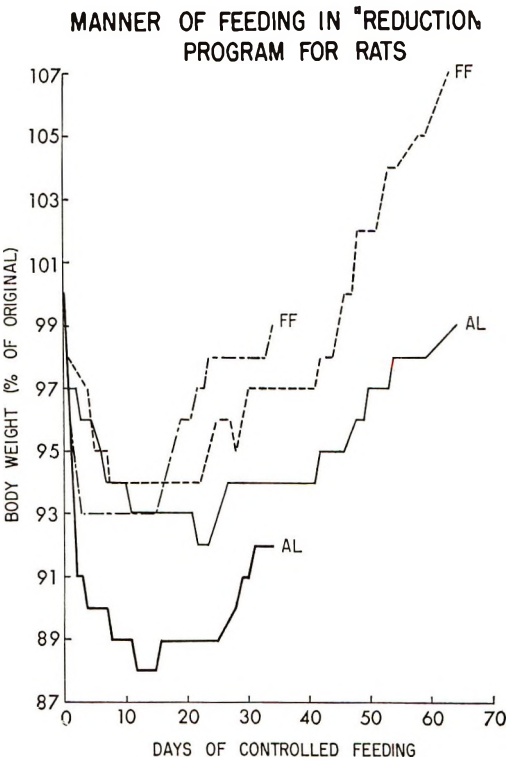

Fig. 2 Body weights of obese rats eating ad libitum or pair-force-fed. Obesity resulted from force-feeding excess calories for a 3-month period, following which the animals were observed for 36 or 64 days.

Experiments 2 and 3. Figure 2 shows that over a 36-day experimental period, obese rats lost more weight and regained less when eating ad libitum than animals ingesting their nutrients on two occasions daily. During the 36 days, the trough of the weight loss occurred at about 12 days, following which the animals gained weight. The periodicity of food intake was reflected in alterations in body composition in that rats with free access to food lost fat pre-

TABLE 2

Influence of periodicity of food intake on body composition of obese rats subject to a weight reduction regimen [1]

	Manner of feeding during weight loss period [2]			
	Ad libitum		Force-fed	
	Pre-starved (3) [3,4]	Not starved (5) [4]	Pre-starved (3) [3,4]	Not starved (5) [4]
Body weight, g				
Original	420	435	430	434
Final	424	418	467	451
Final body composition				
Water,[5] % body wt	61.3 ± 1.1	60.2 ± 0.4	56.3 ± 1.3	57.6 ± 1.1
Fat,[5] % body wt	13.6 ± 1.4	15.8 ± 0.9	23.6 ± 0.8	22.1 ± 1.3

[1] Obesity was produced by force-feeding to excess for a 2-month period.
[2] Measured food intake period was 61 days for the not starved and 54 days for the pre-starved animals. Every force-fed rat was individually pair-fed against an animal with free access to food.
[3] These animals were starved for 7 days prior to food availability.
[4] Number in parentheses refers to the number of animals per series.
[5] Statistical analysis of the data by paired comparison yielded values for the differences between the appropriate groups (ad libitum versus force-fed) of $P < 0.01$ for both water and protein.

dominantly (partially replaced with water and protein) whereas the force-fed animals lost less fat and accumulated less water and protein (table 3). Over the 36 days, an average of 336 g of diet was consumed.

In a similar type of study, increasing the time of observation to 64 days (exp. 3) yielded results that were similar to those seen in the previous study, except that the differences in body composition attendant on the periodicity of food intake became greater (table 4). The animals that had eaten ad libitum weighed the same at the start and end of the study; however, although no change in body weight was noted, a striking rearrangement of body components was observed—a marked loss of body fat and its replacement with water and protein. By contrast, the animals fed by tube gained weight, lost less fat and accumulated less water and protein. In this study, all animals ingested an average of 810 g of diet.

Experiment 4. When the food intake of obese rats was restricted to 263 g over 63 days of feeding, the periodicity of food intake had no influence on either the rate of weight loss or on body composition. The tissues lost under these circumstances were essentially fat, with but minimal amounts of protein being sacrificed (table 5 and fig. 3).

DISCUSSION

The above data demonstrate that, during a weight reduction regimen for obese rats, the magnitude of the load of ingested nutrients and the frequency of its consumption both play a role in the body weight loss and in the alterations in body composition that may ensue. As reported previously (17), and confirmed here, when animals are made obese by the tube feeding of excess calories and are then offered

TABLE 3

Effect of periodicity of food intake on body composition in a weight reduction regimen for obese rats [1]

	Manner of feeding [2]	
	Ad libitum	Force-fed
Body composition		
Original [3]		
Weight, g	478	471
Water, g	216	213
Fat, g	187	184
Protein, g	51	50
Final		
Water, g	264	243
Fat, g	74	115
Protein, g	75	66
Changes [4]		
Weight, g	−36 ± 15.4	−15 ± 6.5
Water, g	+48 ± 8.9	+30 ± 5.3
Fat, g	−113 ± 5.9	−70 ± 3.9
Protein, g	+24 ± 2.4	+16 ± 2.1

[1] Rats were made obese by force-feeding excess calories for a 3-month period.
[2] Each force-fed animal was pair-fed against one with free access to food for 36 days. There were 4 animals/group.
[3] Derived from body composition data of animals killed at start of measured food intake period.
[4] Statistical analyses of the data by paired comparison yielded values for the differences of $P > 0.05$ for water, $P < 0.01$ for fat and $P < 0.05$ for protein.

TABLE 4

Effect of periodicity of food intake on body composition in a weight reduction regimen for obese rats [1]

	Manner of feeding [2]	
	Ad libitum	Force-fed
Body composition		
Original [3]		
Weight, g	486	475
Water, g	233	225
Fat, g	164	161
Protein, g	59	58
Final		
Water, g	290	257
Fat, g	73	142
Protein, g	86	77
Changes [4]		
Weight, g	0 ± 13	+35 ± 9
Water, g	+57 ± 8.6	+29 ± 6.8
Fat, g	−91 ± 6.9	−19 ± 8.6
Protein, g	+27 ± 5.6	+19 ± 0.8

[1] Rats were made obese by force-feeding excess calories for a 3-month period.
[2] Each force-fed animal was pair-fed against one with free access to food for 64 days. There were 4 animals/group.
[3] Derived from body composition data of animals killed at start of measured food intake period.
[4] Statistical analysis of the data by paired comparison yielded values for the differences $P < 0.01$ for water $P < 0.001$ for fat and $P < 0.025$ for protein.

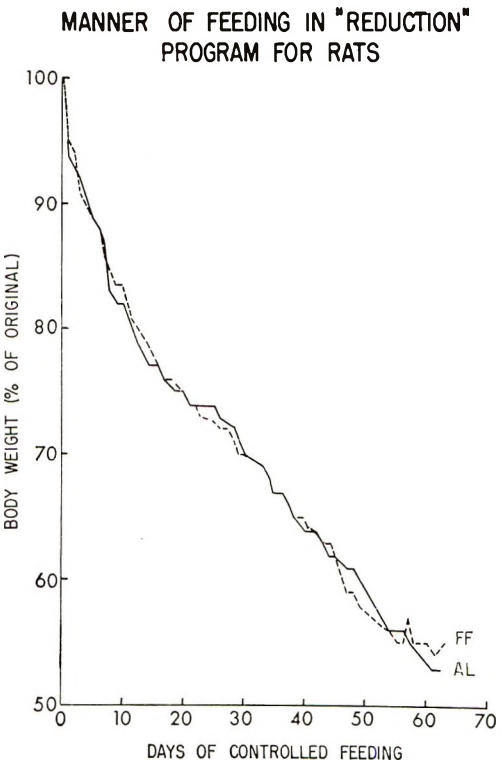

Fig. 3 Body weights of obese rats fed a restricted amount of diet either twice daily or 24 times per day. The total daily food intake of all animals was identical.

nutrients, they voluntarily eat minimal amounts of food until they attain a body weight "normal" for their age and sex. At this time, their food intake increases to approximate the amount eaten by "normal" animals of a similar age and sex. The twice daily force-feeding of parallel quantities of diet to other obese animals results in changes in body composition that conform to previous findings—increased body fat and decreased protein and water (4). The results of the first three experiments are consistent in this respect. When the amount of food eaten was restricted to a quantity less than this "optimal" or "normal" amount (exp. 4), however, the manner of frequency of food ingestion was without effect on both body weight loss and composition of the tissues lost. Findings of a like nature have been noted by Sarett et al. (19) who reported that when obese rats were fed a restricted amount of diet, either ad libitum or alternating 3 days of fasting and 3 days of food availability, the weight loss and body composition changes of all rats were similar.

In an earlier study (4), during a period of growth, rats were force-fed 80% of the amount of food consumed by controls with free access to food. At this subnormal level of nutrition, the force-fed-underfed rats gained weight but less than that gained by the controls. However, the *relative* amounts of water, protein and fat in the new tissue deposited by both groups did not differ; accordingly, total body composition of the animals, calculated on a percentage basis, was the same. Analogous results were obtained during a period of weight loss (exp. 4) when the diet was reduced to approximately 33 to 40% of "normal"; with the ingestion of less than the "optimal" amount of diet, the periodicity of food ingestion was without influence on either weight loss or body composition. Both the relative and absolute losses in body constituents were similar. Hence, during periods of weight gain and weight

loss, there appears to be some minimal load of nutrients (greater than 80% of the "normal" intake) that must be exceeded if changes in body composition, that can be attributed to differences in the rate of ingestion of the diet, are to be manifest.

TABLE 5

Effect of restricted quantity but different feeding frequencies of food intake on body composition in a weight reduction regimen for obese rats [1]

	Manner of feeding [2]	
	Force-fed	Offered food hourly
Body Composition		
Original [3]		
Weight, g	541	546
Water, g	233	234
Fat, g	228	230
Protein,	62	62
Final		
Water, g	200	184
Fat, g	21	31
Protein, g	56	54
Changes [4]		
Weight, g	−246 ± 17.9	−254 ± 6.6
Water, g	−33 ± 6.1	−50 ± 3.6
Fat, g	−207 ± 3.6	−199 ± 0.9
Protein, g	−6 ± 1.7	−8 ± 1.3

[1] Rats were made obese by force-feeding excess calories for a 3-month period.
[2] The group consisted of 4 force-fed animals and 3 fed hourly. All animals received the same total amount of food daily during the 63-day period of controlled feeding.
[3] Derived from body composition data of animals killed at start of measured food intake period.
[4] Statistical analyses of the data revealed no significant differences in the changes of body constituents.

The results obtained allow other conclusions to be reached. With but one exception (20), no one appears to have been able to train rats (and presumably other species) to eat a "normal" amount of food in a restricted time period. Under these conditions, the quantity of nutrients eaten is usually not great enough to exceed the threshold amount apparently required to reveal alterations in body composition. Accordingly, one may be forced to resort to force-feeding if one desires to observe changes dependent on feeding habits. This interpretation of the data may explain some of the discrepancies in the results of different investigators. As a corollary, the results of experiment 4 lend additional support to previous studies suggesting that the body composition changes seen in force-fed animals are not attributable to any "stress" attendant on this feeding procedure, but to the magnitude of the load of nutrients that must be handled per unit of time. Van Putten's (21) studies with hypothalamic hyperphagic rats, which can and do consume "normal" amounts of food in a limited time period, are in accord with this suggestion.

The findings reported here may be important with respect to suggested reducing programs for man. On the basis of a study indicating that starvation is necessary to "de-adapt" enzymes from a "meal-eating" lipogenic pattern (20), Gordon et al. (14) starved his obese humans for several days before placing them on a reduced caloric intake fed 6 times daily. The results of experiment 1, comparing animals subject to starvation before food availability and those not starved suggest that abstinence from food is not only not necessary but may even have adverse effects. Bortz et al. (15) reported that there was no difference in either the rate of weight loss or in urinary nitrogen excretions (which were assumed to reflect the relative loss of body fat or protein) when obese human volunteers ate a 600 kcal diet once or ten times daily. The results of experiment 4 are in complete agreement with the findings of Bortz et al. Hence, both the human and animal studies indicate that with a decreased caloric intake, feeding frequency is without influence on the rate of weight loss or type of tissue lost. Presumably, man, like the rat, will lose fat predominantly under these circumstances.

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Zinc Requirement of Baby Pigs on Casein Diets [1]

S. H. SHANKLIN,[2,3] E. R. MILLER,[2] D. E. ULLREY,[2] J. A. HOEFER [2] AND R. W. LUECKE [4]

Departments of Animal Husbandry and Biochemistry, Michigan State University, East Lansing, Michigan

ABSTRACT In 2 trials to determine their zinc requirement 27 baby pigs were fed diets in which casein supplied the protein. Casein was treated with disodium ethylenediaminetetraacetate to remove zinc and insure a subrequirement dietary level in the first trial. Since deficiency symptoms appeared in pigs on the untreated basal casein diet, in trial 1, this procedure was discontinued in trial 2. Levels of dietary zinc used were 4, 10 and 14 ppm in trial 1 and 10, 14 and 20 ppm in trial 2. All pigs receiving 10 ppm or less of dietary zinc in either trial exhibited symptoms of zinc deficiency, including parakeratotic lesions and a reduction of growth rate, food utilization, serum zinc, calcium and alkaline phosphatase levels, bone size and strength, allometric weight of thymus, tissue zinc level and zinc and calcium balance. The pigs which received 14 ppm or more of dietary zinc in either trial showed no parakeratotic lesions, and rate of growth and food utilization were normal. Pigs receiving 14 ppm of dietary zinc gave evidence of zinc repletion with serum zinc and alkaline phosphatase slowly returning to near normal values for nursing pigs of this age. Values of serum zinc and alkaline phosphatase were more readily restored to normal in pigs receiving 20 ppm of dietary zinc. Zinc retention data indicated that a dietary zinc level of between 14 and 20 ppm was required to maintain total body tissue levels similar to those of the nursing pig.

The zinc requirement of the growing pig fed semipurified rations containing isolated soybean protein has been established by Smith and co-workers (1, 2) at 46 ppm. The zinc requirement of the growing pig receiving semipurified diets containing dried skim milk or casein is less (3) but has not been precisely established. Factors affecting the zinc requirement of growing pigs have been studied, including dietary calcium (4–6), phytate (7), ethylenediaminetetraacetic acid (EDTA) (3), copper (8, 9), histidine [5] and autoclaving of the diet (3, 10). Earlier studies at this laboratory with the baby pig (11) failed to produce specific symptoms of zinc deficiency with purified diets containing vitamin-free casein. Davis et al. (12) and more recently Macapinlac et al. (13) demonstrated that zinc could be effectively removed from protein by treating with the disodium salt of ethylenediaminetetraacetic acid (Na_2 EDTA) and repeated washing with distilled water. This dietary treatment, together with elimination of other sources of zinc contamination, permits the development of extremely low zinc diets.

The purposes of the work reported here were a) to reduce the zinc level of a casein, purified diet sufficiently to produce clear-cut symptoms of zinc deficiency in the baby pig, b) to determine the zinc requirement of the baby pig receiving a purified diet containing casein, and c) to determine the effects of dietary zinc in purified diets containing casein upon zinc, calcium and nitrogen balance in the baby pig.

MATERIALS AND METHODS

Two trials were conducted using 27 Yorkshire-Hampshire crossbred pigs of either sex. Pigs were taken from their dams at 3 to 5 days of age and reared in stainless steel cages as previously reported (11). The purified diet and the methods of adjustment to the diet and zinc depletion were also similar to those described previously (11). At 10 days of age the pigs had become well adjusted to both the basal diet and the environment and were assigned to experimental diets on the bases

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[2] Department of Animal Husbandry.

[3] Present address: A. E. Staley Manufacturing Co., Decatur, Illinois.

[4] Department of Biochemistry.

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of size, sex and litter. Each trial lasted 28 days.

To assure a sufficiently low zinc level in the basal casein diet to produce zinc deficiency symptoms (11), zinc was removed from casein in the first trial by a modification of the method of Davis et al. (12). The vitamin-free or high protein casein was treated by suspending it in deionized distilled water at 120 g/liter. The slurry was stirred mechanically in a plastic container while heating in a water bath at 50°. The pH was adjusted to 4.6, the isoelectric point of the casein, with 0.1 N HCl. Disodium ethylenediaminetetraacetate (Na_2 EDTA) was added at the level of 0.5% of the protein and the mixture was stirred for 30 minutes. The casein was then allowed to settle and the supernatant was siphoned off. The casein was resuspended in deionized distilled water and treated again with Na_2 EDTA as previously described. This procedure was repeated 4 times. The Na_2 EDTA was then removed from the casein by repeated washing with distilled water until the absence of EDTA in the supernatant was indicated by a calcium oxalate precipitate when a drop of a saturated $CaCl_2$ solution was added to 10 ml of supernatant which had been combined with 5 ml of an ammonium oxalate solution and adjusted to pH 11.0 with NaOH. Finally, as much water as possible was removed by filtering, the protein was dried in an oven at 50° and finely ground with a stainless steel grinder before use in the purified diets. The supply of vitamin-free casein was depleted after 2 weeks of the first trial and high protein casein replaced it in all diets for the remainder of the trial. Symptoms of zinc deficiency developed during week 3 in pigs receiving the untreated casein, making treatment of the casein in the second trial unnecessary. Untreated and treated proteins and the diets utilized in the 2 trials were analyzed for zinc concentration and these values are presented in table 1. Samples were dry ashed, the ash taken up with boiling HCl and after appropriate dilution analyzed on an atomic absorption spectrophotometer.[6] Analysis of one sample of casein before and after treating revealed that the Na_2 EDTA had removed 80% of the zinc, 70% of the molybdenum, 80% of the manganese, 85% of the calcium, 90% of the magnesium, 65% of the copper and 50% of the iron.

TABLE 1

Average zinc content of treated and untreated casein and diets

Casein or diet	Zinc
	ppm
Trial 1	
Untreated vitamin-free casein [1]	36
Na_2 EDTA-treated vitamin-free casein diet [2,5]	4
Untreated vitamin-free casein diet [2]	10
Na_2 EDTA-treated vitamin-free casein diet + 10 ppm zinc [2]	14
Untreated high protein casein [3]	28
Na_2 EDTA-treated high protein casein diet [4]	4
Untreated high protein casein diet [4]	10
Na_2 EDTA-treated high protein casein diet + 10 ppm zinc [4]	14
Trial 2	
Untreated high protein casein diet [5]	10
Untreated high protein casein diet + 4 ppm zinc	14
Untreated high protein casein diet + 10 ppm zinc	20

[1] Nutritional Biochemicals Corporation, Cleveland.
[2] Diets for first 2 weeks of trial 1.
[3] General Biochemicals, Inc., Chagrin Falls, Ohio.
[4] Diets for final 2 weeks of trial 1.
[5] Diets used during 5- to 7-day adjustment periods of respective trials.

During both trials blood was withdrawn on 3 occasions (initial, week 2 and final) from the anterior vena cava for the determination of serum zinc, calcium, copper, alkaline phosphatase and serum protein concentrations as well as whole blood measures of hemoglobin concentration, hematocrit and leukocyte population and differential distribution (11).

All pigs in the first trial were group-fed ad libitum for the entire period. Drinking water which had passed through a commercial water softener and which contained no measurable zinc was available ad libitum. In trial 2, all pigs were group-fed ad libitum until skin lesions were observed in the lot receiving the basal diet (10 ppm of zinc) after 18 days on experiment. At this time, all pigs were adapted to individual feeding 3 times daily for a period of 3 days, during which feed and

[6] Jarrell-Ash Model 82-516. Jarrell-Ash Company, Waltham, Massachusetts.

water at each feeding were limited to an amount which was consumed in 10 minutes. Daily food intake was nearly equivalent to that of ad libitum feeding. At the end of this 3-day adjustment period, the pigs were placed in individual stainless steel metabolism cages for a 3-day period of fecal and urinary collections with a constant level of food intake. Pigs were moved from their metabolism cages to the individual rearing cage for each feeding with water and food mixed for rapid and complete consumption. Then their snouts were wiped free of feed and they were quickly returned to the metabolism cages. Urine was collected in 3 N HCl to prevent bacterial contamination or ammonia loss and feces were collected daily, weighed, sampled for nitrogen analysis and the remainder dried at 50°. Total urine volume for each pig was measured, sampled and stored at 3° in acid-washed plastic bottles. Composite dried feces for each pig were weighed, ground and stored in airtight containers. Food, feces and urine were analyzed for nitrogen by the semimicro-Kjeldahl procedure, and for zinc and calcium by atomic absorption spectrophotometry.

At the completion of both trials the pigs were killed to obtain organ and gland weights and tissues for zinc analysis. Two of the deficient animals died before the end of the trial and similar measures were taken at death for these animals. Organ weights were made as in previous studies and compared with body weight (14). Tissues analyzed for zinc included kidneys, liver, heart, thymus, pancreas, skin, hair, testis, bulbourethral gland and complete female genitalia. A modified wet ash procedure (15) was used to digest tissue samples and after appropriate dilution with distilled water, zinc concentration was determined by atomic absorption spectrophotometry. The right femur and right 7th rib were also taken and determinations of weight, specific gravity and zinc concentration were made on both bones. Breaking load of the femur (16) was also determined.

The data were examined by analysis of variance. The statistical significance of treatment differences within each trial was determined by the multiple range test of Duncan (17).

RESULTS AND DISCUSSION

Results of growth, food utilization and blood cellular constituents of pigs in the 2 trials are shown in table 2. Pigs receiving the lowest level of dietary zinc in trial 1 gained weight normally for 1 week, maintained their weight during week 2, then lost weight during the final 2 weeks with all of the pigs succumbing or near death during the final week. Pigs receiving 10 ppm of dietary zinc in either trial gained weight at a normal rate during week 1 and then gained weight at a subnormal rate for the remainder of the trial. Pigs receiving 14 ppm of dietary zinc in trial 1 and 14 or 20 ppm of zinc during trial 2 gained weight at a normal rate throughout the study with a somewhat lesser rate of weight gain in trial 2 during the period of adjustment to individual feeding for the balance study. Food consumption was depressed in pigs of either trial which were receiving levels of 10 ppm or less of dietary zinc. This depression of food consumption was not as severe as the growth depression and consequently resulted in less efficient utilization of food (gain/food). Parakeratotic lesions were apparent during week 2 in all pigs receiving the lowest level of dietary zinc. Lesions became more severe until death or near death during week 4. Less severe parakeratotic lesions became evident during weeks 3 and 4 in all pigs of both trials receiving 10 ppm of dietary zinc. Lesions were not evident in any of the pigs receiving 14 ppm or more of dietary zinc at any time during either trial.

Earlier studies indicated that measures of erythrocyte hematology were not significantly altered by zinc deficiency (11). This was true in these trials as well, although with pigs receiving from 10 to 20 ppm of dietary zinc there was a trend toward a positive relationship between dietary zinc level and either hemoglobin or hematocrit measures. Earlier studies (11) indicated that zinc deficiency produces an alteration in leukocyte differential counts. In trial 1 of the present study leukocyte differential counts made on blood taken 2 weeks after the trial started indicated that zinc-deficient pigs at this age have a re-

TABLE 2

Growth, food consumption and blood analyses of baby pigs

	Trial 1			Trial 2		
Dietary Zinc, ppm	4	10	14	10	14	20
No. of pigs	5	5	5	4	4	4
Initial wt, kg	2.9	2.9	2.9	2.8	2.8	2.8
Daily gain, g	−7	73 [aa]	220 [bb]	95	177 [aa]	200 [aa]
Daily food intake, g	129	224	302	210	230	230
Gain/food	−0.05	0.33	0.73	0.45	0.77	0.87
Pigs with parakeratosis	5	5	—	4	—	—
Blood analyses [1]						
Hemoglobin, g/100 ml	11.5	11.6	11.8	11.9	12.9	14.4
Hematocrit, %	37	36	40	36	41	44
Leukocytes, $10^3/mm^3$	11.3	13.0	15.5 [a]	11.3	10.3	10.8
Lymphocytes, %	27	35	26	45	59	48
Segmented neutrophils, %	22	40 [aa]	60 [bb]	48	33	44
Nonsegmented neutrophils, %	36 [bb]	12 [a]	5	2	2	3
Monocytes, %	11	8	6	4	3	3
Eosinophils, %	0	2	1	1	2	2

[1] From blood samples taken after 2 weeks of trial 1 and after 4 weeks of trial 2.
[a] Significantly greater than least value within the trial ($P < 0.05$). [aa] $P < 0.01$.
[b] Significantly greater than both other values within the trial ($P < 0.05$). [bb] $P < 0.01$.

duced segmented neutrophil count and an elevated band neutrophil count (table 2). In trial 2 there was no significant alteration in the leukocyte differential count even though parakeratotic lesions were evident in all pigs receiving 10 ppm of dietary zinc.

Data from serum analyses are presented in table 3. Measures of serum zinc and alkaline phosphatase reveal that the pigs in trial 1 were quite depleted of zinc stores at the beginning of the trial. This was no doubt due to the use of the zinc-extracted casein in the diet during the adjustment period preceding this trial. Values of serum alkaline phosphatase continued to fall in pigs receiving the lowest level of dietary zinc, becoming extremely low for pigs of this age (18). Serum zinc values continued to fall somewhat further but apparently reached the minimum level necessary to support the life of the pig. Pigs receiving 10 ppm of dietary zinc were scarcely able to maintain their initial levels of serum zinc and alkaline phosphatase. A dietary zinc level of 14 ppm allowed a slow repletion of body zinc, yielding serum zinc and alkaline phosphatase values which approached normal values (18, 19) for nursing pigs of this age. In trial 2 initial serum zinc and alkaline phosphatase values were not nearly as low as in trial 1 because of the use of the untreated casein during the adjustment period. In this trial 10 ppm of dietary zinc failed to maintain initial serum zinc and alkaline phosphatase levels. A dietary zinc level of 14 ppm in this trial, as in the first trial, allowed a slow repletion toward normal values. Pigs receiving 20 ppm of dietary zinc gave evidence of a fairly rapid repletion of the mobilizable body zinc pool. Serum calcium levels were significantly lowered only in the more severely deficient pigs of trial 1. Serum copper, which was measured only in trial 2, was not altered by the level of dietary zinc. Although the ratio of albumin to globulin was decreased by zinc deficiency in this study the effect was not nearly as great as that observed in earlier studies (11) and does not appear to be a very sensitive measure of dietary zinc levels.

Data presented in table 4 verify earlier findings (11) that bone growth in zinc deficiency is affected in direct proportion to the effect upon body growth and that

TABLE 3

Serum analyses of baby pigs

	Trial 1			Trial 2		
Dietary Zinc, ppm	4	10	14	10	14	20
Serum alkaline phosphatase, Bessey-Lowry units						
Initial	2.9	2.9	3.3	10.8	9.1	11.1
2 weeks	0.4	1.0 [a]	4.2 [bb]	1.7	4.3 [aa]	8.2 [bb]
Final [1]	0.6	2.7 [aa]	7.3 [bb]	2.6	6.9 [aa]	10.5 [bb]
Serum zinc, μg/100 ml						
Initial	11	10	19	49	42	42
2 weeks	10	8	28 [bb]	31	35	70 [bb]
Final [1]	6	9	55 [bb]	27	59 [aa]	92 [bb]
Serum calcium, mg/100 ml						
Final [1]	9.5	10.9	13.0 [b]	13.0	13.0	12.8
Serum copper, μg/100 ml [2]						
Initial	—	—	—	179	172	177
2 weeks	—	—	—	187	189	193
Final	—	—	—	185	185	200
Serum proteins,						
g/100 ml [3]	5.0	4.9	4.9	4.8	4.9	5.1
γ-globulin, %	14.1	13.7	14.0	11.4	11.3	11.1
β-globulin, %	10.4	10.6	12.0	12.0	12.3	12.4
α_3-globulin, %	9.9	9.1	7.5	11.4	5.5	7.9
α_2-globulin, %	15.1	14.6	10.1	16.6	13.7	14.8
α_1-globulin, %	7.5	7.3	9.8	8.7	8.1	7.8
Albumin, %	43.0	44.7	46.6	47.5	53.3 [a]	51.9 [a]

[1] Two of the pigs in trial 1 on the lowest level of dietary zinc died during the final week.
[2] Determined only during trial 2.
[3] From blood samples taken after 2 weeks of trial 1 and after 4 weeks of trial 2.
[a] Significantly greater than least value within the trial ($P < 0.05$). [aa] $P < 0.01$.
[b] Significantly greater than both other values within the trial ($P < 0.05$). [bb] $P < 0.01$.

TABLE 4

Bone weight, density, strength and zinc analysis

	Trial 1			Trial 2		
Dietary Zinc, ppm	4	10	14	4	14	20
Bone weight, g						
Femur	19.1	24.3	60.0 [bb]	33.2	48.8 [aa]	50.5 [aa]
Rib	1.51	2.25	5.98 [bb]	3.88	5.29 [aa]	5.34 [aa]
Specific gravity						
Femur	1.14	1.17 [a]	1.19 [b]	1.16	1.18	1.18
Rib	1.22	1.32 [aa]	1.32 [aa]	1.18	1.20	1.23 [a]
Femur breaking load, kg	16	27 [aa]	55 [bb]	30	47 [aa]	48 [aa]
Zinc analysis, ppm of fresh bone						
Femur	57	42	92 [b]	95	92	99
Rib	60	54	46	67	70	94 [b]

[a] Significantly greater than least value within the trial ($P < 0.05$). [aa] $P < 0.01$.
[b] Significantly greater than both other values within the trial ($P < 0.05$). [bb] $P < 0.01$.

breaking strength of bone is directly proportional to bone weight. Pigs receiving 14 ppm or more of dietary zinc had maximum weight, density and strength of bone. Although there was a trend toward higher levels of zinc in bone from pigs receiving the higher dietary zinc levels, the measure does not appear to give consistent results with different bones and does not elicit a sensitive response to level of dietary zinc.

TABLE 5

Relative organ weights and tissue zinc analysis

	Trial 1			Trial 2		
Dietary Zinc, ppm	4	10	14	10	14	20
Body weight, kg	2.1	4.9 [aa]	10.0 [bb]	6.6	9.8 [aa]	10.6 [aa]
Organ weights,% of body weight						
Kidneys	1.00 [aa]	0.98 [aa]	0.58	0.72	0.69	0.61
Heart	0.62 [aa]	0.68 [aa]	0.46	0.58	0.52	0.48
Liver	3.2	4.3 [bb]	2.8	3.1	2.8	2.7
Lungs	1.42 [aa]	1.41 [aa]	1.12	1.25 [a]	1.11	1.04
Stomach	1.19 [aa]	0.96 [a]	0.70	1.07 [b]	0.84	0.87
Pancreas	0.19	0.19	0.20	0.20	0.23	0.24
Thymus	0.03	0.06	0.20 [bb]	0.17	0.25 [a]	0.25 [a]
Spleen	0.08	0.15 [aa]	0.15 [aa]	0.22	0.20	0.18
Thyroid	0.012 [a]	0.013 [a]	0.009	0.012 [bb]	0.007	0.007
Adrenals	0.050 [bb]	0.029 [aa]	0.010	0.020 [b]	0.013	0.014
Testis	0.06	0.11	0.10	0.10	0.09	0.10
Bulbourethral gland	0.03	0.04	0.03	0.04	0.03	0.03
External inguinal lymph nodes	0.12 [aa]	0.16 [aa]	0.04	0.10	0.09	0.10
Zinc analyses, ppm of fresh tissue						
Kidney	14	17	23 [b]	24	26	26
Heart	11	15	22 [b]	20	23	23
Liver	34	24	31	32	37	58 [bb]
Pancreas	29 [a]	20	38 [b]	26	31	31
Thymus	14	18	20 [aa]	21	21	21
Testis	12	15	16 [a]	16	15	17
Bulbourethral gland	23	24	20	14	20	19
Female genitalia [c]	—	—	—	26	27	35
Skin	17	14	28 [bb]	10	11	12
Hair	—	—	—	154	166	195 [a]

[a] Significantly greater than least value within the trial ($P < 0.05$). [aa] $P < 0.01$.
[b] Significantly greater than both other values within the trial ($P < 0.05$). [bb] $P < 0.01$.
[c] Includes vulva, vagina, uterus, ovaries and fallopian tubes.

Allometric and tissue zinc values of organs and glands are presented in table 5. Many of the allometric values of organs and glands in zinc deficiency are due to growth interruptions as observed in other studies (11, 14). These include allometric values of kidneys, heart, liver, lungs, stomach, thyroid, and lymph nodes. The great increase of relative adrenal weight in zinc deficiency is probably a reflection of general physiological stress seen in many nutrient deficiencies (14). The equally great reduction in thymus weight in zinc deficiency may be more specifically related to zinc deficiency (11). A substantial decrease in spleen and testis allometric value was also observed in severe zinc deficiency. None of the allometric data indicate that 14 ppm of dietary zinc in casein diets is inadequate. Tissue zinc concentration of kidney, heart, pancreas, thymus, testis and skin in trial 1 and liver and hair in trial 2 were significantly influenced by dietary zinc concentration.

Data from the balance studies made during trial 2 are plotted in figure 1. Daily zinc retention of pigs receiving 10, 14 or 20 ppm of dietary zinc were 1.8, 3.5 and 5.1 mg. At the time of the collection period pigs receiving 14 or 20 ppm of dietary zinc were increasing in body weight at the rate of about 250 g daily. To maintain an average total body tissue zinc concentration of 20 ppm (20) at this rate of growth a zinc retention of 5 mg daily would be required. This was being achieved by pigs receiving 20 ppm of dietary zinc. Pigs receiving 14 ppm of dietary zinc under these conditions were only retaining a concentration of new body tissue zinc of 3.5 mg ÷ 250 g = 14 ppm. Pigs receiving 10 ppm of dietary zinc met the dilemma of reducing their rate of body weight gain or of diluting their body tissue zinc concentration. Both occurred.

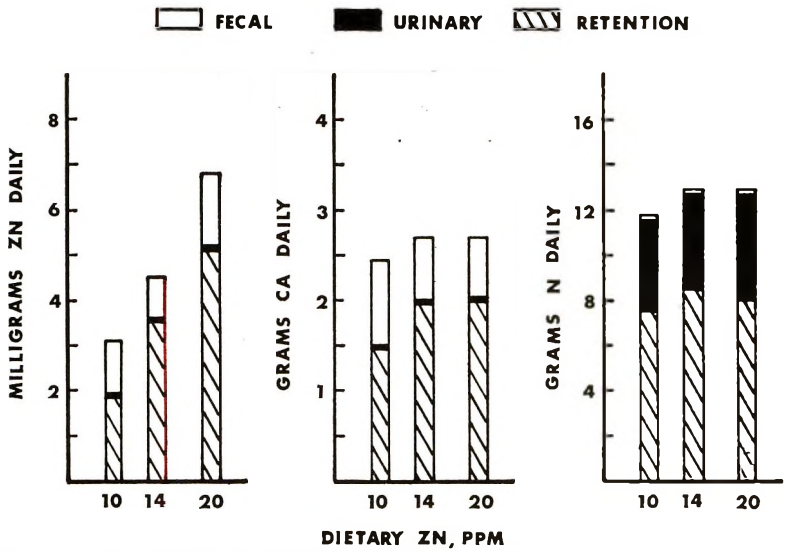

Fig. 1 Zinc, calcium and nitrogen balance as affected by dietary zinc level.

Calcium retention was also significantly less for pigs receiving 10 ppm of dietary zinc. These pigs were retaining only 60% of their calcium intake compared with 75% for pigs receiving 14 or 20 ppm of dietary zinc. Nitrogen balance as measured in this study was not significantly influenced by level of dietary zinc.

It is clear from zinc balance studies that a dietary zinc level of 14 ppm is inadequate to maintain body tissue levels similar to that of nursing pigs (20) but that a dietary zinc level of 20 ppm will provide sufficient zinc retention to meet this criterion. Final values of other criteria of zinc adequacy observed in the pigs receiving 20 ppm of dietary zinc are similar to normal values of naturally reared pigs of similar age. These include measures of growth and serum zinc (19), serum alkaline phosphatase (18), blood hemoglobin (21), blood leukocytes (22), serum proteins (23), tissue zinc (24), organ weights (25) and bone measures (26). Thus, the data presented in this study indicate that the minimum dietary zinc requirement of the baby pig receiving diets in which casein is the source of protein is between 14 and 20 ppm.

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Protein Quality of Wheat and Soybeans After *Rhizopus oligosporus* Fermentation [1]

HWA L. WANG, DORIS I. RUTTLE AND C. W. HESSELTINE
Northern Regional Research Laboratory, Peoria, Illinois [2]

ABSTRACT Protein quality of wheat and soybeans fermented with *Rhizopus oligosporus* has been studied by rat assay methods and amino acid analysis. The fermentation process did not significantly change the essential amino acid composition of wheat or of a mixture of wheat and soybeans. The growth of rats fed fermented wheat improved significantly over those fed unfermented wheat; also the protein efficiency ratio (PER) of wheat was increased by fermentation. These improvements were partly attributed to the increase in availability of lysine in wheat by fermentation. A mixture of wheat and soybeans (1:1) gave a good pattern of amino acids. The mixture as a protein source supported growth well as did casein; also, the fermentation process raised the PER value of the mixture so that it was comparable to casein.

Soybeans have been an important source of protein in Asia for centuries. To improve their flavor, and perhaps also their nutritional value, soybeans are often converted into various food products by fermentation with molds or bacteria. The nutritional value of those fermented foods, however, has not been adequately investigated. In recent years, numerous studies have been made on an Indonesian soybean product, tempeh, which is prepared by fermenting soybeans with a species of *Rhizopus*. These studies (1–6) revealed that fermentation increased total soluble solids, vitamins, free fatty acids, soluble nitrogen, and free amino acids while total nitrogen and amino acid composition remained fairly constant. Results from rat growth experiments (4, 7), however, indicated that fermentation neither increased the growth rate of rats nor improved the protein efficiency ratio (PER) and digestibility of soybeans.

Wheat alone and wheat combined with soybeans were also reported as suitable substrates for preparing tempeh-type products by *Rhizopus oligosporus* NRRL 2710 (8, 9). A study was undertaken, therefore, to evaluate the effects of fermentation on the protein quality of wheat only and wheat combined with soybeans.

EXPERIMENTAL METHODS

Preparation of fermented samples. Pure-culture fermentation was carried out in shallow perforated trays as described by Martinelli and Hesseltine (10). Wheat and soybeans were slightly cracked, washed, and boiled with excess water. The boiling time required for wheat was 12 minutes; for soybeans, 25 minutes (9). The cooked grains were well drained, cooled to room temperature, inoculated with a spore suspension of *R. oligosporus* NRRL 2710, and incubated at 31° for 24 hours. After incubation, the products were steamed for 5 minutes to destroy the mold, then freeze-dried, and ground with a Wiley [3] mill. When wheat and soybeans were combined for fermentation, equal weights of each grain were boiled separately and then mixed. Control samples of wheat, soybeans, and a mixture of wheat and soybeans were treated similarly except that the inoculation step was omitted.

Biological evaluation of protein quality. Protein was evaluated according to AOAC Official Methods of Analysis (11). Each sample to be evaluated was incorporated into a basal diet to provide a 10% level of protein (table 1). On the basis of proximate analysis, the diets were equalized

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[2] This is a laboratory of the Northern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

[3] The mention of firm names or trade products does not imply that they are recommended by the U. S. Department of Agriculture over other firms or similar products not mentioned.

TABLE 1
Protein evaluation diet

Components	*g/100 g diet*
Casein or sample	A to provide 10 g of protein
Cottonseed oil	$8 - \frac{A \times \%\ \text{ether extract}^3}{100}$
Salt mixture [1]	$5 - \frac{A \times \%\ \text{ash}}{100}$
Vitamin mixture [2]	1
Cellulose	$1 - \frac{A \times \%\ \text{crude fiber}}{100}$
Water	$5 - \frac{A \times \%\ \text{moisture}}{100}$
Sucrose	To make 100

[1] Percentage composition of the salt mixture was: NaCl, 13.93; KI, 0.079; KH_2PO_4, 38.91; $MgSO_4 \cdot 7H_2O$, 5.73; $CaCO_3$, 38.14; $FeSO_4 \cdot 7H_2O$, 2.70; $MnSO_4 \cdot H_2O$, 0.40; $ZnSO_4 \cdot 7H_2O$, 0.055; $CuSO_4 \cdot 5H_2O$, 0.048; and $CoCl_2 \cdot 6H_2O$, 0.002.

[2] One gram of vitamin mixture provided the following vitamins: (in mg) vitamin A (dry, stabilized), 2000 (IU); vitamin D (dry, stabilized), 200 (IU); vitamin E (dry, stabilized), 10 (IU); menadione, 0.5; choline, 200; p-aminobenzoic acid, 10; inositol, 10; niacin, 4; Ca D-pantothenate 4; riboflavin, 0.8; thiamine·HCl, 0.5; pyridoxine·HCl, 0.5; folic acid, 0.2; biotin, 0.04; vitamin B_{12}, 0.003; and dextrose to 1 g.

[3] All percentage figures referred to the proximate composition of the sample.

with respect to moisture, fat, ash, and crude fiber. All diets were supplemented with vitamins and minerals known to be adequate for the rat. Groups of 10 male weanling rats of the Sprague-Dawley strain were then fed the appropriate diet and water ad libitum for 4 weeks. The animals were housed individually in cages with a metal-screen bottom. In all the studies casein was used as a reference for comparison. The feeding experiments were conducted for us by the Wisconsin Alumni Research Foundation.

Amino acid analysis. All analyses were performed in duplicate on homogenized samples combined from at least 6 fermentation trays. The samples were defatted by ether extraction. Defatted samples containing approximately 100 mg of protein were hydrolyzed for 24 hours under reflux with 250 ml of constant boiling hydrochloric acid. The acid was removed under vacuum to near dryness with a rotatory evaporator, followed by evaporation with 3 small volumes of added water. The humin was then removed by centrifugation. The clear supernatant was made up to 50 ml with pH 2.2 sodium citrate buffer, and the samples were kept in a frozen state until analyzed. Cystine was analyzed by conversion to cysteic acid as described by Moore (12). Since tryptophan was destroyed by acid hydrolysis, it was determined by 5 N sodium hydroxide hydrolysis of samples in a sealed tube for 20 hours at 100°.

Pepsin and pancreatin digestion (13) was prepared by incubating samples containing 100 mg protein with 1.5 mg pepsin in 15 ml of 0.1 N hydrochloric acid at 37° for 3 hours. The digestion mixtures were then neutralized with 7.5 ml of 0.2 N sodium hydroxide and incubated for an additional 24 hours after the addition of 4 mg pancreatin in 7.5 ml of pH 8.0 phosphate buffer. The undigested proteins and larger peptides were removed by picric acid.

All amino acids, except tryptophan, were analyzed with a Beckman Spinco amino acid analyzer (14). Tryptophan was assayed by a microbiological method (15).

TABLE 2
Effect of fermentation on the proximate composition of wheat and soybeans

Grain	Ash	Ether extract	Protein	Fiber	Carbohydrates
	%	%	%	%	%
Wheat, control	1.7	1.9	17.4	2.6	76.5
Wheat, fermented [1]	1.8	2.0	18.2	3.1	74.9
Soybeans, control	3.4	26.8	47.8	3.9	18.1
Soybeans, fermented	3.3	24.7	48.1	3.1	20.9
Wheat + soybean (1:1) control	2.5	12.5	31.6	2.8	50.7
Wheat + soybean (1:1) fermented	2.6	12.1	33.1	2.7	49.6

[1] Fermentation time with *Rhizopus oligosporus*: 24 hours.

RESULTS AND DISCUSSION

Proximate compositions of wheat and soybeans after R. oligosporus fermentation. As shown in table 2, the fermentation process did not greatly affect the proximate composition of wheat and soybeans. It did increase slightly the percentage of protein of both wheat and soybeans, although it had been previously reported (1, 8) that the total protein of wheat and soybeans remained the same after fermentation. Therefore, this increase in the percentage of protein reflects the decrease of other constituents which the mold might have consumed for growth. The decrease of carbohydrates in wheat after fermentation suggested that the mold had used the carbohydrates as an energy source. On the other hand, *R. oligosporus* is unable to use the carbohydrates present in soybeans (16); instead the mold easily uses the oil as its energy source. Our data, which indicate an increase in carbohydrates and a decrease in ether-extractable substances of soybeans after fermentation, further substantiate previous findings.

Nutritional value of fermented wheat and soybeans. Data on rats fed diets containing fermented or unfermented grains as the protein source are summarized in table 3. Weight gain and food consumption for rats receiving a diet containing fermented wheat were significantly increased over the wheat control group, whereas the weight gain of rats fed diets containing fermented soybeans was not significantly different from the gain of rats on the control diet, but food consumption was less than for the control group. Fermentation also affected the PER of wheat and soybeans differently. The PER of wheat was significantly increased by fermentation (1.71 versus 1.28) whereas that of soybeans remained the same (2.27 versus 2.17). The results on soybeans reported here support previous findings by Hackler et al. (7) and Smith et al. (4).

As a protein source the mixture of wheat and soybeans, either fermented or unfermented, improved significantly both growth response and PER values over either wheat or soybeans alone. Growth response of the rats receiving diets containing the mixture was comparable to that of a diet containing casein as a protein source. The PER value of the unfermented mixture, however, was lower than casein (2.49 versus 2.81), but fermentation increased the value to 2.79, equaling that of casein.

Fermentation time and nutritional value of wheat. Wheat was allowed to ferment for 12, 24, 48 and 72 hours; the products were then incorporated into rations as a protein source to determine whether the duration of incubation affects the protein quality of wheat. The data (table 4) indicate that there was no significant effect on the nutritional value of wheat at 12 hours of fermentation. As the fermentation time was increased to 24 hours, a significant increase in body weight and PER over that of the control was observed. No further improvement in PER was noted, however, when the wheat was allowed to ferment more than 24 hours. On the other hand, the weight gain continued to increase for

TABLE 3

Weight gain, food consumption and protein efficiency ratio of rats fed fermented or unfermented grains as protein source

Protein source	Weight gain	Food consumption	PER
	g	*g*	*g wt gain/g protein consumed*
Casein	98.0 ± 6.6 [1]	347 ± 13 [1]	2.81 ± 0.10 [1]
Wheat, control	37.6 ± 2.7	295 ± 13	1.28 ± 0.05
Wheat, fermented	55.0 ± 1.6 [2]	322 ± 7 [2]	1.71 ± 0.05 [2]
Soybeans, control	76.5 ± 2.3	353 ± 10	2.17 ± 0.03
Soybeans, fermented	72.9 ± 3.3	321 ± 12 [2]	2.27 ± 0.05
Soybeans and wheat, control	97.1 ± 3.2	389 ± 8	2.49 ± 0.04
Soybeans and wheat, fermented	94.2 ± 2.2	338 ± 12 [2]	2.79 ± 0.04 [2]

[1] Standard error of mean.
[2] Significantly ($P < 0.05$) different from corresponding unfermented grain.

animals fed 48-hour fermented wheat, but was less for animals fed the 72-hour fermented product. This difference in weight gain probably reflected food consumption, because the PER values were the same. The length of fermentation time is known to modify the flavor and taste of the product. Our data indicate that both the 24-hour and 72-hour fermented products were not as acceptable to rats as the 48-hour fermented product. On the basis of the PER value and growth of the rats, the optimal fermentation time for wheat by *Rhizopus* was 48 hours.

Changes in amino acid composition after fermentation. The amino acid compositions of wheat and of a mixture of wheat and soybeans before and after 24 hours of fermentation are shown in table 5. In general, the amount of most amino acids was not significantly changed by fermentation; this finding is in agreement with results on soybean fermentation reported by Stillings and Hackler (5), Smith et al. (4) and Murata et al. (6). Apparently, the mold does not depend upon any specific amino acid for growth as suggested by Sorenson and Hesseltine (16).

TABLE 4

Influence of fermentation time on protein quality of wheat

Time	Weight gain	Food consumption	PER
hr	*g*	*g*	*g wt gain/g protein consumed*
0 (control)	29.3 ± 1.7 [1]	235 ± 13 [1]	1.25 ± 0.07 [1]
12	28.9 ± 2.3	225 ± 9	1.28 ± 0.05
24	40.7 ± 2.1 [2]	228 ± 9	1.78 ± 0.07 [2]
48	49.5 ± 2.2 [2]	269 ± 11 [2]	1.84 ± 0.04 [2]
72	39.4 ± 3.0 [2]	226 ± 11	1.73 ± 0.06 [2]

[1] Standard error of mean.
[2] Significantly ($P < 0.05$) different from control.

TABLE 5

Effect of fermentation on the amino acid composition of wheat and of a mixture of wheat and soybeans (1:1)

Amino acid	Wheat		Wheat and soybeans		Mycelium [1] of *R. oligosporus*
	Control	Fermented	Control	Fermented	
	g/16 g N	*g/16 g N*	*g/16 g N*	*g/16 g N*	*g/16 g N*
Lysine	2.69	2.80	4.82	5.26	4.07
Histidine	2.02	1.90	2.15	2.24	1.52
Arginine	4.17	3.83	5.70	4.50	2.47
Aspartic	4.27	4.25	8.00	8.30	4.82
Threonine	2.70	2.70	3.16	3.40	2.56
Serine	4.23	3.86	4.32	3.81	2.95
Glutamic	30.15	26.22	21.08	14.82	4.72
Proline	10.17	9.50	6.63	3.72	1.96
Glycine	3.57	3.36	3.56	3.46	2.68
Alanine	3.15	3.79	3.53	5.28	2.77
Cystine	2.31	2.33	1.83	1.92	1.03
Valine	3.88	4.00	4.30	4.34	3.41
Methionine	1.68	1.62	1.58	1.58	0.89
Isoleucine	3.53	3.47	3.96	4.02	3.22
Leucine	6.36	6.06	6.63	6.03	4.61
Tyrosine	2.89	2.75	3.12	3.20	1.85
Phenylalanine	4.44	3.85	4.59	3.61	2.89
Tryptophan	0.80	0.75	0.85	0.85	0.50
Total amino acids (T)	93.01	87.04	89.81	80.34	48.92
Total essential (E) amino acids	31.28	30.33	34.84	34.21	25.03
E/T	0.34	0.35	0.39	0.43	0.51

[1] Source: Stillings and Hackler (5), p. 1044.

However, a significant change was noted for the fermented mixture of wheat and soybeans, glutamic acid decreased by 30% and alanine increased by 50%. The conversion of glutamic acid to alanine by the action of glutamic-pyruvic transaminase could, in part, account for these changes. If so, the mold not only synthesized the glutamic-pyruvic transaminase, but also elaborated a greater amount of this enzyme when the mold was grown on a soybean substrate rather than on a wheat substrate. Since the mold cannot use the carbohydrates present in the soybeans (16), the amount of pyruvic acid as acceptor is limited when the mold is grown on soybeans. Consequently, the changes in glutamic acid and alanine during either soybean (4–6) or wheat fermentation are not as significant as during fermentation of the mixture. Further studies are needed to demonstrate the elaboration of glutamic-pyruvic transaminase by *R. oligosporus*.

As indicated in table 5, the mycelium of *R. oligosporus* has a much lower amino acid content per 16 g N and a higher ratio of essential amino acids to total amino acids (E/T) than does wheat or a wheat-soybean mixture. The amount of mycelial protein present in the fermented products was perhaps not high enough to alter greatly the amino acid composition of the substrates. Nevertheless, an overall result showed a slight decrease in total amino acids and an increase in E/T of both fermented wheat and fermented wheat-soybean mixture as compared with their respective controls. An increase in E/T of protein can affect the growth rate of the animal and PER value of the protein, especially when rats are fed diets containing a marginal level of protein as in this study.

Our data also suggest that the significant improvement of the PER value of the wheat-soybean mixture over that of wheat can be explained by the complementary effect of a deficient amino acid (i.e., 4.82 g lysine/16 g N in the wheat-soybean mixture as compared with 2.69 g lysine/16 g N in wheat) and also by the increased E/T ratio, 0.34 for wheat versus 0.39 for the wheat-soybean mixture and 0.43 for the fermented mixture. Furthermore, the proportion of each essential amino acid to total essential amino acids in the wheat-soybean mixture closely resembles that in cow's milk (table 6).

Essential amino acids released from wheat and fermented wheat by pepsin-pancreatin digestion in vitro. The increase in the PER value of wheat after fermentation cannot be explained by the amino acid composition, since it was not significantly changed. The effect of fermentation on the availability of the individual amino acids in the protein to the animal was then determined in vitro by pepsin-pancreatin digestion. These studies

TABLE 6

Essential amino acid patterns

Amino acid	Amino acid/total essential amino acids				
	Wheat		Wheat and soybeans		Cow's milk [1]
	Control	Fermented	Control	Fermented	
	mg/g	*mg/g*	*mg/g*	*mg/g*	*mg/g*
Lysine	85	92	138	154	155
Threonine	86	89	91	99	91
Total aromatic amino acids	234	218	222	199	197
Phenylalanine	142	127	132	106	97
Tyrosine	92	91	90	93	100
Total sulfur-containing amino acids	128	130	98	101	65
Cystine	74	77	53	56	17
Methionine	54	53	45	45	48
Valine	124	132	123	127	137
Isoleucine	113	114	114	118	127
Leucine	203	200	190	176	196
Tryptophan	26	25	24	25	28

[1] Source: Report of a Joint FAO/WHO Expert Group on Protein Requirement. Rome, 1965.

TABLE 7
Effect of fermentation on essential amino acids released by pepsin and pancreatin digestion of wheat

Amino acids [1]	Control	Fermented	Total essential amino acids	
			Control	Fermented
	mg/g N	*mg/g N*	*mg/g*	*mg/g*
Lysine	94	118	119	137
Histidine	17	25	22	29
Threonine	136	134	173	156
Valine	57	55	72	64
Total sulfur-containing amino acids	62	71	80	83
Isoleucine	54	62	69	72
Leucine	179	199	229	231
Total aromatic amino acids	186	197	237	228
Total essential amino acids	785	862		

[1] Tryptophan was destroyed during the picric acid procedure and not determined in the enzyme hydrolysates.

(table 7) indicate that the total essential amino acids released from wheat by enzymatic digestion increased about 10% after 24 hours of fermentation. Among the essential amino acids, lysine and histidine increased proportionally more than the other amino acids: 119 to 137 mg lysine/g total essential amino acids, and 22 to 29 mg histidine/g total essential amino acids. Possibly the proteolytic enzyme systems produced by the mold (17) attacked the protein in such a way that more lysine and histidine could be made available by the digestive enzymes, pepsin and pancreatin, of the animals. Since lysine is considered a limiting amino acid of wheat protein, the increase in available lysine would be nutritionally significant and can, in part, account for the increased PER value of wheat by fermentation.

ACKNOWLEDGMENT

We are indebted to James F. Cavins for the amino acid analyses.

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Chemical and Morphological Changes in the Brains of Copper-deficient Guinea Pigs [1,2]

GLADYS J. EVERSON, RUTH E. SHRADER AND TONG-IN WANG
Department of Nutrition, University of California, Davis, California

ABSTRACT The histological findings of the brain, cord, and nerve of copper-deficient guinea pigs at birth and the distribution of phospholipids in a small number of control and copper-deficient animals at birth and 63 days of age were investigated. Cerebellar folia were missing or malformed in some of the copper-deficient animals at birth and throughout the brain there was underdevelopment of myelin. Phospholipid determinations of whole brain supported conclusions established by myelin staining that there was delayed myelination present. The findings have been compared with the syndrome known as hypomyelinogenesis congenita and with the classical description of swayback in lambs.

Symptoms of copper deficiency in the guinea pig include retarded growth, reduced elastin content of aortic tissue with a high incidence of aneurisms and coincident internal hemorrhages, changes in texture and pigmentation of the hair, ataxia, and gross brain abnormalities (1). These neonatal defects were the result of a maternal shortage of copper during gestation. The problem of anemia is less acute in this species and has not been responsible for neonatal deaths in copper-deficient young.

It is the purpose of this paper to report the histological picture of the brain, cord, and nerve tissue of animals described earlier (1) and to relate these observations to those described in the disorder, swayback, found in lambs. Information will also be given concerning preliminary studies on the composition of phospholipids in whole brain of copper-deficient and control guinea pigs during early postnatal growth.

MATERIALS AND METHODS

Histological studies. Brain, spinal cord, and sciatic nerve were removed at autopsy from neonatal guinea pigs born to females fed diets containing either 0.5 to 0.7 ppm or 6.0 ppm copper throughout pregnancy (1). One-half of the brain cut sagittally was stored in glass vials at −4° to be used for phospholipid analyses and the contralateral portion was used for histological examination. Tissues for histology were fixed in 10% neutral formalin for at least 2 weeks before processing. Dehydration in ethanol was followed by clearing in xylol and embedding in paraplast. Tissues were sectioned at 15 μ and were stained with hematoxylin and eosin. Ora's stain (2) and Lockard and Reer's (3) modification of the Luxol fast blue stain were used for demonstration of myelin. The Nauta and Gygax method (4) and the Margolis and Pickett technique (5) were used to determine the presence of degenerating myelin. Cresylecht violet was used to identify Nissl substance. Romanes' silver method was used to study the axon fibers (6). Tissues from 5 newborn young of females fed the low copper ration and 4 young of females fed the control ration were used for detailed histological studies.

Phospholipid determinations. Brain samples from 20 guinea pigs chosen from copper-deficient and control-ration groups were used to investigate the distribution of phospholipids in whole brain at birth and at various intervals up to day 63. The copper-deficient animals included in this work represented the most severely affected young available, based on the degree of stunting of growth, depigmentation and texture changes of hair, and the presence of ataxia at birth.

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[2] This paper includes portions of a thesis submitted to the Graduate Division, University of California (Davis Campus) by Tong-In Wang in partial fulfillment of the requirements for the M.S. degree.

A 15-fold volume of chloroform : methanol (2:1 v/v) was added to the partially defrosted brain samples and the tissue was homogenized at high speed in a Virtis "45" homogenizer.[3] The homogenate was further diluted with chloroform : methanol, equal to a 20-fold dilution of tissue, and duplicate aliquots were taken for moisture determinations. A 20-ml sample was used for lipid analysis. Following centrifugation, the supernatant layer was collected and the residue thoroughly extracted with 10-ml portions of chloroform : methanol, 2:1; 1:2; and 7:1. Aliquots of the combined extracts were evaporated to dryness under nitrogen. The dry crude lipid extract was dissolved in 10 ml of chloroform : methanol : water (60:30:4.5) and the preparation was purified by passing the sample over a 4.6 cm layer of Sephadex according to directions of Wells and Dittmer (7). The collected purified lipid preparation was diluted to provide approximately 4 μg lipid/μl of solution.

Two-dimensional thin-layer chromatography was used for separation of the phospholipids. The absorbent was prepared by mixing 9 parts of silica gel H with 1 part finely powdered magnesium silicate[4] (8). Solvent systems described by Rouser et al. (9) were used without modification. Chloroform : methanol : concentrated ammonium hydroxide, 65:35:5, was used for the first separation. After the plates were dried in a hood for approximately 10 minutes they were put into a second chamber containing chloroform : acetone : methanol : acetic acid : water in the proportions of 5:2:1:1:0.5. Two-dimensional chromatograms were prepared in duplicate for each sample. Phospholipid spots were detected with iodine vapor and encircled with a fine pointed dissecting needle. After much of the iodine had evaporated the spots were removed with a razor blade and individual phospholipids were collected into glass centrifuge tubes. Samples were eluted from the silica gel following the method of Skipski et al. (10). Phosphorus was determined by the method of Chen et al. (11), with modifications in the digestion procedure (12). Aliquots of the purified lipid extract were applied to plates and removed and the total phosphorus recovered was assigned a value of 100%. The distribution of individual phospholipids in whole brain, expressed as percent of total lipid phosphorus was calculated using the customary factor of 4 to represent the percent phosphorus present in each class of phospholipids.

RESULTS

Histological findings. The gross appearance and general characteristics of the brains of the newborn copper-deficient guinea pigs have been previously described (1). The cerebral hemispheres showed reduced surface vascularity and fewer fissures and surface gyri. The brain tissue appeared slightly translucent. Some of the cerebellar folia were missing from the brains of 4 of the low copper young examined, and the defective area was occupied by a fluid-filled vesicle. Of the cerebellar folia, the lingula centralis, nodules and pyramis and a portion of the culmen were found to be present in each case, and to be of sufficient size to be identified grossly. The declive, vermis, and most of the culmen were malformed in all 4 defective animals with the degree of abnormality varying to some extent. Figure 1 shows the normal cerebellar tissue of the newborn guinea pig at 17× magnification. Figures 2 and 3 show the characteristic cerebellar tissue of 2 defective copper-deficient young. Because of the small size of the cerebellar remnants these illustrations were enlarged to 36 diam. Some of these cerebellar folia show no central fibers and the cell strata are difficult to visualize although all cellular elements appear to be represented. Figures 4 and 5 are high power illustrations of cerebellar folia in control and copper-deficient brains to demonstrate the comparative morphology of the cells and fiber tracts. In the deficient folia shown here the architecture approaches the normal picture and the customary subpial granular layer, molecular, Purkinje cell and inner granular layers are identifiable. Central fibers in these folia are present but minimal in number and deficient in myelin. These folia also show some reduction in density of cells and fibers and there

[3] The Virtis Company, Inc., Gardiner, New York.

[4] Allegheny Industrial Chemical Corp., Butler, New Jersey.

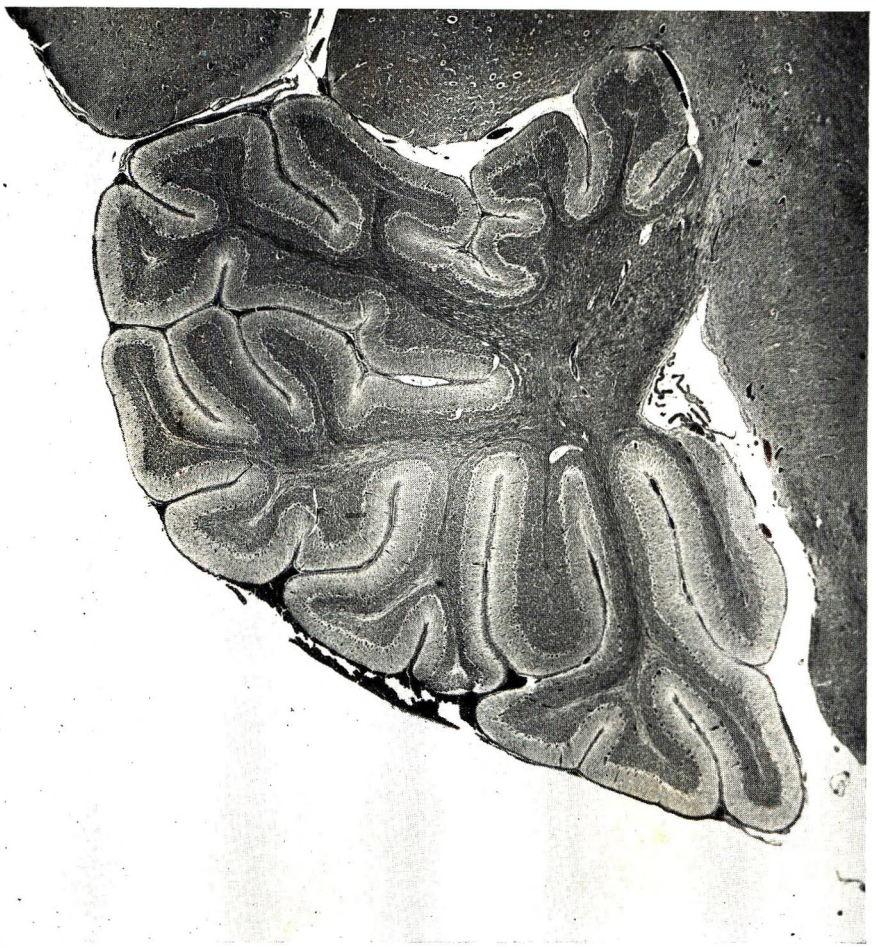

Fig. 1. Cerebellum and part of the medulla of a control newborn guinea pig brain illustrating normal morphology. Luxol fast blue × 17.

appears to be a diffuse interstitial edema. The cells throughout the cerebellum stained poorly in the copper-deficient animals. Some deep cerebellar nuclei were present but owing to the derangement of structure in this area it was not possible definitely to identify these roof nuclei. The band of tissue enclosing the fluid-filled cavity (figs. 2 and 3) consisted of loosely organized ependymal tissue and some compressed neural elements consisting for the most part of glial cells and a few Purkinje cells. No marked gliosis, satellitosis, inflammatory reactions, perivascular cuffing or necrotic debris was observed in the walls or lumen of the intracerebellar cavity. No connection could be traced between these vesicular spaces and the floor of the fourth ventricle.

The midbrain and cerebral cortex showed a marked reduction in myelin in the copper-deficient brains (figs. 6 and 7). All tracts appeared to be present but to have diminished amounts, or to be completely lacking, in myelin. The fibers of the anterior and posterior commissural bands are sparsely myelinated. This was in striking contrast to the complete absence of myelin in the fibers of the corpus callosum. The fibers of the fasciculus gracilis were not myelinated whereas some myelin was present in the pyramidal tract and decus-

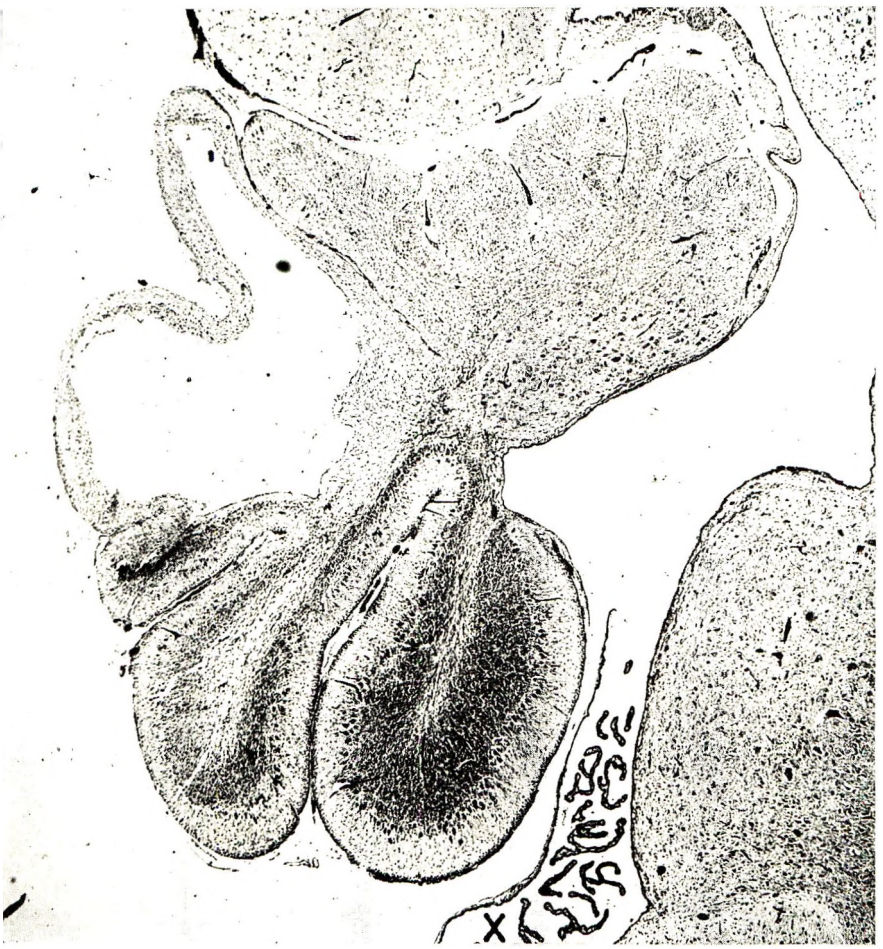

Fig. 2 Cerebellar structure at midline of an abnormal brain from a newborn low copper guinea pig. Luxol fast blue × 36. Choroid plexus marked X.

sating pontine fibers. Also myelin was decreased in amount in the first portion of the cervical cord but was present normally in the thoracic and lumbar segments. Peripheral and spinal nerves also appeared to be myelinated normally in these animals. There was moderate distention of the lateral cerebral ventricles (fig. 7) and the vessels of the choroid plexus appeared to be prominent (figs. 2 and 3). It was not possible to determine whether these vascular structures were hypertrophied and there was no evidence of thickening of the vascular walls or of any type of exudate in relation to the vascular bed.

The cells of the cerebral cortex, midbrain, brain stem nuclei and cord appeared to be normal in structure and to contain normal amounts of Nissl substance and no nuclear aggregations or cell layers were obviously absent or reduced in number.

Distribution of phospholipids in whole brain. Information on brain weight, moisture content, percent of lipid present, and the percent of phosphorus found in the lipid fraction of whole brain is given in table 1. The most striking difference noted between control and copper-deficient animals was the rise in the percent of phosphorus present in the lipid fraction of brain

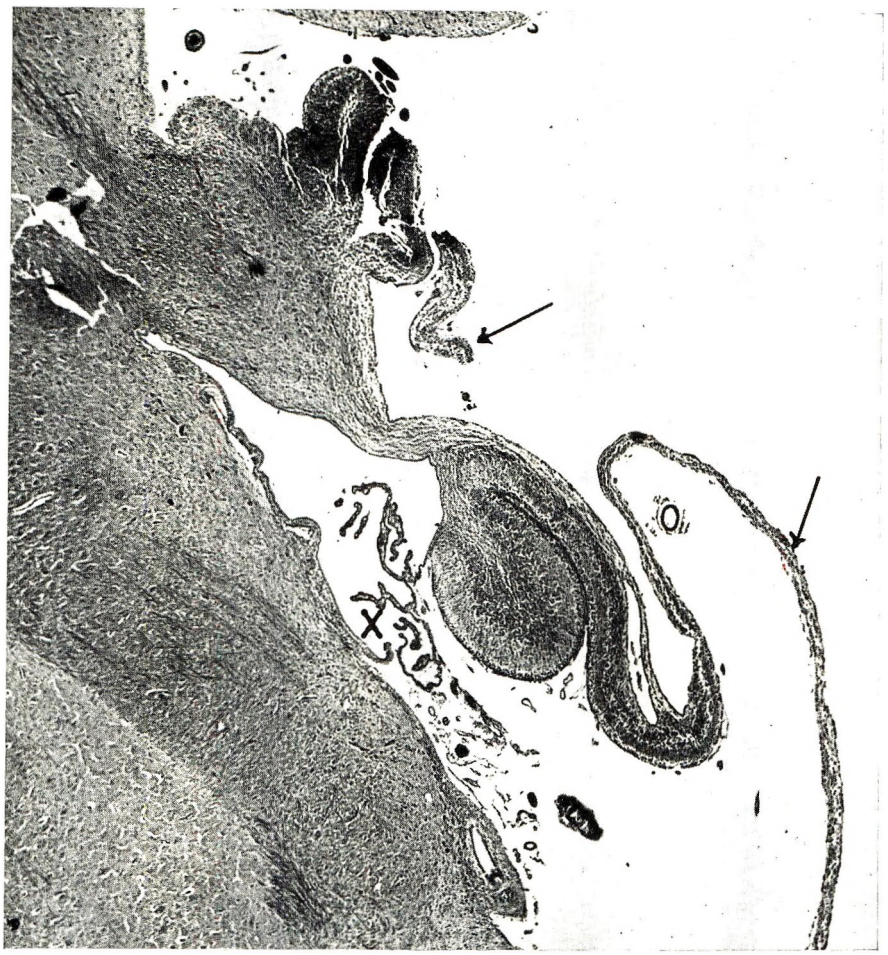

Fig. 3. Cerebellar structure of a second abnormal brain from a newborn low copper guinea pig. Note difference in tissue loss in figures 2 and 3 and the presence in both cases of the most anterior and the most posterior cerebellar folia. The greatest derangement of cellular organization occurred in the anterior lobules. The strand of cellular tissue marked with an arrow in figure 3 enclosed a fluid-filled cavity similar to that present in figure 2. Luxol fast blue × 36. Choroid plexus marked X.

with age. A relatively small increase in lipid phosphorus occurred in deficient animals during the first 63 days after parturition. These data confirmed earlier findings.[5]

The concentrations of individual phospholipids in whole brain of normal and copper-deficient guinea pigs have been summarized in table 2. The major phospholipids present in guinea pig brain are phosphatidyl ethanolamine and phosphatidyl choline. These two classes of compounds made up approximately 70% of the total phospholipid present. In control animals phosphatidyl serine increased from 10% at birth to 12% for animals 63 days of age. Sphingomyelin comprised about 5% of the phospholipid present at birth and rose to slightly over 7% at 28 or 63 days of age. The percent of total phospholipid present as phosphatidyl inositol was small and was not found to change over the age studied.

[5] Chow, Huan-Chang 1962 Influence of maternal restriction of copper on the lipid portion of the brain of guinea pigs. M. S. Thesis. Filed in the library, University of California, Davis.

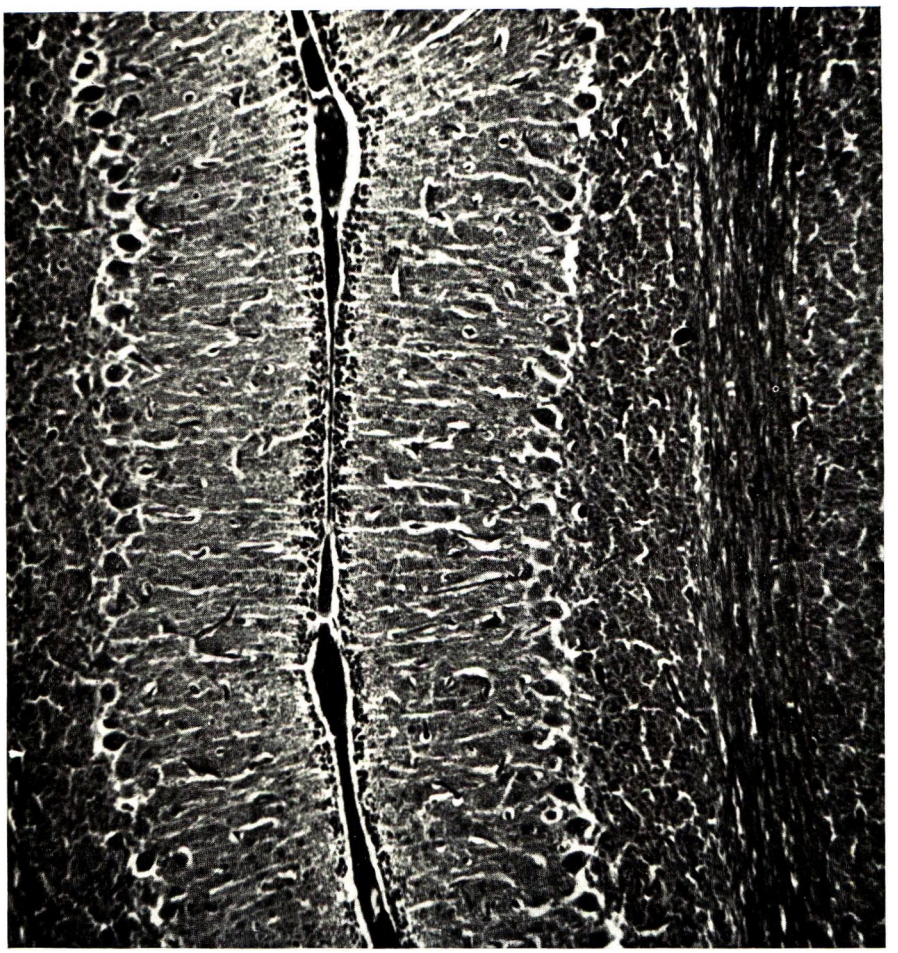

Fig. 4 Normal cerebellar morphology under higher magnification. This illustration shows the periphery of two adjoining leaflets with the central fibers shown at the right. Adjacent layers are well delineated and the inner granular layer is separated from the molecular layer by large well-stained Purkinje cells. The outer layer of granular cells, characteristic of the newborn cerebellum is approximately 5 cells in depth. Luxol fast blue × 120.

In addition to less total phosphorus in the lipid fraction of brain of copper-deficient guinea pigs, the distribution of phospholopids was altered in approximately 50% of the deficient animals tested. Six of the 13 deficient guinea pigs had concentrations of phosphatidyl serine below those of control animals at birth. Seven animals in the deficient group had a disproportionately small amount of sphingomyelin present. The 2 ataxic newborn animals tested (A' and B') of tables 1 and 2 were the most poorly developed from the standpoint of the amount of phosphorus present in the lipid fraction of the brain and in the percent of phosphatidyl serine and sphingomyelin present in total phospholipids.

DISCUSSION

The hope that the guinea pig would prove an advantageous animal in which to study the effect of in utero copper deficiency on brain abnormalities has been realized. Comparison of our observations on copper-deficient newborn guinea pigs, however, with the findings reported by Innes and Saunders (13), Barlow (14)

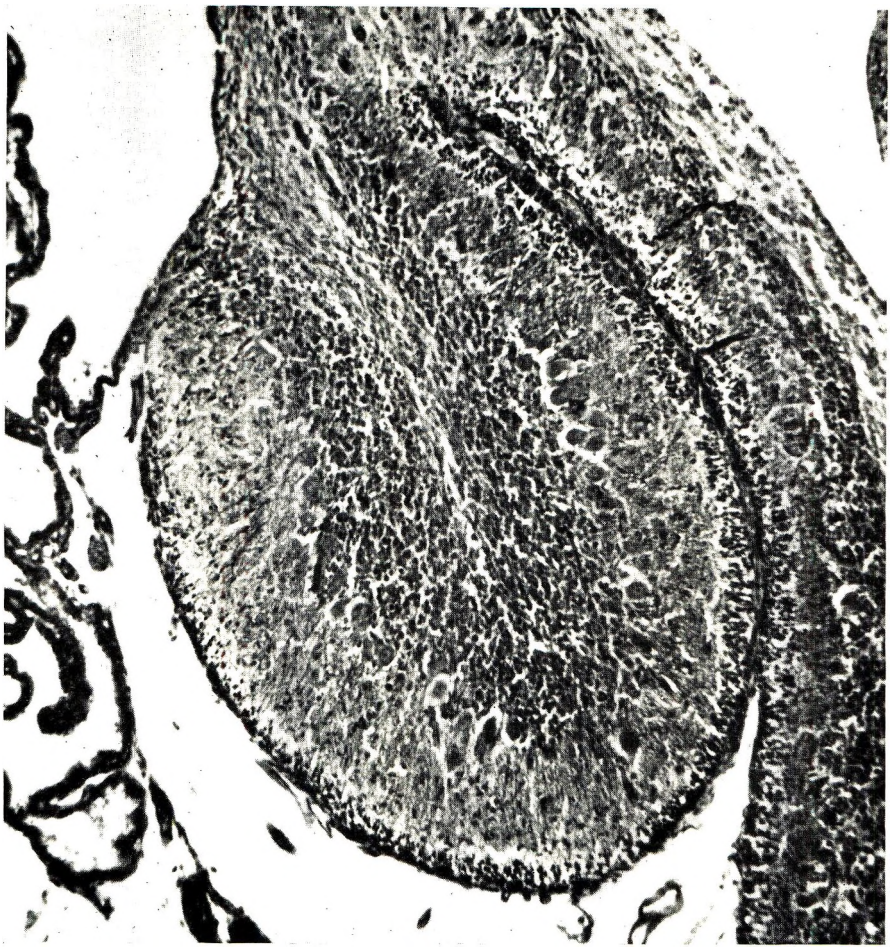

Fig. 5 The most highly organized portion of the cerebellum from the brain shown in figure 3. Myelinated fibers are virtually missing from the central part of the lobule. The granular layer encroaches on the central lobular area and its cells have a less compact and orderly arrangement. The Purkinje cell layer is absent in some areas and contains variable concentrations of cells in others. All existing Purkinje cells appear to contain Nissl material and none appear to be atrophic. The molecular layer shows derangement of the cells and fibers. The extra subpial granular layer appears to be increased in width and cellularity in a portion of the lobule and to be deficient in cells in other areas. Luxol fast blue × 120.

and others for swayback lambs, suggests that great species variability may exist in the response of animals to this nutritional stress.

Innes in reviewing the literature related to the pathology of swayback in lambs has suggested that the brain changes are the result of demyelination. Barlow (14) in a progressive study of the disorder has shown that the myelin which is present in the brain of newborn lambs disappears with increasing age and that the products of degenerating myelin are found in increasing amounts. The classic pathology of copper deficiency in lambs is one of neonatal ataxia, gelatinous cavitation of the cerebral white matter, chromatolysis, neuron necrosis and myelin degeneration in the brain stem and cord (14). Not all of the above changes were encountered in lambs at birth, however, in fact demyelination was recorded earliest between week 1 and 2 (14). Degenerative lesions of the cerebellum, hyalinization of the neurons of the

Fig. 6 Hemisection of the brain of a normal newborn guinea pig showing fiber tracts of the brain and upper cord and the appearance of the normal cerebellum. Luxol fast blue myelin stain × 5.

red nucleus, perivascular edema and gliosis have all been reported to occur in such animals.

The changes which have been observed in the brains of newborn guinea pigs whose dams were fed a diet deficient in copper during growth and gestation do not correspond to the alterations characteristic of the brain of copper-deficient lambs but more nearly resemble those reported to occur in hypomyelinogenesis congenita in sheep (15), calves (16) and in the cat (17). Here, although the morphological derangement of the cerebellar tissues varies greatly between individual cases, the common defect appears to involve the normal development of myelination throughout the brain. In the cerebellum, which is involved in all reported cases of this disorder, alterations ranged from limited cellular damage to loss of neural elements, and from cellular disorganization to complete agenesis of entire folia. There is no consistently present distinctive cellular damage in the cerebral cortex, the brain stem or the cord in this syndrome. No etiologic factors have been identified in any of the congenital disturbances of myelination referred to above and perhaps causal factors differ. The reports of Markson et al. (15) on this disorder in sheep included 36 cases arising in 3 widely separated geographical areas and a range of breeds. Determinations of the copper content of blood and liver tissue taken at the time the defective animals were killed revealed normal copper levels. To what extent copper stores were adequate during gestation is of course unknown. In this regard the observations of Butler and Barlow (18) that there is a marked fall in the copper content of the blood of ewes occurring in the first half of pregnancy, and that in each group of sheep studied there were significant and consistent differences between the blood copper levels of individual animals of similar nutritional history, should be kept in mind.

In the present study only a portion of the females fed the low copper ration delivered young which had gross brain defects. Of the 20 brain samples analyzed for phospholipid composition, approximately 50% of the young from dams fed the low copper diet were found to have some degree of deviation from the normal brain phospholipid pattern of this species. The animals having the most abnormal phos-

Fig. 7 Hemisection of the brain of a newborn low copper guinea pig showing lack of staining of myelinated tracts throughout the brain stem, absence of myelination around fibers of the corpus callosum, and the abnormal structure of the cerebellum. Luxol fast blue × 5.

pholipid patterns also showed demonstrable morphological changes.

Wiener (19) has suggested that genetics may play a role in swayback in sheep and although this postulate in the case of swayback has not been explored widely, our experiments with copper deficiency in guinea pigs also suggest that an inherent

TABLE 1

Brain weight, moisture content, lipid present in brain, and phosphorus present in brain lipid

Age		Brain wt (wet wt)	Moisture content	Total lipid per (wet wt of brain)	Lipid phosphorus per (lipid fraction of brain)
days		*g*	*%*	*%*	*%*
		Guinea pigs receiving the control diet			
Newborn	A	2.5952	80.8	9.13	1.93
	B	2.6555	80.9	9.11	1.97
	C	2.9106	80.5	9.17	1.89
15 days		2.6679	79.7	9.32	2.46
23 days		3.4033	79.5	9.51	2.49
28 days		3.1461	78.4	10.11	2.70
63 days		3.4817	78.6	10.01	2.87
		Guinea pigs receiving the copper-deficient diet			
Newborn	A [1]	1.5526	83.3	7.34	1.68
	B [1]	1.7236	82.1	7.93	1.60
	C	2.1490	80.7	9.00	1.92
	D	2.5098	80.5	9.31	1.66
	E	2.7400	81.1	9.25	1.96
15 days		2.7535	79.9	9.46	1.88
23 days		3.0320	79.4	10.48	1.84
28 days	A	3.2416	77.9	10.80	1.75
	B	3.2026	78.5	10.35	1.88
	C	3.2361	79.3	10.07	1.89
	D	3.3340	78.9	9.77	2.04
40 days		3.4022	79.0	10.66	1.78
63 days		3.6533	77.8	10.63	2.11

[1] Guinea pig with agenesis of the cerebellum.

TABLE 2

Distribution of phospholipids of whole brain of guinea pigs [1]

Age		Phosphatidyl ethanolamine	Phosphatidyl choline	Phosphatidyl inositol	Phosphatidyl serine	Sphingomyelin
days		*%*	*%*	*%*	*%*	*%*
		Guinea pigs receiving the control diet				
Newborn	A	34.2	37.2	3.3	10.6	4.9
	B	36.4	35.7	3.2	10.9	5.1
	C	36.8	35.0	3.3	10.7	4.8
15 days		36.6	34.5	3.5	11.9	6.6
23 days		33.9	32.5	3.5	11.7	6.0
28 days		34.8	33.4	3.2	11.6	7.2
63 days		33.9	32.1	3.0	12.3	7.2
		Guinea pigs receiving the copper-deficient diet				
Newborn	A [2]	37.4	38.4	3.4	7.2	2.5
	B [2]	37.1	36.8	3.4	6.8	2.6
	C	36.2	35.9	3.2	10.5	4.7
	D	36.9	37.7	3.0	10.3	4.6
	E	37.8	37.7	3.4	10.1	4.8
15 days		34.9	33.9	3.3	11.0	6.8
23 days		35.8	33.3	3.3	9.5	4.8
28 days	A	38.1	32.5	3.5	10.3	3.9
	B	36.4	35.0	3.0	10.0	7.0
	C	38.4	34.5	3.3	9.9	4.7
	D	35.5	32.6	3.1	9.7	5.2
40 days		36.4	33.5	3.3	11.7	4.8
63 days		35.1	33.0	3.0	12.3	7.2

[1] Expressed as percent of total lipid phosphorus.
[2] Guinea pig with agenesis of the cerebellum.

genetic susceptibility may condition the response of individuals to reduce amounts of this trace element.

The observation that young from certain dams appeared to be more markedly affected by a decreased copper intake led us to undertake a series of preliminary breeding experiments. In these studies females which had borne defective young while being maintained on the low copper ration were fed a normal stock ration [6] and inbred to males from their previous litters. In 3 generations, representing more than 30 litters of such inbred young, no abnormal progeny have resulted.

As with other developmental defects the degree and perhaps the kind of defect produced in the young appear to be related to the severity and the duration of the stress imposed by the deficiency. The use of a strain of guinea pigs sensitive to reduced dietary copper may in the future make it possible to clarify some of the many remaining questions concerning the role of this trace mineral in the metabolism of myelin formation and destruction.

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[6] Purina Guinea Pig Chow, Ralston Purina Company, St. Louis.

Effect of a Soluble Fraction of Oil Seed Meals on Uptake of ^{65}Zn from Ca·Mg·^{65}Zn·Phytate Complexes by the Chick [1]

J. G. LEASE

Department of Food Science and Biochemistry, College of Agriculture and Biological Sciences, Clemson University, Clemson, South Carolina

ABSTRACT The objectives were to relate the uptake of ^{65}Zn by the chick from certain fractions of phytate-containing oil seed meals to the varying availability of the meal zinc in vivo, and to delineate conditions in vitro which could account for availability of zinc bound by a casein digest, as compared with zinc bound by phytate. The insoluble Ca·Mg·^{65}Zn·phytate complex and the corresponding "soluble fraction" of 61 sesame meal (61 SM); zinc unavailable to the chick, and of Venezuelan sesame meal (VSM); zinc available, were prepared by an in vitro method simulating the chick's initial digestive processes. Inclusion of the soluble fraction of 61 SM with its ^{65}Zn·phytate complex did not significantly increase uptake of ^{65}Zn by blood, liver or tibiae. Inclusion of the soluble fraction of VSM significantly increased uptake from its phytate complex. It was postulated that VSM contained precursors of substances which form a more stable bond with zinc than does phytate during the digestion process; zinc is available from this complex; 61 SM lacks these precursors. The soluble fraction of soybean meal (SBM) contained about 75% of the extracted zinc; uptake of ^{65}Zn from this fraction was comparable to that from $^{65}ZnCl_2$. Inclusion of the unlabeled SBM fraction with the ^{65}Zn·phytate complex of 61 SM or VSM resulted in a significant increase in uptake of ^{65}Zn. When equal parts of 61 SM and SBM were fed, inclusion of the SBM prevented appearance of leg abnormalities and caused a significant increase in weight. It was postulated that SBM contained a substance which could render the zinc of an insoluble phytate complex available in vivo. ^{65}Zn added to a casein digest was bound at intestinal pH; dialysis to water was low but about 50% of the ^{65}Zn dialyzed to blood. Little dialysis of ^{65}Zn to blood or water occurred with phytate-containing sesame digest. It is suggested that during the process of digestion in vivo zinc may be bound in the presence of a number of feedstuffs; the avidity with which zinc is taken up by blood components will influence its absorption.

Some workers have found that phytic acid added to casein diets reduced the availability of zinc to the chick or rat (1–3); others have found little effect (4, 5). Oil seed meals contain both zinc and phytate but a relationship between the availability of their zinc to the chick and the zinc, calcium, magnesium or phytate content was not found (6). When several meals were fractionated by a process in vitro simulating digestion in vivo, zinc was found in an insoluble, nondialyzable Ca·Mg·phytate complex at pH 6.8, the intestinal pH, regardless of the availability in vivo of the zinc of the original meal (7). Very little of the zinc of the phytate complex was taken up by the chick. It was postulated that for zinc to be available from the phytate complex, other substances must be present in the meal which would form a more stable bond with zinc than did phytate. Zinc would be available to the chick from this latter complex.

Although soybean meal contained about the same proportion of zinc to phytate phosphorus as did the meals yielding an insoluble zinc·phytate complex, on digestion in vitro about 75% of the extracted zinc was found in a water-soluble organic complex at intestinal pH. Zinc was dialyzable from this complex. The zinc-containing soluble fraction could also render soluble and dialyzable some of the zinc of the insoluble Ca·Mg·Zn·phytate complex of other meals and was termed a "carrier" (7).

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When casein was digested in vitro and labeled with ^{65}Zn, little dialysis of zinc occurred at intestinal pH (7). This suggested that zinc might be complexed in the intestine by substances other than phytate. Zinc added to casein rations is available to the chick (2).

The objectives were 1) to try to relate the uptake of ^{65}Zn by the chick from specific fractions of phytate-containing meals to differences in availability of the zinc of these meals found in vivo, and 2) to delineate conditions in vitro affecting dialysis of ^{65}Zn bound by casein as compared with ^{65}Zn bound by phytate and to relate these to availability of zinc in vivo.

MATERIALS AND METHODS

Protein sources. The 61 sesame meal [2] and Venezuelan sesame meal [3] served as sources of an insoluble Ca·Mg·^{65}Zn·phytate complex and its complementary soluble fraction at intestinal pH. The meals were chosen from a series of meals tested for availability of their zinc to the chick (6). They were comparable in phytate and zinc content but it was necessary to add 60 mg/kg of zinc to a 61 sesame meal ration before good growth and absence of leg deformities were obtained. Zinc of the Venezuelan sesame meal was available to the chick as shown by absence of leg deformities when no zinc was added to the ration and by the failure of 30 or 60 mg/kg of added zinc to increase growth significantly.

The soybean meal [4] contained approximately 40% as much zinc or phytate phosphorus as the sesame meals (7). The proportions of phytate phosphorus to zinc were about the same. Few leg deformities appeared in chicks given soybean meal rations without supplementary zinc, however, addition of 30 mg/kg of zinc caused a significant increase in weight with the sample of meal used in these trials (8).

Severe leg deformities and poor growth were obtained in chicks fed a low zinc ration containing casein [5] and gelatin (8) or an isolated soy protein [6] (6) as the protein source. Good growth and prevention of leg deformities were obtained by the inclusion of 10 or 15 mg/kg of zinc, respectively.

Preparation of meal fractions. An in vitro method, simulating the chick's initial digestive processes, was used to prepare the various fractions (7). Pepsin was added to a water slurry of the meal adjusted to pH 3. After stirring for 15 minutes at 40°, the supernatant was removed by centrifugation. For the Ca·Mg·^{65}Zn·phytate complex of the sesame meals, ^{65}Zn was added to the supernatant and the pH adjusted to 6.8. The copious precipitate of Ca·Mg·^{65}Zn·phytate which appeared was removed by centrifugation, washed once with water, dried and pulverized.

The "soluble fraction" of a meal was prepared by the same procedure, omitting the addition of ^{65}Zn, and consisted of the supernatant after removal of the phytate precipitate at pH 6.8. The soluble fraction was mixed with the moist Ca·Mg·^{65}Zn·phytate complex, dried and pulverized. The phytate complex plus its supernatant simulated the initial digestion products of a meal in the intestine of the chick.

The equivalent of 2.5 g of meal as the phytate complex, the soluble fraction or a combination of the two was fed in one or two no. 0 gelatin capsules in all trials.

In addition to the basic combination of soluble fraction and insoluble phytate complex of 61 or Venezuelan meal, the phytate complex of 61 sesame meal was also combined with the soluble fraction of an isolated soy protein [6] and variations in treatment of the soluble fraction of Venezuelan sesame meal were made. The soluble fraction of Venezuelan meal was extracted with methanol, described for the soluble fraction of soybean meal below, and the extract combined with the phytate complex. The soluble fraction was also digested with pancreatin and erepsin, 0.25 g of pancreatin plus 0.125 g of erepsin per the equivalent of 2.5 g of meal, at 40° for 4 hours in a water-bath shaker. A methanol extract of the product was made and combined with the phytate complex.

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[2] Obtained from John Kraft Sesame Corporation, Paris, Texas. The decorticated meal contained 150 mg/kg of zinc and 1.00% phytate phosphorus.

[3] Obtained from Protinal Compania Anonima, Valencia, Venezuela. The solvent processed meal contained 120 mg/kg of zinc and 0.99% phytate phosphorus.

[4] 50% protein commercial soybean meal.

[5] High Nitrogen Casein, Nutritional Biochemicals Corporation, Cleveland.

[6] Assay Protein C-1, Skidmore Enterprises, Cincinnati.

Only the soluble fraction of soybean meal, containing the "carrier" properties and about 75% of the extracted zinc (7), was used in the tests. In the trial which measured the uptake of ^{65}Zn from the soluble fraction, the digest was labeled with ^{65}Zn at pH 3 and the soluble fraction removed after adjustment to pH 6.8. Labeling was omitted in tests of the effect of the soluble fraction on uptake of ^{65}Zn from the insoluble phytate complex of the sesame meals.

Two no. 0 gelatin capsules were all a 2-week-old chick could be induced to swallow without regurgitation. To accommodate the supplements of the soluble fraction of soybean meal it was necessary to reduce the bulk. After drying to about 10% moisture, the soluble fraction was macerated with methanol. The process was repeated twice over a 24-hour period so that the final extraction was colorless. The combined labeled methanol extracts were dried and pulverized. The combined unlabeled extracts were concentrated, mixed with the moist phytate complex of 61 or Venezuelan sesame meal and the slurry dried and pulverized.

Retention of some of the properties of the soluble fraction of the soybean meal in the methanol extract was measured. The extract of the labeled soluble fraction was found to retain 94% of the ^{65}Zn activity. The ^{65}Zn was also retained in an organic complex. An aliquot of the extract was dried, reconstituted with water and passed through a gel-filtration resin.[7] After passage of the void volume, the activity appeared in association with ninhydrin-positive substances. Ninety-four percent of the activity was recovered in succeeding aliquots which also contained ninhydrin-positive substances.

To check the retention of the ^{65}Zn-binding property in the methanol extract of the soybean meal in vitro, the dried extract of the unlabeled soluble fraction was reconstituted with water. When added to the Ca·Mg·^{65}Zn·phytate complex of 61 sesame meal, the methanol extract rendered 24% of the ^{65}Zn dialyzable, the original soluble fraction, 29%.

Duplicate samples of a phytate complex or of a soluble fraction were analyzed for zinc, calcium and magnesium by atomic absorption spectrophotometry, and for phytate phosphorus by a modification of the method of Pringle and Moran (6). The nitrogen of the soluble fraction was determined by micro-Kjeldahl digestion followed by nesslerization by a modification of the Koch and McMeekin method (9).

Dialysis in vitro. Casein or Venezuelan sesame meal was digested with pepsin at pH 3, the supernatant removed by centrifugation, labeled with ^{65}Zn and adjusted to pH 6.8 (7). This "pH 6.8 digest" was taken to be the form in which casein or the sesame meal might be found in the initial stages of digestion in the intestine. Addition of L-histidine·HCl, L-arginine·HCl or L-lysine·HCl was made at pH 3 in the proportions of 50 mg to an aliquot of digest equivalent to 10 g of the source. Pancreatin, 100 mg, and erepsin, 50 mg, were added at pH 6.8 to the same amount of aliquot. For the water trials with casein, the digest equivalent to 10 g of protein source, 50 to 60 ml, was dialyzed against 200 ml of water (7). The equivalent of 2.5 g, 12.5 ml, was dialyzed against 30 ml of pig blood. For the Venezuelan meal, the proportions of digest to blood or water were the equivalent of 2.5 g, 17 ml, against 40 ml. Because relatively large quantities of blood were needed to make simultaneous tests with the amino acid addition, pig blood was used.

The dialysis containers were shaken in a water bath at 40° for 4 hours; any dialyzable substances produced by pancreatin and erepsin digestion during this time could continuously dialyze.

Uptake of ^{65}Zn by the chick. The ^{65}Zn uptake by the blood, tibiae and liver was measured at 4, 8 and 24 hours (7). A supplement of $^{65}ZnCl_2$ plus an amount of stable zinc equal to that of a Ca·Mg·^{65}Zn·phytate supplement was included in each trial. This was intended to serve as a control for the uptake of zinc by the various tissues over time. The uptake of ^{65}Zn from the soluble fraction of soybeal meal was compared with that from $^{65}ZnCl_2$ + stable zinc supplement. The uptake of ^{65}Zn from the soluble fraction seemed to be slower than that from the basal $^{65}ZnCl_2$, possibly

[7] Bio-Gel P-10. Bio. Rad Laboratories, Richmond, California. A 2 ml aliquot was filtered through a column 1 × 29 cm.

due to other constituents of the fraction. In subsequent trials, the uptake of ^{65}Zn from a phytate complex was compared with that from the phytate complex plus the soluble fraction rather than with the basic $^{65}ZnCl_2$ supplement.

The data for the tissue uptake, expressed as percent of ^{65}Zn given, were analyzed for variance as a factorial experiment. The data were subjected to the *F* test (10). The values denoting the least significant different (LSD) between means at $P < 0.01$ and $P < 0.05$ were calculated. In some experiments the uptake of the 3 tissues was added together for any one of the three time periods to give a combined tissue uptake for the specified time. This was not subjected to statistical analysis and was only used in interpretation of the individual tissue uptakes.

Feeding tests with whole meals. One-day-old broiler-type cockerels were fed rations (table 1) containing Venezuelan sesame meal, soybean meal, 61 sesame meal or a combination of equal parts of 61 sesame meal and soybean meal for a 3-week period. Growth, leg deformities and the zinc content of the tibia were measured.

TABLE 1

Composition of basal rations

	g/kg			
61 sesame meal	344 [1]	—	—	185 [2]
Venezuelan sesame meal	—	454 [1]	—	—
Soybean meal	—	—	400 [1]	185 [3]
L-Lysine·HCl	9	9	—	0.25
MHA [4]	—	—	3.3	0.10
Choline·chloride (70%)	3	3	3	3
Vitamin mix [5]	5	5	5	5
Mineral mix [6]	60	60	60	60
Corn oil [7]	52	49	100	71
Sucrose	527	429	436	501

[1] Supplied 20% protein to the diet.
[2] Supplied 10.7% protein to the diet.
[3] Supplied 9.3% protein to the diet.
[4] The calcium salt of methionine hydroxy analogue at 90% concentration, Dupont, Wilmington, Delaware, was used as a source of methionine.
[5] Provides per kilogram of diet: (in mg) menadione bisulfite sodium 4.5; biotin 0.2; pyridoxine·HCl 5; folic acid 5; riboflavin 10; Ca D-pantothenate 30; thiamine (mononitrate) 10; niacin 50; vitamin B_{12} 0.02; and 1500 ICU vitamin D_3; 10,000 IU vitamin A (vitamin A palmitate); 50 IU vitamin E (*d*-α-tocopheryl acetate).
[6] Provides per kilogram of diet: (in g) $CaHPO_4 \cdot 2H_2O$, 27.2; $CaCO_3$, 14.0; K_2HPO_4, 11.1; NaCl, 6.0; (in mg) $MgCO_3$, 175; Fe citrate, 333; $MnSO_4 \cdot H_2O$, 333; KI, 2.6; and $CuSO_4 \cdot 5H_2O$, 33.4. Since addition of 450 ppm Mg to 61 sesame meal rations decreased the availability of the zinc of the sesame meal (8), the added magnesium was purposely kept low. The sesame meal rations contained 0.23%, the soybean meal 0.16% and the Venezuelan sesame meal ration 0.36% Mg, by analysis.
[7] Corn oil was added so that the total lipid content of the ration was 10%.

RESULTS AND DISCUSSION

Unavailability of the zinc of 61 sesame meal and availability of the zinc of Venezuelan sesame meal to the chick was confirmed by the feeding trials (table 2). Chicks given the basal 61 sesame meal ration showed severe leg deformities, poor growth and a low tibia zinc content. Addition of 30 mg/kg of zinc caused a significant increase in weight ($P < 0.05$) and an increase in tibia zinc. Addition of 60 mg/kg of zinc caused a further significant increase in weight ($P < 0.05$); few leg deformities appeared. The zinc content of the tibia was further increased. Zinc of Venezuelan sesame meal was available to the chick as demonstrated by lack of a significantly different weight gain ($P < 0.05$) when 30 or 60 mg/kg of zinc were added to the ration. Leg deformities were not found in any of the groups; tibia zinc content was high in comparison with that of the chicks given 61 sesame meal.

The addition of 30 mg/kg of zinc increased growth significantly when the soybean meal ration was fed (8). A lesser amount of zinc was added to ascertain if some of the zinc of soybean meal were available to the chick. In the in vitro trials about 50% of the zinc of the meal had been extracted and about 75% of this appeared in the soluble fraction (7). A basal soybean meal ration contained 32 mg/kg of zinc (table 2). If partition of the zinc of the meal on digestion in vivo followed the in vitro pattern, about 12 mg/kg could be furnished by the soluble fraction. It has been suggested that the optimum requirement of zinc for a casein diet is 15 mg/kg (11). A supplement of 8 mg/kg of zinc was used with the soybean meal ration to provide a possible maximum of 20 mg/kg of "available zinc."

The addition of 8 mg/kg of zinc to the ration caused a significant increase in weight ($P < 0.05$); the addition of 30 mg/kg did not cause a greater significant in-

TABLE 2

Growth, leg deformities and tibia zinc at three weeks of age of chicks fed Venezuelan sesame, soybean meal, 61 sesame or 61 sesame + soybean meal rations [1]

Meal	Added zinc [2]	Zinc of ration [3]	Weight [4]	Leg score [5]	Tibia zinc [6]
	mg/kg	*mg/kg*	*g*		*μg/g bone ash*
		Experiment 1			
61 sesame	0	50 ± 2	201 [a]	3.1	99 ± 10
61 sesame	30	76 ± 6	307 [b]	2.6	142 ± 0
61 sesame	60	108 ± 0	359 [c]	0.9	192 ± 33
61 sesame + soybean	0	40 ± 0	307 [b]	0.8	113 ± 1
61 sesame + soybean	30	74 ± 2	368 [c]	0.7	234 ± 29
61 sesame + soybean	60	100 ± 0	375 [c]	0.5	322 ± 26
		Experiment 2			
Venezuelan sesame	0	56 ± 0	233 [a]	0.2	378 ± 19
Venezuelan sesame	30	79 ± 1	267 [a]	0.3	422 ± 12
Venezuelan sesame	60	122 ± 2	268 [a]	0.6	475 ± 21
		Experiment 3			
Soybean	0	32 ± 6	342 [a]	0.3	101 ± 3
Soybean	8	37 ± 2	371 [b]	0.7	156 ± 1
Soybean	30	55 ± 2	392 [b]	0.6	294 ± 1

[1] One-day-old broiler type cockerels, in duplicate groups of 10, were housed in stainless steel cages, given deionized water, and weighed weekly.
[2] As $ZnCO_3$.
[3] By atomic absorption spectrophotometry, average and range of an aliquot from each replicate cage.
[4] Within an experiment, weights followed by the same letter are not significantly different ($P < 0.05$).
[5] Graded as zero to 4 in order of increasing severity.
[6] A.O.A.C. method (16) for bone ash. The tibiae of 3 or 5 chicks from each duplicate group of each level of zinc were composited. The values represent the average and range of the 2 composite groups.

crease (table 2).[8] This suggested that some of the zinc of the soybean meal was available to promote growth in the presence of phytate.

Uptake of ^{65}Zn

The 61 sesame meal phytate complex and soluble fraction. Little of the ^{65}Zn of the phytate complex was taken up by any of the tissues (table 3). When the soluble fraction was included, the mixture simulating the digestion products of 61 sesame meal in the intestine of the chick, no significant increase appeared in uptake of ^{65}Zn by any of the tissues. It is postulated that a component, which upon digestion formed a more stable complex with zinc than phytate and which could render zinc available to the chick, either was lacking or was bound so that the chick's digestive processes did not free it.

Venezuelan meal phytate complex and soluble fractions. Although the zinc of Venezuelan meal is available to the chick (table 2), in the in vitro studies the addition of the soluble fraction did not increase dialysis of ^{65}Zn from the phytate complex (7). The action of pancreatin and erepsin caused little increase in dialysis. This suggested that the soluble fraction contained the precursor of the carrier; further digestion in vivo could free the carrier per se.

Since the carrier of soybean meal was soluble in methanol, a trial to measure a similar solubility of the possible precursor was included.

The soluble fraction was also digested in vitro with pancreatin and erepsin. If the action of these enzymes contributed to freeing the carrier in vivo, the carrier in the fraction pretreated with these enzymes could appear and act on the phytate-bound ^{65}Zn sooner in vivo than the carrier eventually produced in vivo from the untreated fractions. An increased absorption and tissue deposition of ^{65}Zn by 4 hours could result from the predigestion treatment.

Uptake of ^{65}Zn from the phytate complex was low except for the blood at 4

[8] In 2 later experiments, addition of 10 mg/kg of zinc caused a significant ($P < 0.05$) increase in weight. Addition of 30 mg/kg did not cause a further significant increase. Unpublished data.

TABLE 3

Effect of the "soluble fraction" of 61 sesame meal or of isolated soy protein on the uptake of ^{65}Zn from the Ca·Mg·^{65}Zn·phytate complex of 61 sesame meal [1]

Hours	Blood			Liver			Tibiae		
	4	8	24	4	8	24	4	8	24
$^{65}ZnCl_2$ [2]	4.08	3.41	2.89	9.35	8.31	9.47	3.35	3.40	4.30
Phytate complex [3]	0.83	0.66	0.71	1.40	1.70	1.77	0.27	0.46	0.98
Phytate complex plus the soluble fraction of 61 sesame meal [4]	1.54	0.92	0.87	1.99	3.29	2.18	0.38	0.83	1.13
Phytate complex plus the soluble fraction of an isolated soy protein [5]	2.54 **	2.01 **	1.16	4.24 **	6.04 **	3.54 *	0.86 *	1.46 **	1.45

[1] Average uptake for 5 chicks per time period per supplement as percent of ^{65}Zn fed.
[2] Each chick received 1.1 μCi of ^{65}Zn (3.84 mμCi/mg) plus 0.20 mg of stable Zn as a water solution of $ZnSO_4$.
[3] Each chick received a supplement, equivalent to 2.5 g of meal, which contained 1.1 μCi ^{65}Zn and in milligrams, 0.22 stable Zn, 5.95 Mg, 2.30 Ca, 0 N and 6.0 phytate P.
[4] The phytate complex and soluble fraction, each equivalent to 2.5 g of meal, were combined and fed to each chick. The combined supplement contained 1.1 μCi ^{65}Zn and in milligrams, 0.22 stable Zn, 9.06 Mg, 2.30 Ca, 9.7 N and 6.0 phytate P.
[5] The phytate complex and the soluble fraction of isolated soy protein, each equivalent to 2.5 g of the source, were combined and fed to each chick. The combined supplement contained 1.1 μCi ^{65}Zn and in milligrams, 0.31 stable Zn, 8.25 Mg, 4.60 Ca, 10.8 N and 6.0 phytate P.
* Significantly different ($P < 0.05$) from the Ca·Mg·^{65}Zn·phytate complex.
** Significantly different ($P << 0.01$) from the Ca·Mg·^{65}Zn·phytate complex.

hours (table 4). By error, in preparation of the phytate complex the usual water-wash was omitted; the relatively high uptake of the blood at this time may reflect the uptake of some $^{65}ZnCl_2$ which adhered to the complex. Later tissue uptakes were not higher than that of a comparable phytate complex which had been washed (table 6); any free $^{65}ZnCl_2$ apparently had only a transient effect.

Inclusion of any of the 3 soluble fractions caused a significant increase in uptake of ^{65}Zn from the phytate complex by the blood, liver or tibiae at 8 hours (table 4). The effect of the methanol extracts on uptake by the tibiae was extended to 24 hours. The combined ^{65}Zn content of the tissues at 24 hours was calculated to ascertain whether the lack of a significant increase in uptake at 24 hours by the tibiae of the group given the basic soluble fraction was due to a lower combined tissue content or to a difference in tissue distribution. The combined tissues of the group receiving the basal phytate dosage contained 4.71% of the dosage, those of the untreated soluble fraction, 8.02%, those of the methanol extract, 7.98% and those of the predigested soluble fraction, 8.25%. These data suggest that while tissue distribution varied somewhat at 24 hours, the effect of any of the 3 soluble fractions was to increase the uptake of ^{65}Zn from the phytate complex.

Inclusion of the predigested soluble fraction caused a highly significant increase ($P < 0.01$) in uptake of ^{65}Zn by the blood at 4 hours in contrast with the 2 other soluble fractions (table 4). Similarly, at 4 hours these chicks had the highest combined tissue uptake, 9.71% as compared with 8.75 and 8.04%. Although the differences are small, the trend through the first 4 hours was to an increased rate of uptake of ^{65}Zn when the soluble fraction was predigested with pancreatin and erepsin. As noted above, only small differences in combined tissue uptake were present at 24 hours indicating that the amount of ^{65}Zn taken up was little affected. The trend suggests that the action of pancreatin and erepsin in vivo might be a step in freeing the carrier.

It is postulated that Venezuelan sesame meal contained a precursor of substances

TABLE 4

Effect of the "soluble fractions" of Venezuelan sesame meal on tissue uptake of ^{65}Zn from its insoluble Ca·Mg·^{65}Zn·phytate complex [1]

Hours	Blood			Liver			Tibiae		
	4	8	24	4	8	24	4	8	24
$^{65}ZnCl_2$ [2]	5.66	4.27	3.36	11.04	12.84	6.21	4.97	5.15	5.73
Phytate complex [3]	2.43	0.88	0.93	2.90	2.70	2.26	0.74	1.05	1.52
Phytate complex plus the soluble fraction [4]	3.16	2.69 **	1.58	3.78	5.54 **	4.30 *	1.10	2.23 **	2.18
Phytate complex plus MeOH extract of the soluble fraction [5]	2.86	1.78 *	1.95 **	4.60	4.94 *	3.42	1.29	1.96 *	2.61 **
Phytate complex plus MeOH extract of the soluble fraction predigested with pancreatin and erepsin [6]	3.48 **	2.74 **	1.95 **	4.82	5.98 **	3.72	1.41	2.31 **	2.58 **

[1] Average uptake for 5 chicks per time period per supplement as percent of ^{65}Zn fed.

[2] Each chick received 1.95 μCi of ^{65}Zn (3.84 μCi/mg) plus 0.16 mg of Zn as a water solution of $ZnSO_4$.

[3] Each chick received a supplement equivalent to 2.5 g of meal which contained 1.95 μCi of ^{65}Zn and, in mg, 0.16 stable Zn, 4.63 Mg, 3.24 Ca and 7.31 phytate P.

[4] The phytate complex and soluble fraction, each equivalent to 2.5 g of meal, were combined and fed each chick. The combined supplement contained 1.95 μCi of ^{65}Zn and, in milligrams, 0.16 stable Zn, 11.13 Mg, 4.15 Ca, 7.31 phytate P and 63.8 N.

[5] The phytate complex was combined with the methanol-soluble portion of the soluble fraction, each equivalent to 2.5 g of meal, and fed to each chick. The combined supplement contained 1.95 μCi of ^{65}Zn and, in milligrams, 0.18 stable Zn, 5.80 Mg, 3.90 Ca, 7.31 phytate P and 18.0 N.

[6] The soluble fraction was digested with pancreatin and erepsin, extracted with methanol and the methanol extract combined with the phytate complex, each equivalent to 2.5 g of meal. The combined supplement contained 1.95 μCi of ^{65}Zn, and in milligrams, 0.16 stable zinc, 5.80 Mg, 3.49 Ca, 7.31 phytate P and 41.4 N.

* Significantly different from the Ca·Mg·^{65}Zn·phytate complex ($P < 0.05$).

** Significantly different from the Ca·Mg·^{65}Zn·phytate complex ($P < 0.01$).

which on digestion in vivo bound the zinc of an insoluble Ca·Mg·Zn·phytate complex in a new complex from which zinc was available to the chick. The data suggest that digestion in vivo by pancreatin and erepsin may aid in liberation or formation of the latter zinc-binding complex.

Soybean meal soluble fraction. In contrast with the sesame meals, after digestion in vitro about 75% of the extracted zinc was present in the soluble fraction (7). Apparently further digestion was not necessary to free a factor which formed a more stable bond with zinc than phytate.

Significant differences in ^{65}Zn uptake between the chicks receiving the soluble fraction and those receiving the basal $^{65}ZnCl_2$ dosage were found only with the blood at 4 hours and the tibiae at 4 and 8 hours (table 5). The higher absorption at 4 hours from the soluble fraction could be due to an increased absorption of ^{65}Zn or to a difference in time at which the peak uptake of the two groups occurred. The combined tissue content of the basal group at 4 hours was calculated to be 19.06% of the dose given, that of the soluble fraction, 17.86%. The tibia uptake of the group given the soluble fraction was significantly lower at this time. Considering both uptakes, it appeared that at 4 hours the higher uptake by the blood of the soluble fraction group represented a point on the upswing of ^{65}Zn absorption whereas the peak of absorption of the basal group had been passed.

In many trials in this laboratory, ^{65}Zn uptake from $^{65}ZnCl_2$ by the tibiae has increased over the 24-hour period. It is thought that the high, anomalous uptake at 8 hours for the basal group was due to chick variation. Another group of chicks in this experiment, that received a supplement which did not cause a significant difference in ^{65}Zn uptake by the tissues at any of the times, had an uptake of 5.85% for the tibiae at 8 hours. If this value is used, no significant difference in tibia uptake at 8 hours is found. By 24 hours, the uptake of the combined tissues of the chicks receiving the basal dosage contained 16.14% of the amount given; those of the chicks receiving the soluble fraction, 15.81%. Although initial absorption may

TABLE 5

Tissue uptake of ^{65}Zn from the "soluble fraction" of soybean meal [1]

Hours	Blood			Liver			Tibiae		
	4	8	24	4	8	24	4	8	24
$^{65}ZnCl_2$ [2]	4.15	3.33	3.21	9.83	10.45	7.13	5.08	6.64	5.80
Soybean meal soluble fraction [3]	6.34 **	3.70	2.63	8.59	10.73	7.41	2.93 **	4.41 **	5.77

[1] Average uptake for 4 ($^{65}ZnCl_2$) or 5 chicks per time period per supplement as percent of ^{65}Zn fed.

[2] Each chick received 1.3 μCi of ^{65}Zn (3.84 μCi/mg) plus 80 μg of stable zinc as a water solution of $ZnSO_4$.

[3] After 3 successive extractions, the combined methanol extracts of the dried soluble fraction were dried. Each chick received a supplement equivalent to 2.5 g of meal, which contained 1.31 μCi of ^{65}Zn and in milligrams, 0.08 stable zinc, 0.80 Mg, 1.15 Ca, 56.0 N and no detectable phytate P.

** Significantly different ($P < 0.01$).

have been slower from the soluble fraction, by 24 hours the ^{65}Zn content of the groups was comparable; there was not a significant difference in uptake between any of the tissues measured.

It is postulated that the zinc present in the soluble fraction of soybean meal is bound in a complex from which it is available to the chick. The availability of some of the zinc of soybean meal, shown in the feeding trials, is thought to be attributable to the zinc bound in the soluble fraction.

Effect of the soluble fraction of soybean meal on preformed zinc·phytate complexes. In vitro studies had shown that the soluble fraction of soybean meal rendered some of the zinc of the phytate complexes of 61 or Venezuelan sesame meal dialyzable (7). The capacity of the soluble fraction of soybean meal to increase the uptake of ^{65}Zn from these phytate complexes by the chick was measured (table 6). The objective was to determine whether the zinc-binding factor of soybean meal could compete with phytate only under the conditions of digestion of soybean meal or whether it had a more general action.

Inclusion of the unlabeled soluble fraction of soybean meal with the insoluble ^{65}Zn·phytate complex of 61 sesame or Venezuelan sesame meal resulted in a significant increase in the uptake of ^{65}Zn by the blood and liver at 4 and 8 hours, and by the tibiae for the 3 time periods (table 6). Under the conditions of digestion in vivo, the carrier of soybean meal could compete with phytate so that some of the zinc of an insoluble phytate complex was absorbed and deposited in the tibiae.

A diet containing equal parts of soybean meal and 61 sesame meal was fed to determine if soybean meal per se would render phytate-bound zinc available (table 2). When 61 sesame meal was the sole source of protein and when no zinc was added severe leg deformities and poor growth were found. When soybean meal was included, although the zinc content of the ration was slightly lowered, leg deformities were not found and growth was comparable to that obtained when the sesame meal ration contained an added 30 mg/kg of zinc. Addition of 30 mg/kg of zinc to the combined meal ration resulted in growth and an increase in the zinc content of the tibiae comparable to that obtained when 60 mg/kg of zinc was added to the sesame meal ration. Since the soybean meal only contributed about 10 mg/kg of zinc, it appeared that the soybean meal per se could make some of the zinc of the 61 sesame meal available to the chick to promote growth, prevent leg deformities and, when some inorganic zinc was added, to increase the tibia zinc content.

The proportions of phytate phosphorus to zinc were calculated to be about 66 to 1 for the ration containing the combined meals and 69 to 1 for the sesame meal ration. The sesame meal contributed 0.59 g of calcium/kg of ration and the combined meals, 0.70 g/kg. The increased availability of zinc when soybean meal was included was not due to a variation in the proportions of zinc to phytate phosphorus or to a decreased calcium content of the ration.

During digestion, a component of soybean meal apparently formed a bond with the zinc of 61 sesame meal which was more stable than that of the phytate of the meal; zinc was available to the chick from the new complex. The carrier property of soybean meal for the zinc of an insoluble phytate complex was operative in the presence of ration ingredients as well as in the fractions prepared by in vitro methods.

The 61 sesame meal phytate complex and the soluble fraction of isolated soy protein. The relatively low added zinc requirement of chicks given this isolated soy protein [6] suggested that some of the carrier properties of soybean meal might have been retained in its manufacture. The inclusion of the soluble fraction (table 3) caused a highly significant increase ($P < 0.01$), in uptake of ^{65}Zn by the blood and liver at 4 and 8 hours, a significant increase ($P < 0.05$) by the liver at 24 hours, and by the tibiae at 4 hours ($P < 0.05$) and 8 hours ($P < 0.01$), but not at 24 hours. This suggested that the soy protein could render some of the zinc of the phytate complex available. An approximation of the comparative capacities of the soluble fraction of isolated soy and of soybean meal was made. Since uptake of ^{65}Zn from the phytate complex varied in the two experiments, the capacity of each fraction to increase uptake over that of the basal phytate dosage was calculated for each ex-

TABLE 6

Effect of the "soluble fraction" of soybean meal on tissue uptake of ^{65}Zn from the insoluble Ca·Mg·^{65}Zn·phytate complex of Venezuelan or 61 sesame meal [1]

Hours	Blood			Liver			Tibiae		
	4	8	24	4	8	24	4	8	24
$^{65}ZnCl_2$ [2]	5.19	3.67	3.09	8.79	11.91	12.11	3.84	5.54	6.35
Phytate complex [3] of 61 sesame meal	0.72	0.75	0.73	0.59	1.85	2.51	0.40	0.77	1.57
Phytate complex of 61 sesame meal + soluble fraction [4]	4.41 **	2.28 **	1.63	4.44 *	5.54 **	4.31	1.59 **	1.78 *	3.04 **
Phytate complex of Venezuelan sesame meal [5]	1.84	1.21	1.10	2.12	3.34	3.36	0.50	1.37	2.08
Phytate complex of Venezuelan sesame meal + soluble fraction [6]	3.36 **	3.02 **	2.24	5.58 **	6.51 *	7.11 **	2.05 **	2.39 *	3.92 **

[1] Average uptake for 5 chicks per time period per supplement, as percent of ^{65}Zn fed.

[2] Each chick received 1.6 μCi of ^{65}Zn (3.84 μCi/mg) plus 0.20 mg of stable Zn as a water solution of $ZnSO_4$.

[3] Each chick received a supplement, equivalent to 2.5 g of meal, which contained 1.56 μCi of ^{65}Zn and in milligrams, 0.21 stable Zn, 5.26 Ca, 7.70 Mg and 6.00 phytate P.

[4] The phytate complex and the soluble fraction of soybean meal, each equivalent to 2.5 g of meal, were combined and fed to each chick. The combined supplement contained 1.56 μCi of ^{65}Zn and in milligrams, 0.29 stable Zn, 6.40 Ca, 7.98 Mg, 6.00 phytate P and 56.0 N.

[5] Each chick received a supplement, equivalent to 2.5 g of meal, which contained 1.56 μCi of ^{65}Zn and in milligrams, 0.18 stable Zn, 4.75 Ca, 5.45 Mg and 7.31 phytate P.

[6] The phytate complex and the soluble fraction of soybean meal, each equivalent to 2.5 g of meal, were combined and fed to each chick. The combined supplement contained 1.56 μCi of ^{65}Zn and in milligrams, 0.25 stable Zn, 5.90 Ca, 5.74 Mg, 7.31 phytate P and 56.0 N.

* Significantly different ($P < 0.05$) from the Ca·Mg·^{65}Zn·phytate complex of the corresponding meal.

** Significantly different ($P < 0.01$) from the Ca·Mg·^{65}Zn·phytate complex of the corresponding meal.

periment. Using the uptake by the blood at 4 hours as an indication of absorption, the soybean meal caused an increase of 3.69% (table 6), the isolated soy protein, 1.71% (table 3). Inclusion of the soybean meal fraction made about twice as much of the ^{65}Zn of the phytate complex absorbable at 4 hours as did inclusion of the isolated soy fraction. By 24 hours, the inclusion of the soybean meal fraction caused an increase of 4.17% in combined tissue uptake as compared with 2.69% when the isolated soy protein fraction was included. The soybean meal fraction caused not only an increase in absorption of ^{65}Zn over that of the isolated soy protein but also increased retention. It appeared that some samples of isolated soy protein may contain sufficient carrier properties, presumably derived from soybean meal, to reduce the binding of zinc by phytate in vivo.

Dialysis of ^{65}Zn from casein or Venezuelan sesame meal digests at intestinal pH. The interest in dialysis of the casein digest lay in the similarities of performance in vitro and in vivo of Venezuelan sesame and casein although phytate was not a factor in the casein tests. Dialysis of zinc did not occur from the digest of either at intestinal pH, yet zinc added to casein rations or the zinc of Venezuelan meal was available to the chick. One possibility was that absorption in vivo of zinc added to casein rations was mediated by intermediates in a manner similar to that of foregoing experiments occurring with Venezuelan meal in vivo. Phytate-containing meals might not be unique in a requirement for several binding agents of varying stability to be operative before zinc was absorbed in vivo.

A second possibility was concerned with the nature of the in vitro dialyzing medium. In in vitro trials with strips of rat intestine, inorganic zinc was readily taken up by the intestine but was slow to appear in the water-based serosal medium used (12). Injected zinc has been shown to be bound by blood components (13). The avidity with which blood components bound zinc could influence the speed with which zinc passed through the intestine in vivo or through a dialysis membrane in vitro. ^{65}Zn from $^{65}ZnCl_2$ was found to pass rapidly through the intestinal wall to the blood and to the tissues in the preceding trials in vivo. In all trials, absorption of ^{65}Zn from either the basal $^{65}ZnCl_2$ or the various fractions was not greater by 8 or 24 hours as shown by the lower blood values for these times. This suggested that the intestinal mucosa was not antagonistic to the passage of zinc. The availability of zinc from a casein ration could be due to formation of a more stable complex with blood components than with components of the casein digest.

Trials, in which blood was used as a dialyzing medium, were made to determine if the zinc-binding components of the blood could influence dialysis of ^{65}Zn bound in either the casein digest or in the phytate-containing Venezuelan meal digest.

Dialysis of ^{65}Zn from casein: The zinc bound in the casein digest at intestinal pH was not present as an insoluble precipitate. As a first step, additions were made to the digest rather than trying to fractionate the various components. Amino acids are produced by further digestion of casein in vivo. Histidine forms complexes with zinc (14) and has been shown to prevent the appearance of leg deformities in chicks fed an isolated soy protein ration (15). As a product of digestion in vivo of casein and as an amino acid which might play a role in zinc metabolism the effect of histidine additions was measured. Trials with arginine and lysine were included to differentiate between effects due to addition of other basic amino acids.

The action of pancreatin and erepsin caused a small but significant ($P < 0.05$) increase in the dialysis of ^{65}Zn to water (table 7). Addition of histidine further significantly increased dialysis but addition of lysine or arginine had little effect. Histidine could form a more stable bond with some of the zinc of the casein digest to produce a water-soluble complex. The increase caused by histidine was not cumulative with that due to the action of pancreatin and erepsin. This suggested that only part of the zinc bound in the casein digest was in a form which could be rendered water soluble by complexing with either histidine or the products of the action of pancreatin and erepsin.

The high capacity of blood components to bind zinc was shown by the large in-

TABLE 7

Dialysis of ^{65}Zn of digests of casein or Venezuelan sesame meal to water or to blood percent dialyzed after 4 hours at 40°

	Dialysis to water		Dialysis to blood	
	Casein [1]	Venezuelan sesame meal [2]	Casein [3]	Venezuelan sesame meal [2]
Digest at intestinal pH	2.0 [c]	2.57 [e]	46.7 [b]	6.34 [c]
Additions:				
Pancreatin and erepsin	11.2 [b]	4.59 [d]	49.9 [b]	9.77 [a,b]
Histidine	25.0 [a]	5.95 [c,d]	72.9 [a]	8.23 [b]
Histidine + pancreatin and erepsin	24.8 [a]	5.03 [c,d]	66.0 [a,b]	10.64 [a]
Arginine	1.4 [c]		48.4 [b]	
Arginine + pancreatin and erepsin	11.6 [b]		54.0 [a,b]	
Lysine	2.1 [c]		51.9 [b]	
Lysine + pancreatin and erepsin	15.0 [b]		58.6 [a,b]	

[1] The digest, equivalent to 10 g of casein (50–60 ml) was dialyzed against 200 ml of deionized water. By Duncan's multiple range test, values which did not differ significantly ($P < 0.05$) have the same letter.

[2] The digest, equivalent to 2.5 g of meal (17 ml) was dialyzed against 40 ml of water or pig blood. Values which did not differ significantly ($P < 0.05$) have the same letter.

[3] The digest, equivalent to 2.5 g of casein (12.5 ml), was dialyzed against 30 ml of pig blood. Values which did not differ significantly ($P < 0.05$) have the same letter.

crease in dialysis of ^{65}Zn when blood was used as the dialyzing medium (table 7). Addition of histidine per se caused a further significant increase in dialysis but when digestion with pancreatin and erepsin accompanied the dialysis, the presence of any of the amino acids did not cause a significant increase. It appeared that the capacity of the blood components to complex the zinc bound in the casein was sufficient to account for availability of the zinc in vivo without the necessity to postulate an intermediate complex.

Dialysis of ^{65}Zn from the Venezuelan meal digest: Addition of histidine to the ^{65}Zn·phytate complex did not increase dialysis of ^{65}Zn significantly over the low dialysis caused by pancreatin and erepsin digestion (table 7). Histidine could form a water-soluble, dialyzable complex with the zinc of the casein digest but it did not compete with phytate.

The blood components bound very little of the zinc of the phytate complex in comparison with the casein digest. Digestion with pancreatin and erepsin, or addition of histidine, or a combination of the two caused small, significant increases in dialysis. The differences, however, between dialysis to water or to blood for any of the treatments were small compared with those obtained with the casein digest. It appeared that phytate formed a more stable bond with zinc than did the blood components. The postulate that intermediate complexes were necessary before appreciable absorption of zinc took place in vivo from the phytate complex of Venezuelan meal appeared to be valid.

The binding of zinc at intestinal pH by the casein digest, the low dialyzability to water and the high dialyzability to blood leads to the postulate that in the intestine the zinc of casein rations is not present as ionic zinc but bound in a water-insoluble complex. Zinc can be made available to the chick through formation of a more stable complex with blood components than that of the casein digest. The intestinal mucosa may play either an active or passive role. The high binding capacity of blood for the zinc of the casein digest plus the fact that zinc *is* readily available to the chick suggests that, irrespective of the binding action of the intestinal mucosa, in the processes of absorption in vivo zinc can be bound by blood components. With Venezuelan sesame meal, however, it appears that a water-soluble complex is not formed during the early stages of digestion in the

intestine nor do blood components complex appreciable amounts of the phytate-bound zinc. Formation of intermediate complexes which will render the zinc either water-soluble or capable of being bound by blood components is necessary. The 61 sesame meal lacks the precursor of these complexes. Complexing of zinc so that it was less water-soluble and dialyzable at intestinal pH also occurred when ^{65}Zn was added to egg albumen, 14% dialysis, and to fish meal, less than 1% (7). These data suggest that in studies of zinc absorption, consideration should be given to the possibility that zinc is not present in the intestine in an inorganic form in the presence of many ration ingredients and that the capacity of the blood to bind the zinc of the complexes may greatly influence absorption of zinc from the intestine.

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Effect of Cortisol on Growth, Food Intake, Dietary Preference and Plasma Amino Acid Pattern of Rats Fed Amino Acid Imbalanced Diets [1]

PHILIP M.-B. LEUNG, QUINTON R. ROGERS AND A. E. HARPER
Department of Physiological Sciences, School of Veterinary Medicine, University of California, Davis, California, and Department of Biochemistry, University of Wisconsin, Madison, Wisconsin

ABSTRACT Since cortisol is known to influence plasma amino acid concentrations and amino acid metabolism in general, rats fed amino acid imbalanced diets have been given cortisol, and growth, food intake, dietary choice and plasma amino acid concentrations have been measured. Food intake and growth of rats fed a threonine- or a histidine-imbalanced diet were depressed. However, food intakes of rats injected with cortisol (1 mg/day) and fed the imbalanced diets were not depressed. When rats were offered a choice between the threonine-imbalanced diet and a protein-free diet, they chose the protein-free diet almost immediately, whereas cortisol-injected rats preferred the imbalanced diet. It has been shown that the most limiting amino acid — threonine — in the plasma of rats fed the threonine-imbalanced diet fell below that of the control and the fasting level. Cortisol caused an elevation of the concentration of threonine and of other indispensable amino acids in the plasma of rats fed the imbalanced diet. The increase in the concentration of amino acids in the plasma of cortisol-injected rats, possibly through some redistribution of amino acids within the body, may account for the prevention, by cortisol, of the food intake depression and the change in food preference of rats ingesting the imbalanced diet.

Addition of an imbalancing amino acid mixture to a low protein diet causes a reduction in food intake and retardation of growth of rats. One of the earliest and most consistent biochemical changes observed in rats fed an imbalanced diet is a marked reduction in the concentration of the most limiting amino acid in blood plasma (1, 2). The possibility exists that alteration in the food intake of rats ingesting the imbalanced diet is caused directly by the change in plasma amino acid pattern (3).

If the plasma amino acid pattern does, directly or indirectly, influence food intake regulation of rats, then modification of the plasma amino acid pattern by some means, such as the administration of a glucocorticoid, should affect the food intake of rats ingesting an imbalanced diet. Since elevation of plasma amino acid concentrations by cortisol has been reported by Friedberg and Greenberg (4), Bondy (5), Lotspeich (6) and Kaplan and Shimizu (7), and since cortisol administration usually causes no depression of food intake of rats fed a nutritionally adequate diet (8), the present studies were undertaken to examine the effect of cortisol on food intake, growth and food preference of rats ingesting an imbalanced diet. To ascertain the effect of cortisol administration on the changes in plasma amino acids, rats trained to eat a single meal daily were injected with cortisol and the plasma-free amino acids were determined in blood samples taken at various intervals during a 24-hour period after feeding a balanced or imbalanced diet.

EXPERIMENTAL

Weanling or young male rats of Holtzman or Sprague-Dawley strains were used in all experiments. They were housed in individual cages with screen bottoms. In the food selection studies, cages of double the usual size were used and extra care was taken to prevent food spillage by using Fisher cups inside a no. 303 tin can in which a one and one-half inch hole was placed about 3 inches from the bottom of

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the can. The rats were fed as described below and water was supplied ad libitum.

Two types of amino acid-imbalanced diets were used. The control diet for the threonine-imbalance studies contained 6% casein supplemented with 0.3% DL-methionine, while that for the histidine-imbalance studies contained 5% casein supplemented with 0.3% L-methionine and 0.2% L-threonine. The threonine-imbalanced diet was prepared by adding 5.4% of a mixture of indispensable amino acids lacking threonine to the 6% casein diet.[2] The histidine-imbalanced diet was prepared by adding 3.6 or 5.4% of the amino acid mixture lacking histidine to the 5% casein control diet.[3] All diets contained salt mixture,[4] 4%; corn oil,[5] 5%; vitamin mixture,[6] 0.5%; choline chloride, 0.2% and dextrin-starch (1:2) mixture as the carbohydrate to make 100%. The "corrected" diets were prepared by adding 0.45% DL-threonine and 0.225% L-histidine·HCl to the threonine-imbalanced or histidine-imbalanced diets, respectively. The amino acids were added at the expense of the mixed carbohydrate.

Cortisol[7] (hydrocortisone acetate), 1mg/rat/day, was administered intraperitoneally or subcutaneously just prior to feeding the animals. Rats receiving the threonine-imbalanced diet were injected at 3 to 4 PM, whereas those fed the histidine-imbalanced diet were injected at 5 to 6 PM daily.

In the plasma amino acid study, rats trained to accept a single 2-hour daily feeding were used. They were trained to eat a 15% casein diet for 2 weeks and then were fed the 6% casein control diet for 3 weeks. They were then selected on the basis of uniformity of body weight and food consumption. The animals were injected with cortisol (1.5 mg/rat) intraperitoneally before being fed either the control or imbalanced diet. The control group was pair-fed against the imbalanced group. Rats fed each of the diets ate approximately 8 g of food. Systemic blood (by cardiac puncture) was taken from 5 rats fed each diet 2, 6, 9, 13, 18 and 24 hours after the feeding period. Blood samples from animals in each group were pooled and the plasma obtained after centrifugation was added to 5 times its volume of 1% picric acid for preparation of a protein-free filtrate as described by Stein and Moore (9). After removing the picric acid with a column of Dowex 2-X8 resin (50 to 100 mesh chloride form), the deproteinized filtrates were analyzed for amino acids using a Technicon amino acid analyzer.

RESULTS

Food intake and change in weight of young rats (80–90 g) fed the imbalanced diet containing 6% casein and 5.4% of amino acid mixture lacking threonine and injected with either saline or cortisol intraperitoneally are shown in table 1 and figure 1. The usual depression in growth and food intake was observed in rats fed the imbalanced diet. The food intake and growth of rats fed the imbalanced diet and injected with cortisol were not depressed even in the early part of the experiment. The total gain in weight of the cortisol-injected imbalanced group was 80% of that of the saline-injected control and 86% of that of the cortisol-injected control. The saline-injected rats ingesting the im-

[2] The mixture of indispensable amino acids devoid of threonine provided: (in percent) DL-tryptophan, 0.32; L-leucine, 1.12; DL-isoleucine, 1.6; DL-valine, 1.68; L-histidine·HCl, 0.48; DL-phenylalanine, 0.72; and L-lysine·HCl, 1.20.

[3] The 3.6% and 5.4% amino acid mixtures lacking histidine contained, respectively: (in percent) L-methionine, 0.2 and 0.3; L-phenylalanine, 0.6 and 0.9; L-leucine, 0.6 and 0.9; L-isoleucine, 0.4 and 0.6; L-valine, 0.4 and 0.6; L-lysine·HCl, 0.6 and 0.9; L-arginine·HCl, 0.4 and 0.6; L-threonine, 0.3 and 0.45; and L-tryptophan, 0.1 and 0.15.

[4] The salt mixture contained: (in percent) $CaCO_3$, 29.29; $CaHPO_4 \cdot 2H_2O$, 0.43; KH_2PO_4, 34.31; NaCl, 25.06; $MgSO_4 \cdot 7H_2O$, 9.98; Fe ($C_6H_5O_7 \cdot 6H_2O$, 0.623; $CuSO_4$, 0.156; $MnSO_4 \cdot H_2O$, 0.121; $ZnCl_2$, 0.020; KI, 0.0005; $(NH_4)_6 MO_7O_{24} \cdot 4H_2O$, 0.0025; and $Na_2SeO_3 \cdot 5H_2O$, 0.0015 The salt mix was prepared by and purchased from General Biochemicals, Inc., Chagrin Falls, Ohio. The salts at 5% of the diet provided: (in percent of element) Ca, 0.592; P, 0.394; K, 0.493; Na, 0.493; Cl, 0.760; Mg, 0.049; Fe, 0.0049; Cu, 0.0019; Mn, 0.00195; Zn, 0.0004; I, 0.000019; Mo, 0.000005; and Se, 0.0000025.

[5] Mazola Oil, Corn Products, Co., New York.

[6] 0.5% of the vitamin mixture in the diet provided the rats with 0.44% sucrose plus the following vitamins: (in mg/kg diet) thiamine·HCl, 5; riboflavin, 5; niacinamide, 25.0; Ca D-pantothenate, 20; pyridoxine·HCl, 5; folic acid, 0.5; menadione, 0.5; *d*-biotin, 0.2; vitamin B_{12} (0.1% in mannitol), 30; ascorbic acid, 50 (added to prevent thiamine destruction); vitamin E acetate (25% in a mixture of gelatin, sugar and starch), 400; vitamin A acetate and vitamin D_2 (325,000 USP units of A per gram and 32,500 USP units of D_2 per gram in a mixture of gelatin, sugar and starch), 12.31. (The thiamine, niacinamide, folic acid, menadione and vitamin B_{12} were purchased from Nutritional Biochemicals Corporation, Cleveland, and the others were purchased from Hoffmann-La Roche, Inc., Nutley, New Jersey.)

[7] Cortisol (Hydrocortisone acetate) in aqueous suspension was purchased from ABCO Dealers, Inc., New York.

TABLE 1

Food intake of rats fed the control, threonine-imbalanced, or corrected diet, and injected intraperitoneally with saline or cortisol

Diet	Treatment	Avg daily food intake [1]		
		Days		
		1–3	3–8	8–14
			g	
Control (6% casein plus 0.3% DL-methionine)	Saline	8.4	8.8	9.3
Control	Cortisol	10.5	11.2	9.5
Imbalanced (control plus 5.4% amino acid mix minus threonine)	Saline	4.7	5.3	6.0
Imbalanced	Cortisol	10.8	9.3	9.4
Corrected (imbalanced plus 0.45% DL-threonine)	Saline	9.3	10.6	10.0
Corrected	Cortisol	11.4	10.6	10.1

[1] Five rats per group.

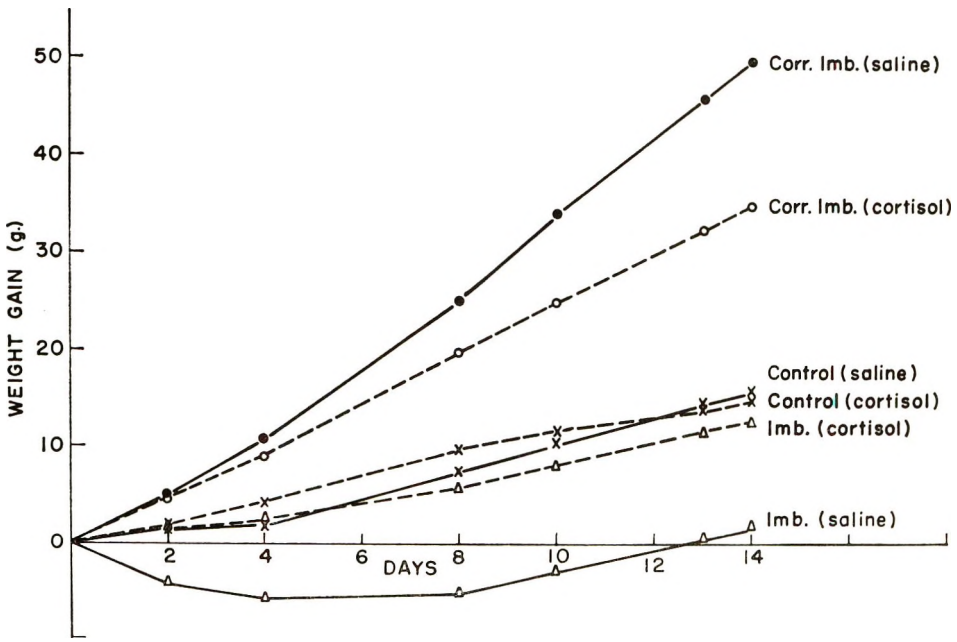


Fig. 1 Effect of cortisol on growth of rats fed ad libitum the control, imbalanced or corrected diet. Control diet: 6% casein plus 0.3% DL-methionine; imbalanced diet: 6% casein plus 0.3% DL-methionine and 5.4% amino acid mixture lacking threonine; corrected diet: imbalanced diet plus 0.45% DL-threonine; saline: saline-injected (intraperitoneally); cortisol: cortisol-injected (intraperitoneally).

balanced diet, on the other hand, gained only 6.7% and 7.2% of the respective saline-injected and cortisol-injected controls. The food intake of the imbalanced group injected with cortisol was 101% and 93%, respectively, of those of the saline-injected and cortisol-injected control groups. Although the food intake of rats ingesting the corrected diet and injected with either saline or cortisol was within the same range, the weight gain of the cortisol-injected group was depressed 23% below that of the saline-injected corrected group. The experiment was re-

peated using slightly younger animals of Sprague-Dawley strain (10 rats/group, 60–80 g) and similar results were obtained except that the cortisol-injected rats ingesting the corrected diet grew at more nearly the same rate as the saline-injected animals.

When the histidine-imbalanced diet was fed to rats (5 rats/group) injected with cortisol (subcutaneously), a similar stimu-

TABLE 2

Food intake of rats fed the control, histidine-imbalanced, or corrected diet, and injected subcutaneously with saline or cortisol

Diet	Treatment	Avg daily food intake		
		Days		
		1–2	2–6	6–14
			g	
Control (5% casein plus 0.3% L-methionine and 0.2% L-threonine)	Saline	9.0	12.4	12.0
Control	Cortisol	14.2	12.5	13.3
Imbalanced (control plus 5.4% amino acid mixture minus histidine)	Saline	3.1	4.7	8.0
Imbalanced	Cortisol	4.6	6.8	10.0
Corrected (imbalanced diet plus 0.225% L-histidine·HCl	Saline	9.4	10.9	13.3
Corrected	Cortisol	9.0	11.1	11.8

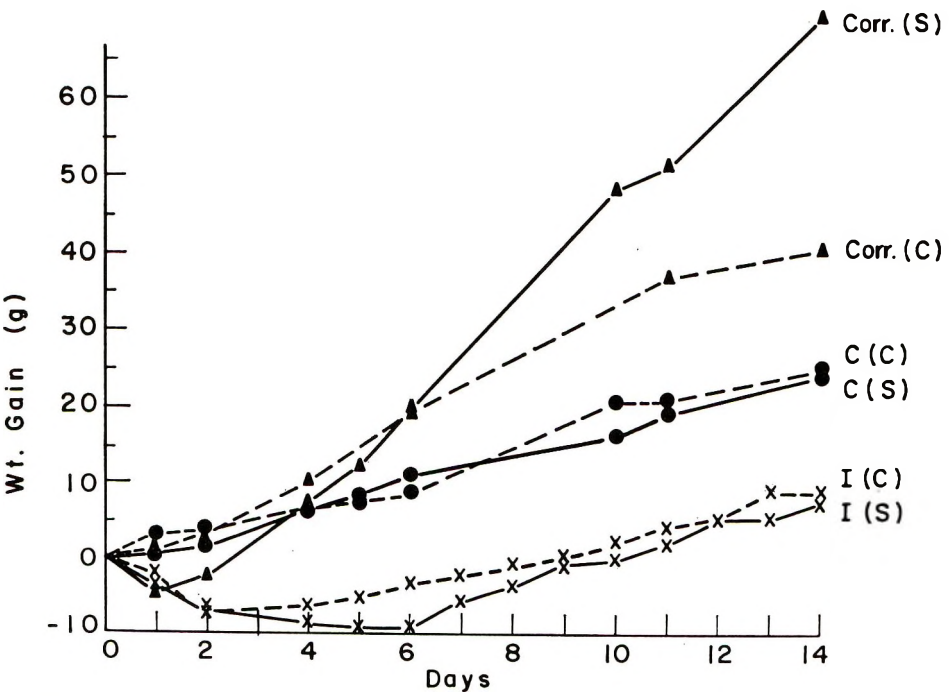


Fig. 2 Effect of cortisol on growth of rats fed ad libitum the control, imbalanced or corrected diet. Control diet: 5% casein plus 0.3% L-methionine and 0.2% L-threonine; imbalanced diet: control diet plus 5.4% amino acid mixture lacking histidine; corrected diet: imbalanced diet plus 0.225% L-histidine·HCl; saline; saline injected (subcutaneously); cortisol: cortisol injected (subcutaneously).

lation of food intake was observed, even though the rats did not gain more weight than those injected with saline. The cortisol-injected control group consumed more food than the saline-injected group but again the gains in weight of both groups were similar. The amounts of food consumed by the cortisol- and saline-injected groups fed the corrected diet were similar, but the cortisol-injected group suffered a 43% depression in growth as compared with the saline-injected corrected group (table 2 and figure 2).

Figures 3 and 4 show the 24-hour food intake pattern of rats (8 rats/group) fed either the control or the threonine-imbalanced diet and injected with either saline or cortisol just prior to feeding. All animals were held without food for 18 hours before they were fed. The food intake of the saline-injected rats ingesting the imbalanced diet was depressed within a few hours, with the greatest depression occurring between 5 and 8 hours. The food consumption of the imbalanced group remained below that of the control group throughout the 24-hour period. However, the food intake of the cortisol-injected rats fed the imbalanced diet was not depressed. Also, cortisol injection had little, if any, effect on the food intake pattern of rats fed the control diet.

The effect of cortisol on food selection of rats offered a protein-free diet and the threonine-imbalanced diet is shown in figure 5. Cortisol was injected intraperitoneally (1 mg/rat) daily. Two out of 5 rats selected the imbalanced diet on day 1. The other 3 rats did not make this selection until day 3. At the end of 2 weeks, when all rats were consuming only the imbalanced diet, saline was substituted for

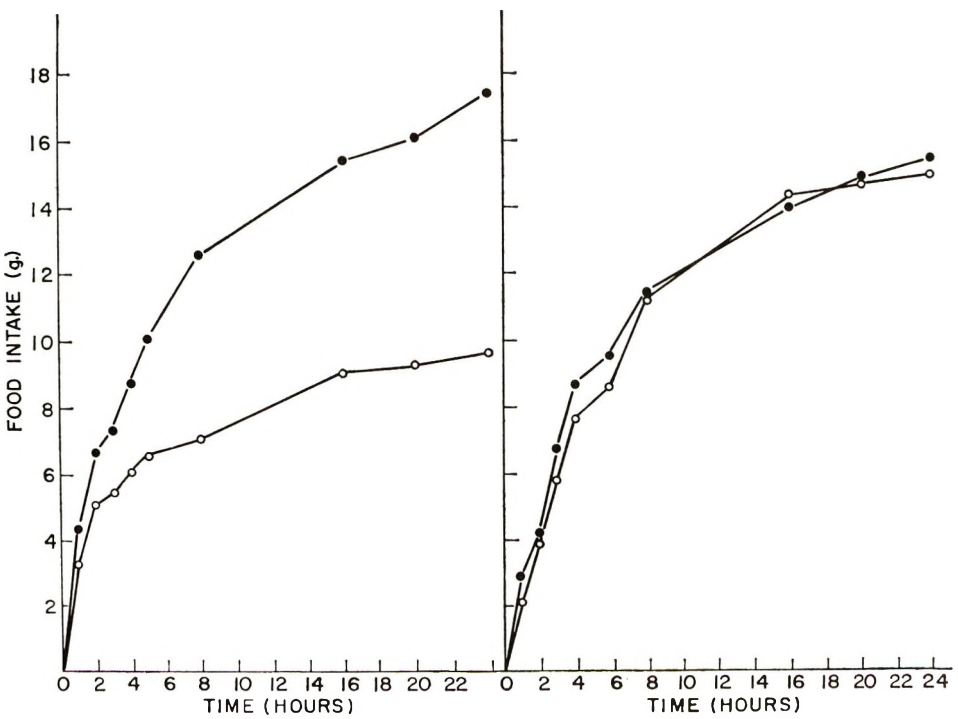


Fig. 3 Twenty-four-hour food intake pattern of saline-injected rats fed the control or the threonine-imbalanced diet.·————· control diet; ○————○ threonine-imbalanced diet.

Fig. 4 Twenty-four-hour food intake pattern of cortisol-injected rats fed the control or the threonine-imbalanced diet. Control diet: 6% casein plus 0.3% DL-methionine; threonine-imbalanced diet: control diet plus 5.4% amino acid mixture lacking threonine. ·————· control diet; ○————○ threonine-imbalanced diet.

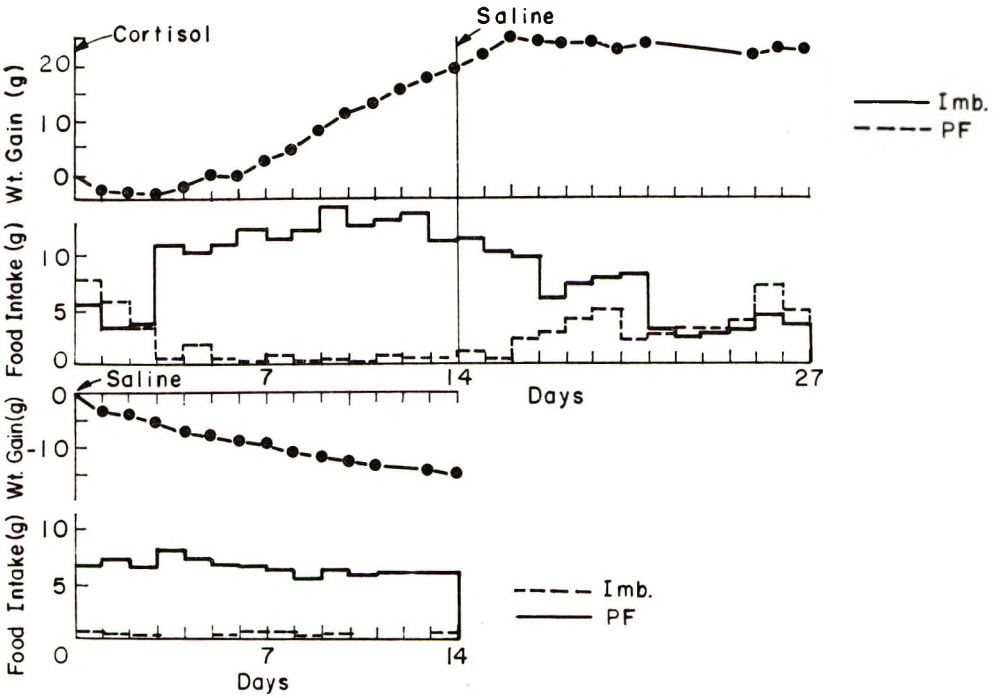


Fig. 5 Changes in body weight and food preference of saline- and cortisol-injected (intraperitoneally) rats fed a protein-free diet and an imbalanced diet simultaneously. Imbalanced diet: 6% casein plus 0.3% DL-methionine and 5.4% amino acid mixture lacking threonine; protein-free diet: same composition as the control diet except that protein and methionine were replaced by mixed carbohydrate (dextrin–starch 1:2).

cortisol. After this only 1 rat continued to consume the imbalanced diet almost exclusively throughout the rest of the experimental period and its total food intake fell; the rest of the animals reduced their consumption of the imbalanced diet gradually over a period of 1 week, and began to consume the protein-free diet in increasing quantities until the end of the experiment. Saline-injected rats selected the protein-free diet immediately when they were offered the choice between the protein-free and the imbalanced diet.

To determine whether rats can discriminate between the threonine-imbalanced diet and the corrected diet (imbalanced supplemented with 0.45% DL-threonine), rats (5/group) with and without injection of cortisol were offered a choice between the 2 diets as shown in figure 6. The saline-injected animals invariably chose the corrected diet over the imbalanced diet and made the choice very quickly, whereas the cortisol-injected animals consumed both diets without showing any specific preference during the 2 weeks. At the end of 2 weeks, cortisol was substituted for the saline injection, and the animals abandoned their preference for the corrected diet after 3 days and started consuming approximately equal quantities of the two diets.

To study the chronic effect of cortisol administration on rats ingesting the imbalanced diet, 2 groups of rats, which had previously been used in the threonine-imbalance experiment were fed the imbalanced diet ad libitum for an additional 4 weeks after completion of the 2-week growth and food intake study. One group of animals continued to receive cortisol injections (intraperitoneally), while the other was injected with saline. After this 6-week period, they were offered a choice between the protein-free diet and the threonine-imbalanced diet for 2 weeks. As

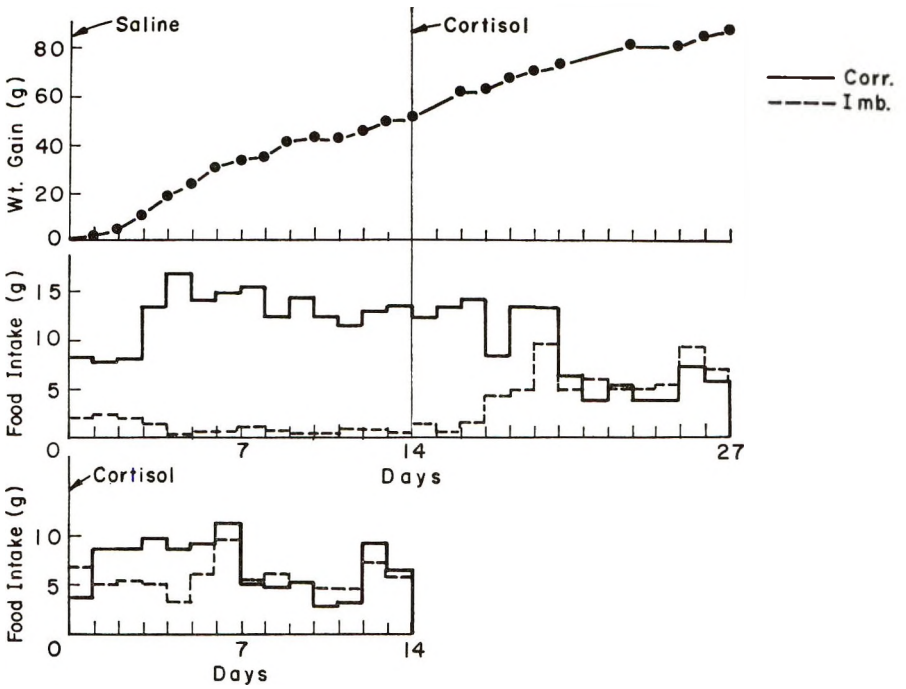


Fig. 6 Changes in body weight and food preference of saline- or cortisol-injected (intraperitoneally) rats fed the imbalanced diet and the corrected diet simultaneously. Imbalanced diet: 6% casein plus 0.3% DL-methionine and 5.4% amino acid mixture lacking threonine; corrected diet: imbalanced diet plus 0.45% DL-threonine.

shown in figure 7, the saline-injected group consumed mostly the protein-free diet throughout the experimental period. Five of the 10 rats selected the protein-free diet almost immediately, while the rest of the animals still consumed some of the imbalanced diet all through the experimental period. The cortisol-injected group, however, chose the imbalanced diet on the first day and continued to do so until the end of the experiment.

The chronic effect of cortisol administration was examined further in another trial. The rats were first fed the histidine-imbalanced diet containing 5.4% amino acid mixture lacking histidine for 2 weeks and were injected with cortisol or saline subcutaneously. Then the amino acid mixture was reduced to 3.6% for another 10 days and cortisol and saline injections were continued intraperitoneally. The rats were then allowed to choose between a protein-free diet and the imbalanced diet containing 3.6% amino acid mixture minus histidine. As shown in figure 8, rats not injected with cortisol invariably refused the imbalanced diet and consumed primarily the protein-free diet. Similar results were obtained when a more severly imbalanced diet containing 5.4% of the imbalancing amino acid mixture was substituted for the less severely imbalanced one on day 11 of the experiment. The cortisol-injected rats immediately rejected the protein-free diet and consumed mostly the imbalanced diets throughout the 4-week experimental period, even though the more severely imbalanced diet was introduced on day 11. The changes in body weight of rats followed closely the changes in food selection, in that the rats lost weight when they selected the protein-free diet and gained weight when they selected the imbalanced diet.

The rates of stomach emptying of meal-fed rats fed the control or the threonine-imbalanced diet were similar.

The changes in the plasma concentrations of amino acids over a 24-hour period in rats that had been injected with cortisol

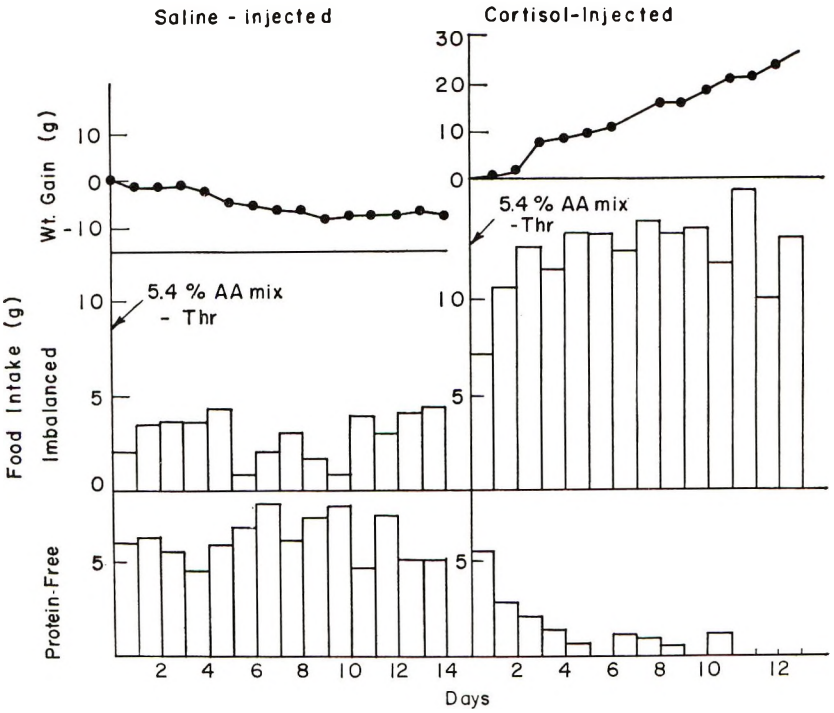


Fig. 7 Effect of long-term saline or cortisol administration (intraperitoneally) on growth and food preferences of rats fed a protein-free diet and the threonine-imbalanced diet simultaneously and injected either with saline or cortisol (10 rats/group). Threonine-imbalanced diet: 6% casein plus 0.3% DL-methionine and 5.4% amino acid mixture lacking threonine; protein-free diet: same composition as the control diet except that protein and methionine were replaced by mixed carbohydrate (dextrin–starch 1:2).

and fed about 8 g of either the control or the imbalanced diet are shown in figure 9. Following cortisol injection, an elevation of the most limiting amino acid, threonine, was observed in the plasma above the fasting level. Although the concentration of threonine in the plasma of the imbalanced group was lower than that of the control until 18 hours after the feeding period, the value was well above the fasting level at all times. The plasma threonine concentration of the imbalanced group began to increase 6 hours after the feeding period reaching the highest point in 18 hours and declining toward the fasting level at the end of the 24-hour period. The plasma threonine concentration of the control group also increased after 6 hours, reaching a maximum in 13 hours and falling steadily toward the fasting level thereafter. The concentration of methionine was similar in the plasma of both groups 9 hours after feeding. The concentrations of methionine 2 and 6 hours after feeding, however, were considerably higher in rats fed the imbalanced diet.

All of the amino acids added to cause the imbalance, except lysine, showed an early peak 2 hours after feeding the imbalanced diet. The changes of the plasma valine and phenylalanine concentrations for the imbalanced group were similar, in that, they increased sharply to the maximum value at 2 hours and declined rapidly by 6 and 9 hours, respectively. The plasma valine and phenylalanine concentrations of the control were relatively constant throughout the 24-hour period.

Leucine concentration also rose rapidly in the plasma of rats fed the imbalanced diet and remained higher than the control throughout. Changes in the isoleucine concentration in the plasma of the imbalanced group were similar to those for leucine. The

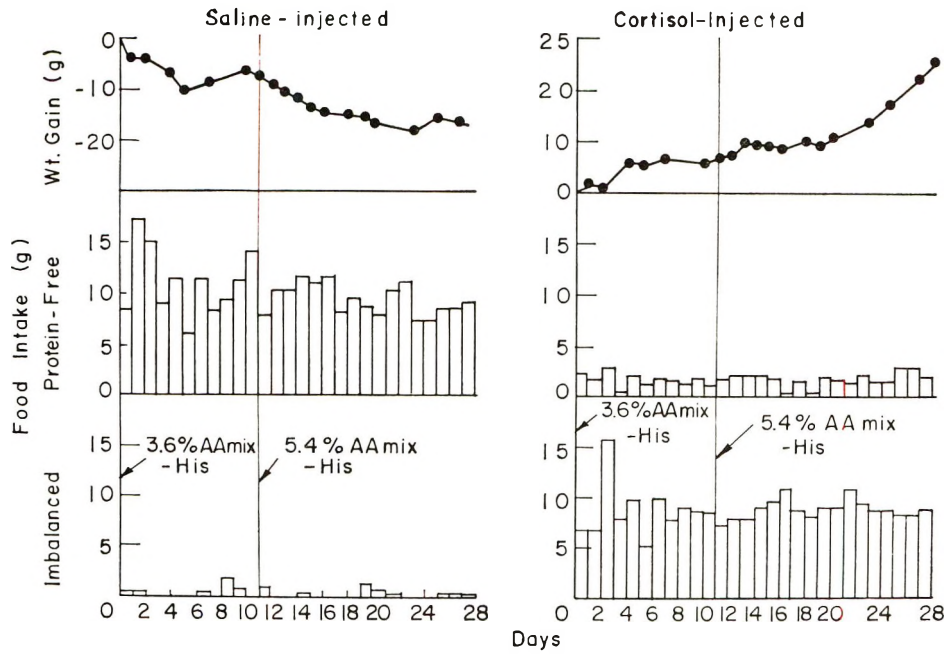


Fig. 8 Effect of long-term saline or cortisol administration (intraperitoneally) on growth and food preference of rats fed a protein-free diet and the histidine-imbalanced diet simultaneously (5 rats/group). Histidine-imbalanced diet: 5% casein plus 0.3% L-methionine, 0.2% L-threonine and 3.6% or 5.4% amino acid mixture lacking histidine.

lysine concentration in the plasma of the imbalanced group increased less rapidly after feeding. The lysine concentration of the control group was below that of the imbalanced group at all times. The histidine concentration of the group fed the imbalanced diet increased shortly after feeding, but fell below that of the control at 6 hours. The control histidine values were quite constant.

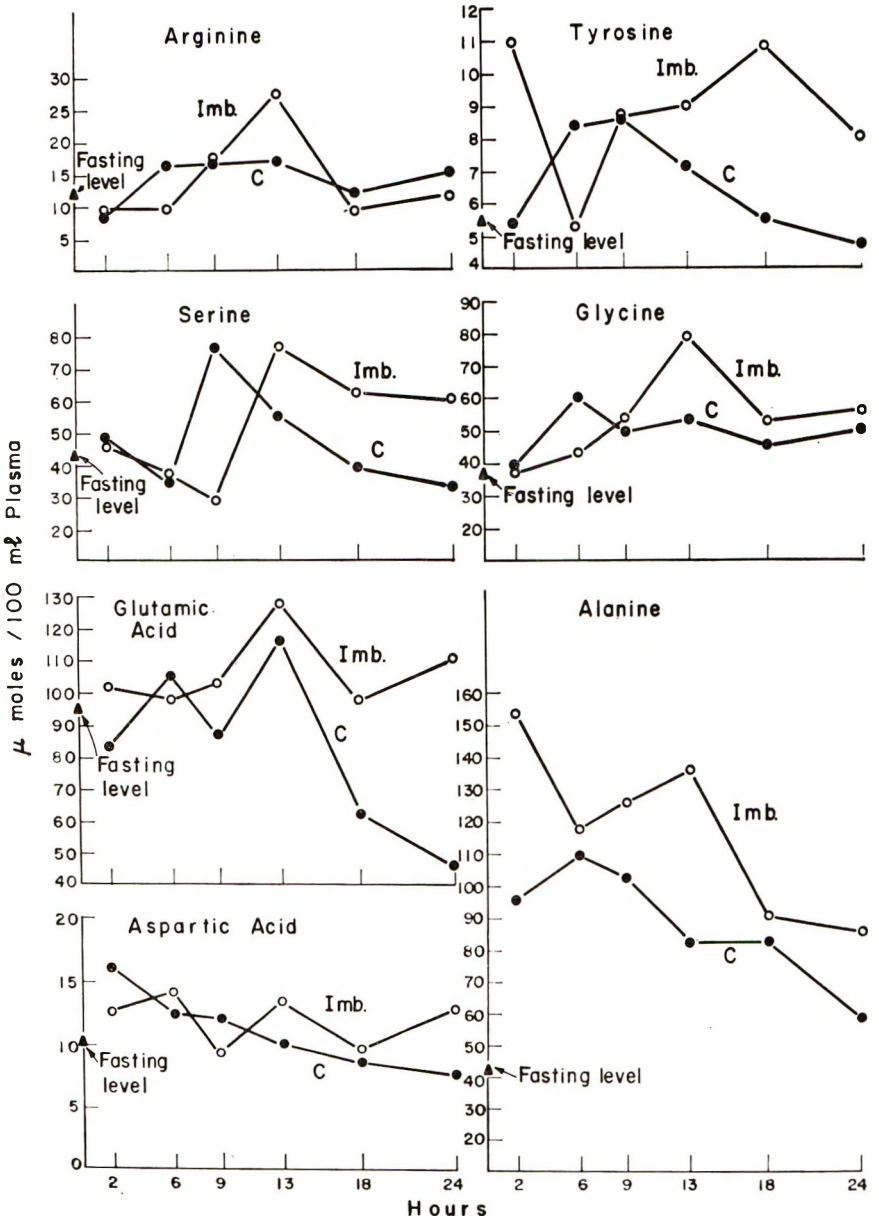
The concentrations of arginine and glycine for the imbalanced groups were higher than for the controls at 13 hours. No marked changes were observed in the plasma concentrations of arginine and glycine for the control groups.

Serine, tyrosine, alanine and glutamic acid exhibited irregular patterns in groups fed either of the 2 diets. Aspartic acid in the plasma of the control or imbalanced group did not show pronounced peaks, and the values were either slightly above or below the fasting value throughout the 24-hour period.

DISCUSSION

The food intake of rats fed the threonine-imbalanced diet increased when they were injected with cortisol; the increase in food intake was accompanied by an increased growth rate. Similar results were obtained with rats fed the histidine-imbalanced diet and injected with cortisol but the stimulations of food intake and growth were smaller. This indicates that the effect of cortisol in stimulating food intake and growth may depend upon the catabolic characteristics of the amino acid that is most limiting in the imbalanced diet. The slow catabolic rate of threonine, as pointed out by Charkey et al. (10) and demonstrated by Ousterhout (11), as compared with that of histidine, may account for the difference in magnitude of food-intake stimulation of cortisol-injected rats ingesting the 2 different imbalanced diets.

In some instances in the present study, the expected benefit from increased food intake may be offset by the catabolic effect of cortisol, which is apparently dependent


Arginine
Imb.
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Tyrosine
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Serine
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Glycine
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Glutamic Acid
Imb.
C
Fasting level
Alanine
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Aspartic Acid
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Fasting level
μ moles / 100 ml Plasma
Hours
2 6 9 13 18 24

Figure 9

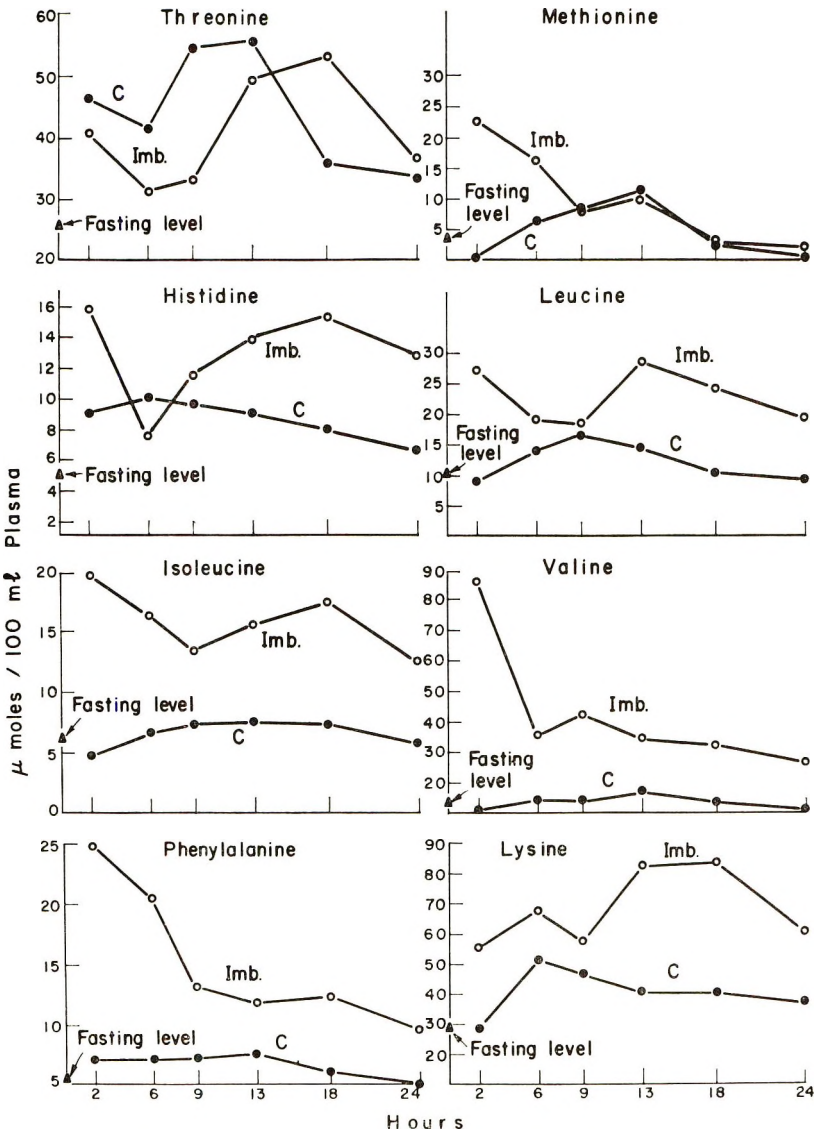

Fig. 9 Effect of cortisol on amino acid concentrations in plasma of rats trained to eat a single 2-hour meal daily and fed the control or the imbalanced diet. C: Control diet (6% casein plus 0.3% DL-methionine); Imb: imbalanced diet (control diet plus 5.4% amino acid mixture lacking threonine). All rats were injected (intraperitoneally) with 1.5 mg of cortisol just before feeding.

on the dose and route of administration. As reported by Wells and Kendall (12) cortisol (injected subcutaneously) is known to inhibit growth of animals. Bellamy (8) also found that cortisol (injected subcutaneously) caused a rapid inhibition of growth, but that it had no significant effect on food intake of rats fed a nutritionally adequate diet. He suggested that cortisol may produce biochemical changes leading to inhibition of growth which are independent of an effect on food intake. Greenspan et al. (13) compared two modes of administration (i.e., subcutaneous versus intraperitoneal) and found that intraperitoneal injections did not cause weight loss and adrenal atrophy, whereas the same dose of cortisol injected subcutaneously produced both weight loss and adrenal atrophy. This may account for the fact that the rats fed the histidine-imbalanced diet and injected with cortisol subcutaneously, suffered growth depression although the food intake was increased. Since the present study agrees with earlier reports by other workers that cortisol treatment elevates plasma amino acids (4–7), and since intraperitoneally injected cortisol is rapidly absorbed and excreted (13, 14), it might be expected that the shorter duration of an effective cortisol level produced by intraperitoneal injection would modify the blood amino acid pattern, but might not provide a sustained stimulus long enough to produce the maximum catabolic effect. Certainly a significant generalized amino acid catabolic effect could not have occurred without affecting growth since the low protein content of the control diet severely limits growth.

It has been suggested that some undesirable physiological or biochemical change may be associated with the sharp drop in the concentration of the most limiting amino acid in blood plasma of rats ingesting the imbalanced diet, and that these changes may be responsible for the depression in food intake (3). Hence, the observations that cortisol-injected rats selected the imbalanced diet over the protein-free diet and that they failed to discriminate between the corrected and the imbalanced diets suggest that the specific physiological or biochemical effect produced by the ingestion of the imbalanced diet is abolished by cortisol treatment. The results of the food selection studies show that rats injected with cortisol preferred the imbalanced diet over the protein-free diet as long as cortisol was administered. Of interest in relation to this is that the plasma concentration of the most limiting amino acid did not fall below the fasting level when cortisol was injected.

The variation among rats in adapting to the threonine-imbalanced diet can be seen from the results of the food selection study. Half of the saline-injected animals fed the imbalanced diet ad libitum for 4 weeks selected the protein-free diet immediately when they were offered the choice, while the other half did not reject completely the imbalanced diet throughout the experimental period. These results indicate that, although the rat will adapt and grow when fed the imbalanced diet for several weeks, the "metabolic response" to the amino acid imbalance is still evident, though perhaps less severe.

The 24-hour food intake pattern of rats injected with cortisol shows that cortisol administration (intraperitoneally) was effective in stimulating the food intake of rats ingesting the imbalanced diet within a short period of time. This result, then, explains the selection of the imbalanced diet by cortisol-injected rats on day 1 of the choice experiment. Cortisol had little, if any, effect on the food intake pattern (24 hour) of rats consuming the control diet, indicating that cortisol acted to enhance specifically the food intake of rats fed the imbalanced diet and that it apparently induces a higher intake of the different imbalanced diets through similar mechanisms—possibly by raising the level of the most limiting amino acid in the plasma. On the other hand, cortisol is known to affect amino acid metabolism in many ways, especially by inducing some of the enzymes in amino acid catabolism; this could also affect the plasma amino acid pattern of rats ingesting the imbalanced diet. Although the results of the plasma amino acid studies are in accord with the hypothesis (3) that the plasma amino acid pattern may influence the regulation of food intake, more information is needed to establish with certainty whether cortisol altered the feeding behavior of rats in-

gesting the imbalanced diet through this or some other mechanism.

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Effect of Dietary Fluoride on Food Intake and Plasma Fluoride Concentration in the Rat [1]

GAD SIMON [2] AND J. W. SUTTIE
Department of Biochemistry, University of Wisconsin, Madison, Wisconsin

ABSTRACT The effect of excessive amounts (200–600 ppm) of dietary fluoride on the food intake and plasma fluoride concentration of rats of various ages was investigated. In older animals, the decline in food intake was partially corrected within a week even though the plasma fluoride concentration remained elevated. When the same amounts of fluoride were fed to younger animals, a longer exposure to the diet was required before an improvement in food intake was seen. The ability of the rat to increase its consumption of the high fluoride diet following an initial drop in consumption suggests some type of adaptation to the elevated plasma and soft tissue fluoride concentrations.

The effects of ingestion of excessive amounts of dietary fluoride by animals have been extensively studied and reviewed (1–3), and one of the major physiological responses repeatedly noted is a decline in growth rate. This decline is the result of a marked decrease in food consumption by the animals, and some studies have specifically indicated that the degree to which the consumption is decreased is lessened as the period of exposure is extended (4). It has been shown also that the ingestion of a high fluoride diet by the rat results in an increase in plasma fluoride content (5), and that the decline in food consumption, at least in short-term experiments, is inversely correlated with this increased plasma fluoride.

This study was designed to investigate the response of food intake to dietary fluoride in more detail, and to further study the relationship between dietary fluoride, plasma fluoride, and food intake.

METHODS

Female rats of the Holtzman strain [3] of varying ages were maintained in individual cages in an air-conditioned room with a 12-hour light-dark cycle. They were fed a semipurified diet (table 1) to which fluoride was added as NaF and were given distilled water ad libitum.

Dietary intake was measured daily, and plasma samples for fluoride determination were obtained in the morning by a heart puncture within 1 hour of the end of the night dark period. Fluoride analyses of the plasma were carried out by the method of Singer and Armstrong (6). To clarify the graphic presentation of the data, food intake values for successive 3-day periods have been averaged, and these, rather than individual day consumptions, have been plotted on the figures.

RESULTS

The data presented in figure 1 indicate the response of 120 g female rats to diets containing varying amounts of fluoride. There was an immediate effect on growth at 400 or 600 ppm F, but little if any effect at 200 ppm. At an ingestion level of 400 ppm F there appeared to be a period of about 1 week during which growth was stabilized, followed by a growth rate which was comparable to that of control rats. At 600 ppm F there was an actual loss of weight, and initial weight was not regained for about 2 weeks. The growth rate subsequent to this was only slightly below that of the controls. The graphs of actual food intake at the various ingestion levels indicate that there was a sharp drop in food

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[2] Present address: Israel Institute for Biological Research, POB 19, Ness Ziona, Israel.

[3] Obtained from the Holtzman Company, Madison, Wisconsin.

TABLE 1

Composition of experimental diet

Ingredient	% of diet
Casein	23.5
Sucrose	66.0
Salts B[1]	5.0
Corn oil	5.0
Vitamin premix [2]	0.5
	100.0

Supplementary additions

1.0 choline·HCl/kg diet in 25% ethanol solution

0.125 g α-tocopherol/kg diet in corn oil

NaF added to the fluoride diets at the level indicated

[1] Harper, A. E. 1959 J. Nutr., 68: 405.

[2] The vitamin premix contained per kilogram: inositol, 20 g; Ca-pantothenate, 4 g; niacin, 2 g; menadione, 0.8 g; riboflavin, 0.6 g; thiamine·HCl, 1.2 g; pyridoxine·HCl, 0.5 g; biotin, 20 mg; folic acid, 40 mg; vitamin B_{12}, 2 mg; vitamin A, 800,000 IU; and vitamin D, 200,000 IU.

intake which reached its minimum within a few days, and then increased until it was near the control values. It is apparent that after a few weeks on the diets containing 400 or 600 ppm fluoride, food intake, on a body weight basis, was elevated over that of the controls, and was comparable to the intake of the control rats when they were that size.

The results of 2 similar experiments, which indicate that weanling rats appear

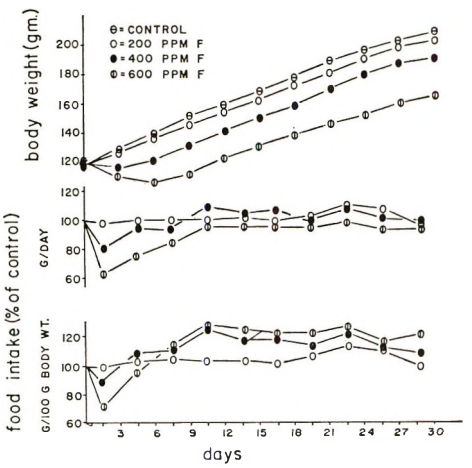

Fig. 1 Growth rate and food intake of female rats (120 g) fed fluoride in the diet, 12 rats/group. Note that in this and subsequent figures, the food intake of the experimental groups is expressed as a percentage of the control group and no curve for the control group is included. The food intake of the control group averaged 11.7 g/day (9.1 g/100 g body weight) at the start of the experiment and 13.3 g/day (6.5 g/100 g body weight) at the end.

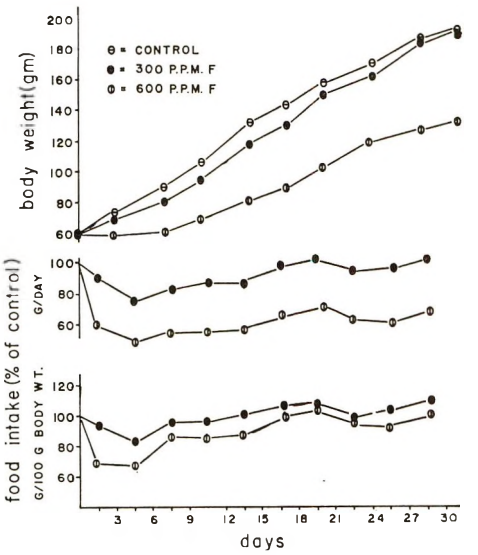

Fig. 2 Growth rate and food intake of weanling female rats fed fluoride in the diet, 10 rats/group. The food intake of the control group averaged 7.9 g/day (10.8 g/100 g body weight) at the start of the experiment and 11.6 g/day (6.4 g/100 g body weight) at the end.

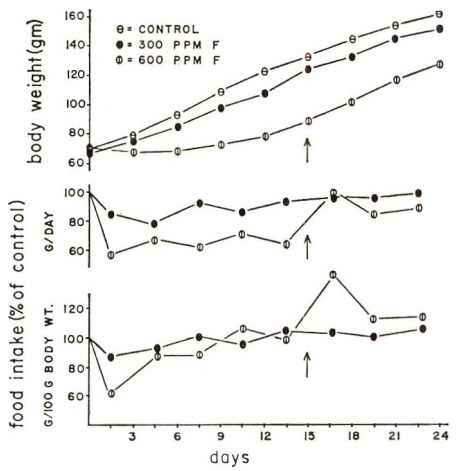

Fig. 3 Growth rate and food intake of weanling female rats fed fluoride in the diet. At day 15, indicated by the arrow, the rats fed fluoride were given the control diet, 15 rats/group. The food intake of the control group averaged 9.6 g/day (12.4 g/100 g body weight) at the start of the experiment and 12.9 g/day (8.2 g/100 g body weight) at the end.

to be more severely affected by the fluoride, are shown in figures 2 and 3. In weanling animals the food intake of those fed 600 ppm F did not recover with time to the extent that it did in the older animals, and even on a body weight basis did not exceed that of the controls. The observation that food intake, on a g/100 g body weight basis, of the animals fed fluoride approached or even exceeded that of the control animals in these experiments did not indicate that fluoride was no longer exerting an appetite-depressing effect. This is illustrated in figure 3, where it can be seen that the removal of fluoride from the diet at day 15 caused a pronounced increase in food consumption of the rats fed 600 ppm F even though they had previously been consuming about the same number of grams of diet/100 g body weight per day as had the controls.

As previous experiments (5) have indicated a close relationship between decreased food intake and plasma fluoride level, it was of interest to follow the changes in plasma fluoride with time. Rats which were 260 g at the start of the experiment were fed the control diet, 300 or 600 ppm F for a period of 42 days. Blood samples were obtained at day 3, day 6, and then at approximately weekly intervals. As the heart punctures were frequent enough to have an effect on food consumption and body weight, only the plasma fluoride data from this experiment are presented in table 2. At all periods sampled, the plasma fluoride concentration of rats receiving either 300 or 600 ppm F was higher than the controls. Although there was some variation from one sampling

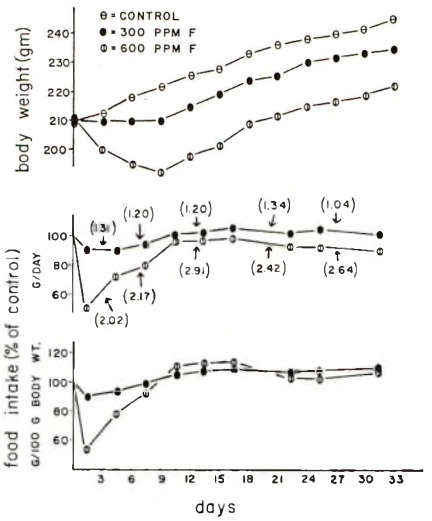

Fig. 4 Growth rate, food intake, and plasma fluoride concentrations in female rats (210 g) fed fluoride in the diet. The food intake of the control group averaged 13.1 g/day (6.1 g/100 g body weight) at the start of the experiment and 13.5 g/day (5.6 g/100 g body weight) at the end. The numbers in parentheses are the plasma fluoride concentrations of 6 rats removed from that group at the times indicated by the arrows. The SE of the mean for the values indicated ranged from 5 to 10% of the mean and the plasma fluoride concentrations determined for the control rats were similar to those seen in table 2. Food consumption and body weight data are based on 36 rats/group at the start of the experiment, and 6 remaining at day 33.

period to another, the plasma fluoride concentration of the 300 ppm F rats did not appear to increase following the initial sample which was obtained at day 3, and tended to average about 1.2 ppm for the entire experiment. In rats fed 600 ppm F, the plasma fluoride concentration appeared

TABLE 2

Plasma fluoride concentrations of rats fed fluoride in the diet [1]

Days on experiment	Control diet	300 ppm F	600 ppm F
		ppm F in plasma	
3	0.31 ± 0.06	1.26 ± 0.10	1.19 ± 0.09
6	—	1.40 ± 0.11	2.10 ± 0.23
13	—	1.28 ± 0.19	2.18 ± 0.18
20	0.40 ± 0.08	1.16 ± 0.07	3.04 ± 0.30
27	—	0.93 ± 0.12	2.61 ± 0.17
35	0.16 ± 0.03	1.13 ± 0.13	2.88 ± 0.21
42	—	1.27 ± 0.07	3.31 ± 0.43

[1] Female rats (260 g) were fed the control or fluoride diets ad libitum. Blood samples were obtained from half of the 16 rats in each group at each sampling period indicated. Values are expressed as mean ± SE.

to increase for about 2 to 3 weeks, and then maintained a concentration near 3 ppm.

This experiment was repeated with sufficient number of rats (200 g) in each starting group so that animals could be removed from the experiment at the time the blood was drawn. This removed the influence of the blood loss on food intake, and growth curves and food intake (fig. 4) can be compared with the data in figures 1–3. It was apparent that the effect of fluoride on these rats was similar to that seen in rats weighing 120 g (fig. 1). The drop in food intake was severe enough to cause a temporary loss of weight in the animals fed 600 ppm F, but when growth was resumed, it was at a rate which was comparable to that of the control rats, and food intake on a g/100 g body weight basis was similar to that of the controls.

The plasma fluoride concentrations seen in the experiment were similar to those shown in table 2. The rats fed the 300 ppm F diet maintained a plasma fluoride concentration of about 1.2 ppm from day 3 to the end of the experiment, while the rats consuming 600 ppm F demonstrated some increase in plasma fluoride concentration for the first 2 weeks and then remained rather stable.

DISCUSSION

These data indicate in some detail the effect of one particular toxic agent, in this case fluoride, on food consumption. It is apparent that the drastic, sharp decline in dietary intake which has often been noted in a fluoride toxicity is in a sense a temporary response, and that food intake returns to more normal levels after a period of reduced consumption. In fact, animals not exposed to fluoride until they were over 100 g were able, after a short period of retardation, to maintain a growth rate which was similar to that of the controls. The data in figure 3 however, show that even though food intake may appear to be stabilized at near the control level in animals fed fluoride, it can temporarily increase to much higher levels if the fluoride is removed from the diet.

Previous work from this laboratory (5) has shown that a concentration of about 3 ppm F in the plasma of the rat was associated with a drastic curtailment of food intake. The rats fed 600 ppm F in these experiments, however, were able to increase their food intake during a period when the plasma F concentration was increasing and approaching this value. The previous studies were of a few days duration only, and the performance of the animals in these current experiments would indicate that there is some type of adaptation to the high fluoride level in the plasma and soft tissues. We have previously postulated (5) that the relationship between food intake and plasma F is an indirect one, mediated through an effect on tissue enzyme activity which results in a shift in the concentration of some metabolite(s), and that this altered balance of tissue metabolites, rather than the increase in fluoride concentration, is the signal for the depressed dietary intake. The ability of the animal to increase its dietary intake in the presence of a continued high tissue fluoride concentration could then be explained by an alteration of metabolic pathways, resulting in a return of metabolite concentrations to more nearly normal. That alterations in at least some metabolic pathways do occur is evident from changes in carbohydrate (7) and lipid [4] metabolism demonstrated in the rat fed fluoride. An alternate explanation would be that the effect of fluoride is on the mechanisms involved in the short-term regulation of food intake only, and that if this is interfered with for a significant period, those factors involved in the long-term regulation of body weight (8) are able to reverse the depression in food intake.

It appears significant that the response of food intake to the inclusion of this toxic agent in the diet is very similar to that which has been observed in an amino acid imbalance (9) and in amino acid toxicities (10). Here again animals adapt to the nutritional disorder, they gradually increase their intake of such diets as they continue to ingest them. Further study of the response of animals to diets such as these might well provide insight into the regulation of food intake in the normal animal.

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Deposition of Linoleic and Linolenic Acid in Rat Adipose Tissue [1]

JEANNETTE H. FORSYTH,[2] ROBERTA SHAFTEL AND D. M. HEGSTED
Department of Nutrition, Harvard School of Public Health, Boston, Massachusetts

ABSTRACT Studies were designed to determine whether there was competition between linoleic and linolenic acid deposition in adipose tissue of rats. Diets containing different amounts and ratios of linoleic and linolenic acid were fed to adult rats. The deposition of these and other fatty acids into the epididymal fat pad was measured by comparing the fatty acid content of the left pad at the beginning and the right pad at the termination of the experiment. The deposition of these fatty acids appeared to be linearly related to the amount eaten, when the total fat content of the diet and the time of the experiment were the same, and no interrelation or competition between these fatty acids in the formation of adipose tissue could be shown.

The metabolism of the unsaturated fatty acids in heart and liver are interrelated. Holman (1) has summarized the evidence indicating that all or nearly all of the unsaturated acids inhibit the elongation or desaturation of other fatty acids; however, the situation in adipose tissue has not been extensively studied. In the work of Mohrhauer and Holman (2) various amounts of linolenic and linoleic acids were fed to rats and the composition of the adipose tissue examined. The feeding of linolenate was associated with lower percentages of linoleic acid in the adipose tissue and the percentage of oleic acid was decreased by feeding either linolenate or linoleic acid. As we have shown (3), however, changes in the percentage composition of adipose tissue can be deceptive. Unless the total amount of adipose tissue remains constant, the percentage of a fatty acid in the adipose tissue may fall, remain constant, or increase while the fatty acid is being actively deposited in adipose tissue. Any change in the percentage of a specific fatty acid is affected by the rates of deposition or mobilization of other fatty acids in the tissue as well as those of the specific fatty acid itself.

It would not be surprising if linoleic and linolenic acids competed with each other in the formation of the adipose tissue triglycerides. The studies in this paper were undertaken to determine whether this was true.

MATERIALS AND METHODS

The studies were done with male rats weighing between 200 and 250 g which had been fed laboratory pellets. The experimental procedure was the same as previously described (3). At the start of the experiment the left epididymal fat pad was removed and analyzed for total fat and fatty acid composition. The animals were then given the experimental diets ad libitum, food intake was measured, and the remaining fat pad was obtained for analysis after 13 or 14, or 28 or 29 days, when the animals were killed. Four rats were killed at each time period from each group. The difference in the content of specific fatty acids in the right and left fat pad is a measure of the increase or decrease of that particular fatty acid during the experimental period. No attempt was made to evaluate the quantities of fatty acids present in only trace amounts.

The purified diets [3] contained either 10 or 20% of safflower or linseed oil or mix-

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[2] Present address: Assistant Professor of Biology, Simmons College, Boston, Massachusetts.
[3] The 20% diets contained: (in percent) casein, 20; glucose, 55.6; salts IV (4), 4; oil mixture, 20; choline, 0.2; and L-cystine, 0.2. The 10% oil diets contained 65.6% glucose. Each 100 g of diet was supplemented with the following: 0.4 mg thiamine; 0.4 mg pyridoxine·HCl; 0.8 mg riboflavin; 4 mg niacin; 0.2 mg biotin; 2.5 mg calcium pantothenate; 0.1 mg folic acid; 0.015 mg vitamin B_{12}; 0.5 mg vitamin K_1; 100 mg α-tocopherol; 9,750 USP units vitamin A; and 975 USP units vitamin D_2.

TABLE 1

Fatty acid composition of dietary fats containing linseed and safflower oil

Diet no.	Composition of mix		Fatty acid composition				
	Safflower	Linseed	16:0	18:0	18:1	18:2	18:3
	%	%	%	%	%	%	%
1 and 5	100.0	—	6.0	2.7	12.5	78.8	—
2 and 6	66.6	33.3	6.2	3.4	14.5	57.1	18.4
3 and 7	33.3	66.6	5.5	3.7	17.8	37.8	35.4
4 and 8	—	100.0	6.1	3.7	19.0	15.2	55.9

TABLE 2

Percentage composition of adipose tissue [1]

Group	Diet		Time on experiment	Fatty acid composition of adipose tissue						
	Kind of oil	Amount of oil		14:0	16:0	16:1	18:0	18:1	18:2	18:3
		%	*days*	%	%	%	%	%	%	%
	Chow (before experiment)			2.40	22.60	5.20	6.50	38.70	22.00	2.30
1	Safflower	20	14	1.00	16.56	4.21	3.26	21.76	53.21	—
			29	0.77	13.45	3.16	2.93	19.28	60.39	—
2	2/3 safflower 1/3 linseed	20	14	1.46	15.46	4.00	3.29	24.87	41.39	10.04
			29	0.91	13.26	3.39	3.39	22.74	44.80	11.87
3	1/3 safflower 2/3 linseed	20	14	1.01	15.17	3.79	3.84	26.33	29.99	19.85
			29	0.70	12.06	2.86	3.38	23.82	33.56	23.91
4	Linseed	20	14	0.98	14.99	3.81	3.95	27.57	17.15	31.53
			29	0.72	11.75	2.92	3.62	24.85	17.64	38.50
5	Safflower	10	13	1.12	19.16	5.84	3.43	24.87	46.25	—
			28	1.09	17.91	5.64	3.25	22.94	48.39	—
6	2/3 safflower 1/3 linseed	10	13	1.25	19.11	5.59	3.93	28.91	33.60	7.60
			28	1.33	19.88	6.74	3.32	25.60	34.68	8.46
7	1/3 safflower 2/3 linseed	10	13	1.36	21.80	6.91	4.23	29.70	22.70	13.31
			28	1.75	17.25	5.24	3.49	26.09	27.71	19.22
8	Linseed	10	13	1.37	20.73	6.58	4.30	30.54	15.74	20.74
			28	1.17	18.18	7.10	3.46	29.42	12.29	28.38

[1] Tissue from 4 rats in each group analyzed individually.

tures of these. These oils differed primarily in their content of linoleic and linolenic acid and thus provided diets with varying levels of these two fatty acids (table 1). The content of the remainder of the fatty acids was similar in the diets although linseed oil contained slightly more oleic acid than safflower oil. Since the diets were fed at either 10 or 20% of oil and for approximately 2 and 4 weeks, the intakes of linoleic and linolenic acid varied substantially. To keep oxidation of the polyunsaturated fatty acids at a minimum, the diets were supplemented with a high level of α-tocopherol and they were prepared fresh each week and stored in a freezer. Food cups were cleaned daily and filled with fresh food.

RESULTS

The percentage composition of the adipose tissue at the start of the experiment and after feeding the experimental diets for 2 to 4 weeks are shown in table 2. The diets caused a substantial fall in palmitic (16:0) and oleic (18:1) acid content accompanied by increases in either linoleic (18:2) or linolenic (18:3) acid. The feeding of linseed oil which contained approximately 15% linoleic acid resulted in a slight decrease of this fatty acid in the adipose tissue. The changes in linoleic

and linolenic acid content were also dependent upon the amount of fat in the diet, the changes being less pronounced with the diets which contained 10% fat (groups 5–8) than with those which contained 20% fat (groups 1–4). Palmitoleic acid (16:1) content remained at approximately beginning levels with the 10% fat diets but fell with the feeding of the 20% fat diets. Most of the changes in percentage composition were accomplished during the first 2 weeks of the experiment.

These data may give the impression that there is a replacement of palmitic and oleic acid by the polyunsaturated fatty acids and that deposition of the polyunsaturated fatty acids occurs to only a limited extent after week 2 of the experiment. An inspection of table 3 which gives the amounts of fatty acids deposited in the epididymal fat pad during the 2- week and 4-week periods is more meaningful. It is apparent that deposition of all fatty acids continued between the second and fourth weeks with the growth of the fat pad. This was true of palmitic and oleic acid even though the percentage of these fatty acids in the adipose tissue decreased from that found in the original fat pads. Similarly, the deposition of linoleic and linolenic acid continued at nearly equivalent rates during the early and later parts of the experiment even though the percentage composition showed little change.

Comparison of the average amounts of linoleic and linolenic acid deposited in the fat pad with the amounts eaten indicates that linoleic acid was slightly more efficiently deposited during the early part of the experiment and that both linoleic and linolenic acid were retained somewhat more efficiently in groups 5–8 which received the diets containing the lower level of fat. This occurred despite the fact that the animals gained less weight and the growth of the fat pads was slightly less than in the animals in groups 1–4. However, the deposition of linoleic and linolenic acid per gram of these fatty acids consumed appeared to be essentially the same at comparable times and with the same level of fat in the diet. The data from animals in groups 1–4 and 5–8 which were killed at the same time were combined and the regression equations relating the amount eaten to the amount deposited were calculated. These equations are shown in figure 1. The results indicate a linear relationship between fatty acid consumption and deposition. The regression coefficient is a better estimate of the efficiency of fatty acid deposition than the mean values given in table 3. Linoleic acid was deposited in the fat pad at a rate of approximately 23.5 mg/g eaten during the first 2 weeks with diets containing 20% fat compared with 36 mg/g eaten with the diets containing 10% fat. Lower values were found during the 4-week period.

Linolenic acid was also more efficiently deposited on the lower fat diets but the rates of deposition were comparable during the 2- week or 4-week period. Linolenic acid was slightly, but not markedly, less efficiently retained than linoleic acid.

DISCUSSION

The data emphasize the conclusion drawn in the previous paper (3) that changes in percentage composition of adipose tissue may be completely misleading. The fall in percentage of palmitic and oleic acid under the conditions studied here might lead one to calculate loss rates of these acids or turnover rates which would be completely unrealistic since it is clear that deposition of these fatty acids occurred during the time the percentage of these fatty acids in the tissue fell. The fall in the percentage of palmitic and oleic acid in these studies simply reflected a slower rate of deposition than the deposition of other fatty acids, mainly linoleic and linolenic acid. Similarly, a calculation of the turnover rate of linoleic or linolenic acid from the percentage composition would lead to a value with little meaning since the percentage composition was nearly stabilized after 2 weeks but the actual deposition continued at almost the same rate between weeks 2 and 4 of the experiment.

Under the conditions of these experiments, in which the amount of adipose tissue was rapidly increasing, the deposition of linoleic and linolenic acid was influenced by the total amount of fat in the diet and the time the diet was fed. When the total fat content of the diet was the same and the time the same, the deposition of either fatty acid appeared to be linearly related

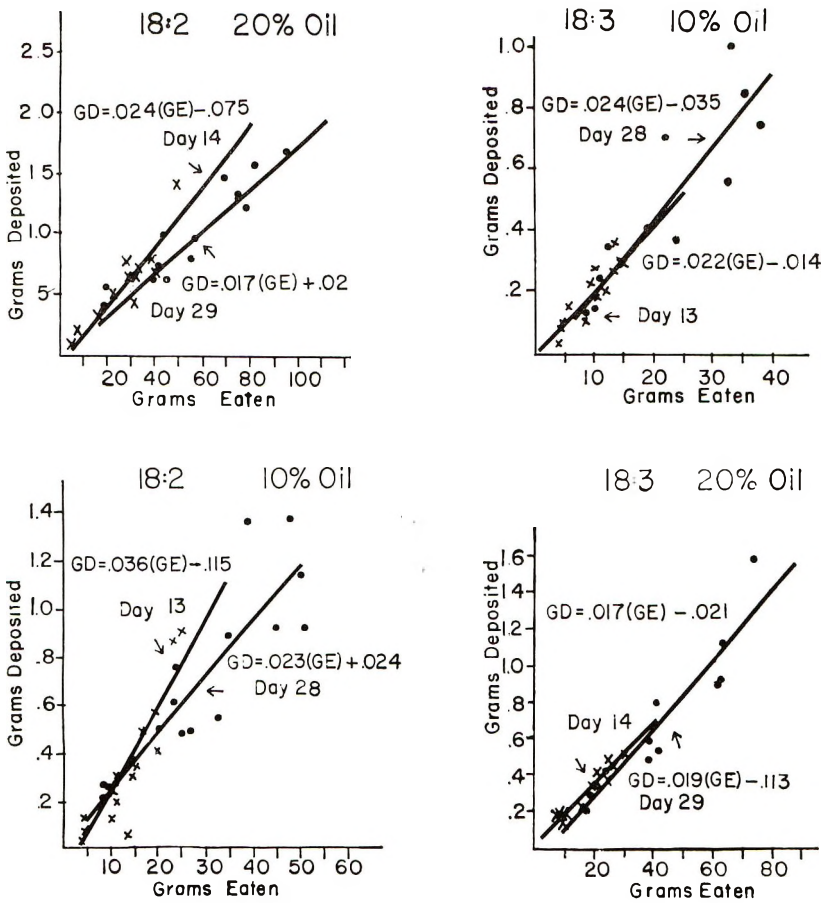

Fig. 1 Grams of 18:2 and 18:3 deposited (GD) per epididymal fat pad in relation to grams eaten (GE) in animals fed diets with different fat content at different periods of time.

to the amount eaten and this was not influenced by large variations in the ratio of linoleic acid to linolenic acid in the diet. Thus, no evidence of competition between these fatty acids in the formation of adipose tissue could be shown. All the diets fed contained substantial amounts of linoleic acid. Since linoleic acid is an essential nutrient, one might expect it to be more efficiently retained when the amounts in the diet approach critical levels, and the conclusions drawn from these experiments may not hold under other conditions.

In these studies there were marked effects of the diets upon the deposition of palmitic, palmitoleic, and oleic acid. Since palmitoleic acid was not present in the dietary fat, this acid must originate from synthetic mechanisms, presumably from palmitic acid. Palmitic and oleic acid may originate either from the diet or from synthesis. Hence, it is difficult to interpret the changes observed. The deposition of all of these fatty acids, however, was lower on the higher fat diets (groups 1–4) than on the lower fat diets (groups 5–8). Consequently, less of these fatty acids were deposited on the higher fat diets even though the intake was higher. This effect might be explained either by selective exclusion of the dietary fatty acids from the adipose tissue or by a decrease in synthesis. It is of some interest that the ratio of palmitic to palmitoleic acid is approximately the same in groups 1 through 4 and groups 5 through 8. Thus, whatever the effect of

TABLE 3

Fatty acids deposited in epididymal fat pad

Diet	Day killed [1]	Calories eaten [1]	Body wt gain [1]	Fat pad gain [1]	Fatty acids deposited [1]							
					16:0	16:1	18:0	18:1	18:2	18:3	18:2 [2]	18:3 [2]
			g	*g*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg deposited/g eaten*	
1	14	1252	94.5	1.396	203	57	27	215	901	—	21.6	—
	29	2514	176.0	2.109	243	59	44	308	1458	—	17.8	—
2	14	1260	84.5	1.197	139	40	20	214	616	167	20.9	17.5
	29	2724	191.5	2.283	254	70	57	426	1150	316	17.7	15.0
3	14	1276	81.7	1.120	132	40	27	219	380	326	18.7	17.1
	29	2745	184.8	2.059	187	46	53	412	752	606	17.5	15.0
4	14	1172	68.0	1.004	114	31	28	222	151	457	20.3	16.8
	29	2847	186.8	2.480	238	60	75	544	414	1141	22.9	17.1
5	13	1145	90.0	1.141	189	73	21	203	664	—	31.1	—
	28	2682	172.0	1.964	324	113	46	363	1097	—	22.5	—
6	13	1165	69.0	1.027	174	63	24	229	421	113	27.0	22.6
	28	2501	144.0	2.167	426	163	54	473	812	218	23.8	19.9
7	13	1174	70.0	0.998	213	81	25	243	229	202	22.1	20.8
	28	2626	155.0	2.012	322	107	53	447	583	491	25.6	23.0
8	13	1040	57.0	0.762	147	58	18	181	80	277	21.7	20.5
	28	2699	176.0	2.344	404	175	66	641	240	798	25.4	23.0

[1] Mean based on individual values from 4 rats.
[2] Based on grams of fatty acid eaten.

the polyunsaturated fatty acids are upon these two fatty acids, linoleic and linolenic acid, would appear to act in the same way. On the other hand, oleic acid was deposited in increasing amounts as the linolenic acid content of the diet was increased. It is doubtful that the difference in the dietary content of oleic acid was sufficient to account for these effects and linoleic acid may be more effective than linolenic acid in inhibiting the deposition of oleic acid.

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Proceedings of the Thirty-second Annual Meeting of the American Institute of Nutrition

TRAYMORE HOTEL, ATLANTIC CITY, NEW JERSEY
APRIL 15–20, 1968

COUNCIL MEETINGS

The Council of the American Institute of Nutrition met Sunday evening, April 14, and Monday morning and evening, April 15. In addition, on the call of President Briggs, a special meeting of the Council was held at 3:00 PM on April 16, to consider the appointment of a new editor of the Journal of Nutrition. The actions of the Council were presented at the Institute business meetings and are included in the report of those meetings, published below.

SCIENTIFIC SESSIONS

A total of 370 abstracts of papers was received by the AIN; 48 were transferred to other societies; 56 were accepted from other societies, making a total of 378 papers for which AIN was responsible. Five of these were not programmed but were published in the Abstracts Volume of Federation Proceedings. Three hundred seventy-three papers were programmed and arranged into 30 regular AIN and 3 intersociety (atherosclerosis) sessions. In addition, two informal conferences were held, the 33rd Poultry Nutrition Conference, and the 9th Ruminant Nutrition Conference. Three AIN symposia were programmed this year:

1. Regulation of Food Intake. *Chairman,* Jean Mayer
2. Nutrition and Metabolic Regulation. *Chairman,* A. L. Black
3. Nutritional Anemias. *Chairman,* V. N. Patwardhan

BUSINESS MEETINGS

Business meetings were held on Wednesday, April 17 and Friday, April 19. President G. M. Briggs presided at both meetings.

I. *Proceedings of 1967 Meeting*

The Proceedings as published in the Journal of Nutrition, *92:* 507–520, 1967 were approved.

II. *Elections*

A total of 701 ballots was received. The tellers, Drs. M. R. S. Fox and P. L. White reported the following results:

President-elect:
Boyd L. O'Dell

Treasurer:
Hartley W. Howard

Councilor:
Gerald F. Combs

Nominating Committee:
Harry P. Broquist
Hans Fisher
Lavell M. Henderson
Ruth L. Pike
Robert E. Shank

III. *Revision of AIN Bylaws*

The proposed revisions of the AIN Bylaws were approved at the first business meeting. The changes (insertions) are italicized below.

Article 1, Section 1. Procedure for Active Membership. Nominations for membership in the American Institute of Nutrition shall be made on, and in accordance with, forms furnished by the secretariat. Nominees must be sponsored by two active *or emeritus* members, each of whom shall write a supporting letter in favor of the candidate. The information on each nominee, in the requisite number of copies, shall be received by the secretariat on or before the deadline date fixed by the Council. The forms shall in turn be sent to the Membership Committee which will consider the qualifications of each nominee and report its recommendations to the Council. The names of nominees, approved

by the Council, shall be presented to the membership at the annual business meeting. A majority of the votes cast shall elect.

Article 1, Section 4. Emeritus Members. A member in good standing for at least five years *upon reaching age* 65, *or* upon retirement from regular employment because of age or disability, may apply to the secretariat for Emeritus Member status. Emeritus Members are exempt from dues but retain all privileges of membership except elective office. They may subscribe to Federation and/or Institute publications at members' rates.

IV. *Membership*

As of April 15, 1968, there were 1,143 members of the Institute: 1,044 active, 116 retired, and 23 honorary members, this being a net increase of 43 members since last year. Ten members retired during the year. The following members resigned from the Institute: Drs. W. P. Boger (ASCN), L. M. Corwin, S. J. Gray (ASCN), Philip Handler, A. L. Lehninger, and F. E. Shideman. The Clinical Division reports a total membership of 203.

Notice of the deaths of the following members was received since our last annual meeting:

Damon V. Catron, November 4, 1967
Casimir Funk, November 19, 1967
Wendell H. Griffith, February 5, 1968
Albert C. Groschke, May 15, 1967
Stanley Levey, November 17, 1967
Clive M. McCay (Charter Member), June 8, 1967
Elmer V. McCollum (Charter Member), November 15, 1967
Martha Potgieter, April 4, 1968
Joseph H. Roe (Charter Member), May 18, 1967
Howard F. Root (Charter Member), November 17, 1967
Harry Steenbock (Charter Member), December 25, 1967

By direction of the Council the following resolutions were introduced into the Proceedings for permanent record.

RESOLVED: That the American Institute of Nutrition, assembled in Atlantic City, New Jersey, at its annual meeting April 17, 1968, place in its minutes for permanent record this statement of deep regret and sorrow at the loss by death of its distinguished member, Dr. Casimir Funk, on November 19, 1967 in his 84th year.

Casimir Funk, born in Warsaw, Poland in 1884, received his basic academic training in the Swiss Universities of Geneva and Bern where he earned his Ph.D. in Chemistry in 1904. He spent two years at the Pasteur Institute in Paris and in 1906 he went to Berlin where for some time he was an assistant to Emil Abderhalden. In 1910, he was appointed a scholar at the Lister Institute in London and it was here that he established his world-wide reputation by his early and highly original investigations on the cure of polyneuritis in pigeons. In what was largely a pioneer effort, he undertook to isolate from rice polishings the substance which cured polyneuritis. The crystalline material which he obtained, although not a pure substance, was highly efficacious and Funk coined the word "vitamine" for the active ingredients in this preparation. He boldly propounded the theory that the human diseases of beriberi, scurvy, pellagra, and, possibly, rickets were "dietary deficiency diseases," a term which he also introduced into nutritional literature. The influence of his concept was greatly extended through the publication of his book, *Die Vitamine*, first published in 1914 and translated into English in 1922.

While Dr. Funk, in his later work, did not continue in what might be considered the mainstream of nutritional investigation, his contributions to the vitamin era will never be forgotten. His memory will long be honored by all those working in the science of nutrition.

RESOLVED: That the American Institute of Nutrition, assembled in Atlantic City, New Jersey, at its annual meeting, April 17, 1968, place in its minutes for permanent record this statement expressing deep regret and sorrow at the loss by death of its distinguished member, Wendell Horace Griffith, fellow and past-president of the AIN, on February 5, 1968 in Baltimore, Maryland, at the age of seventy-two. Wendell Griffith was born in Churdan, Iowa, the son of a Methodist minister. He received his undergraduate training at Greenville College in Illinois from which he graduated with a B.S. degree in 1917. His subsequent graduate studies at the University of Illinois, interrupted for a year of military service as Lieutenant in the U. S. Army Sanitary Corps, 1918–1919, led to his Ph.D. degree in biochemistry in 1923. Moving to the University of Michigan with Professor H. B. Lewis, he spent a year there as instructor and then joined Professor E. A. Doisy in the Department of Biochemistry, Saint Louis University School of Medicine. His association with the latter institution extended over a quarter century, interrupted by leave to serve in the Army of the United States as Chief, Nutrition Branch, Preventive Medicine Division, Office of the Theatre Chief Surgeon, European Theatre of Operations, 1942–1946. From St. Louis he went to Galveston, Texas, as Professor and Chairman, Department of Biochemistry and Nutrition, University of Texas School of Medicine. He completed his academic career as Chairman, Department of Biological Chemistry, UCLA where he served with great distinc-

tion from 1951 until his retirement as Professor Emeritus in 1963. During the last years of his life he acted as Director, Life Sciences Research Office for the FASEB with headquarters at Beaumont in Bethesda, Maryland.

Wendell Griffith was a distinguished biochemist and nutritionist, a lucid and stimulating lecturer, and a superb administrator. Among his many contributions to the nutritional literature his publications on the untoward effects of choline deficiency on the kidney of the young rat are classics. The influence of his 40 years of teaching and research training is reflected by the quality of his many students and the esteem in which he was held by them and by his faculty associates. He was a tireless worker and gave of his time, energy and knowledge to innumerable scientific and professional societies, federal agencies and international organizations. An example of such involvement was his acceptance of appointment as Nutrition Advisor in India 1959–1960 under the sponsorship of United Nations, UNICEF, and FAO. Undoubtedly his most important and distinguished nonacademic service was that performed for the armed forces in the European Theatre of Operations, 1942–1946. In this assignment he, and a group of energetic and competent colleagues which he organized, became responsible for the continued nutritional surveillance of the rations provided for U. S. and allied troops then stationed in the United Kingdom and later committed to combat on the continent. This monumental task included the assessment of nutritional adequacy and wholesomeness of the food, not only for the fighting soldiers, but also for hospitalized casualties and recovered prisoners, and earned for Griffith the awards of Bronze Star and Legion of Merit. He retired as a Colonel, the highest rank then attainable in the Medical Service Corps.

He served AIN well, as president in 1950 and as a member of numerous committees, being chairman of the Fellows Committee at the time of his death.

RESOLVED: That the American Institute of Nutrition, assembled at Atlantic City, New Jersey, at its annual meeting, April 17, 1968, place in its minutes for permanent record this statement of deep regret at the loss by death of one of its distinguished Charter Members, Clive M. McCay, on June 8, 1967 in Englewood, Florida.

Dr. McCay was born in Winamac, Indiana on March 21, 1898. He received the A.B. degree from the University of Illinois, the M.S. degree from Iowa State University and the Ph.D. degree in biochemistry from the University of California. He obtained a National Research Council fellowship for two years of study under Dr. L. B. Mendel at Yale University. In 1927 he was appointed Assistant Professor in the Laboratory of Animal Nutrition at Cornell University, and became a full professor in 1936. Aside from absences in the U. S. Navy and on sabbatic leaves he continued at Cornell until ill health forced his retirement in 1962.

While at Yale, McCay conceived the idea of studying the influence of nutrition on aging. He discussed the idea with Dr. Mendel who told him: "You do it, you are a young man." McCay accepted the challenge and began, around 1930, his studies with rats. It was shown that a diet restricted only in calories, while causing growth retardation, greatly extended the life span and postponed the biochemical and pathological changes of aging. In the mid-forties he began studies with dogs, giving special attention to the aging process. His thirty years of investigations in this broad field resulted in many pioneer contributions and brought him international recognition and renown.

McCay's broad interests stimulated studies with animal species ranging from insects to cows. He was the author of over 150 technical papers, and contributed sections to books and reports dealing with nutrition and aging. His book, *Nutrition of the Dog*, was first published in 1943, with a second edition in 1949. He was an inspiring teacher and his seminars on the history of nutrition were particularly stimulating because of his own great interest and vast knowledge in this field.

He was a member of many scientific societies including the American Institute of Nutrition. He served as president of AIN in 1951.

Dr. McCay had a warm personality and a real interest in people. His death is mourned by a wide circle of friends as well as by his colleagues and graduate students.

RESOLVED: That the American Institute of Nutrition, assembled at Atlantic City, New Jersey, at its annual meeting, April 17, 1968, wishes to place in its record this statement of regret and sorrow at the passing of one of its Charter Members, Elmer Verner McCollum.

Dr. McCollum was, in a very real sense, the founder of experimental nutritional research in the United States. He pioneered in the use of the white rat as a tool for nutritional studies. With this animal in his studies of growth failure resulting from feeding purified diets, he went on to discover vitamins A and D. The white rat became the basis for Dr. McCollum's assay for evaluating the biological value of foods. His many contributions to the field of mineral metabolism and requirements will stand as landmarks in nutritional pathology. He was one of the first to see the unique and important contribution that could result from the collaborative approach of medically and biochemically oriented scientists to nutritional problems.

Throughout Dr. McCollum's long and distinguished career, he was a consultant to both national and international agencies. One of the earliest contributions of this nature was his work with Herbert Hoover during World War I in developing plans for feeding the military

refugees in Europe. His enormous capacity for concentrated work, his keen ability to get at the heart of a problem, and his unfailing determination to uphold scientific principles explain why he was sought after by both governmental and industrial organizations for help and advice.

Not only was Dr. McCollum an outstandingly great investigator, but he also was preeminently successful in translating experimental nutritional findings into the everyday practice of physicians and dentists, as well as laymen. He was untiring in his efforts to bring "the newer knowledge of nutrition" to those who would benefit most from its application. It was in his monthly articles for a popular magazine that he put forth a number of commonly accepted nutritional concepts, the best-known one being "protective foods."

His thoughtfulness for his students and all who came in contact with him only reinforces the permanence of the niche he carved for himself in the minds and hearts of all who knew him. The twinkle in his eye, his unfailing sense of humor, and his kindliness set the stranger at ease and made his younger associates feel that here was a person with whom they could readily talk.

His passing is like the felling of a great tree in a primeval forest of greatness.

RESOLVED: That the American Institute of Nutrition, assembled at Atlantic City, New Jersey, at its annual meeting, April 17, 1968, place in its minutes for permanent record this statement of deep loss and sorrow at the passing of one of its distinguished Charter Members, Joseph Hiram Roe.

Dr. Roe's entire academic career was spent in the School of Medicine, The George Washington University. He was Professor of Biochemistry there since 1922 and Chairman of the Department for 31 years. Dr. Roe served the American Institute of Nutrition as Secretary, member of the Council, and on numerous committees. He was recognized for his development of methods for the determination of many compounds of biochemical and nutritional interest. His most important contributions were to the methodology, metabolism, and nutritional requirements of ascorbic acid. He excelled as a teacher, consultant, and administrator. He was kind and generous in his dealings with everyone and was affectionately known to all as "Joe Roe."

RESOLVED: That the American Institute of Nutrition, assembled at Atlantic City, New Jersey, at its annual meeting, April 17, 1968, place in its minutes for permanent record this statement of regret and sorrow at the loss by death of one of its distinguished Charter Members, Howard Frank Root, on November 17, 1967.

Dr. Root, born August 28, 1890, in Ottumwa, Iowa, received his college and professional training at Harvard: A.B. 1913, M.D. 1919. His scholarship was recognized by his election to both Phi Beta Kappa and Sigma Xi. His hospital training, except for one brief period at the Johns Hopkins Hospital, was obtained in Boston.

In 1920 Dr. Root joined the Joslin Clinic and the staff of the New England Deaconess Hospital, the two institutions with which he was actively affiliated for his entire and long professional career. Dr. Root's interest in nutrition, and his contributions to nutrition were all in relation to his overriding interest in the treatment and care of the patient with diabetes. This was initially kindled by his former Chief, the late Dr. Elliot Joslin. Joslin and Root, probably more than any other two physicians in the world, were responsible for insisting on rigid and exact control of the intake of carbohydrates by the diabetic patient. Educating "countless" numbers of patients, house staff, and postdoctoral fellows in nutrition and its importance in the treatment and care of the patient with diabetes was probably his greatest contribution to nutrition.

Dr. Root was coauthor in five editions of *Treatment of Diabetes Mellitus*, and coauthor or author of numerous chapters in various texts, monographs, encyclopedias including the standard nutrition text entitled *Modern Nutrition in Health and Disease*.

RESOLVED: That the American Institute of Nutrition, assembled at Atlantic City, New Jersey, at its annual meeting, April 17, 1968, place in its minutes this statement of deep regret and sorrow at the passing of one of its Charter Members and Fellows, Harry Steenbock.

The State of Wisconsin claims Dr. Harry Steenbock as one of her most illustrious sons, native born, educated in her schools, and, aside from Wanderjahre at Yale University and at the University of Berlin, one whose whole life of scholarship was spent as a faculty member of the University of Wisconsin. Through his researches in mineral metabolism, the anemias, rickets, and the vitamins, Harry Steenbock added luster to the University's fame. He was a pioneer and leader in one of the most explosive periods of nutritional knowledge in history, and his accomplishments were recognized by many awards and honors, nationally and internationally.

In a long life of accomplishment in nutritional research, undoubtedly the greatest single feat was his discovery of the role of ultraviolet radiation in the activation of vitamin D. Harry Steenbock was, indeed, the trapper of the sun. But with this triumph, this shy and diffident man showed his sensitive awareness of the social impact of his discovery; and, mindful of the social waste of scientific discovery when it is left unattended, or worse, when exploited by the few to the detriment of many, Harry Steenbock did a courageous and imaginative act. He patented his vitamin D

discovery, and with it he founded the Wisconsin Alumni Research Foundation. To that child of his genius he turned over his patent rights, and with the revenues which it garnered, and at a time when the world bowed in a great economic depression, the Wisconsin Alumni Research Foundation fostered science at the University of Wisconsin and nurtured her scholars. The demonstration of the power of the fruits of science to nourish the body of science: that is Harry Steenbock's memorial.

V. *New Members*

The Membership Committee considered the qualifications of 98 nominees. The following 80 nominees recommended by the Council were approved for membership at the business meeting:

NEW MEMBERS — 1968 *

Ahrens, Richard A.	Marshall, Mary W.
Barth, Karl M.	Martin, John L.
Bayless, Theodore M. (C)	Martin, William G.
Beare-Rogers, Joyce L.	Mahlum, Daniel D.
Bierman, Edwin L. (C)	May, Jacques
Beisel, William R. (C)	McCullough, Marshall E.
Benevenga, N. J.	McGuigan, James E. (C)
Bessman, Samuel P.	Metcalf, William (C)
Bhagavan, Hemmige N.	Monson, William J.
Borgman, Robert F.	Moore, Marian E.
Brink, Marion F.	Moran, Edwin T., Jr.
Caldwell, Anne B. (C)	Morris, Manford D.
Chinn, Kenneth S. K.	Nelson, Ralph A. (C)
Davis, Richard F.	Nesheim, Robert O.
Donefer, Eugene	Olson, James A.
Eagles, Juanita A.	Peterson, Malcolm L. (C)
Doisy, Richard J.	Phillips, William E. J.
Dreyfus, Pierre M. (C)	Picon-Reategui, Emilio
Edozien, Joseph C. (C)	Prather, Mary E. S.
Elliott, John M.	Reddy, Bandaru S.
Ellis, William C.	Rosenweig, Norton S. (C)
Floch, Martin H. (C)	Ross, Margaret L. (C)
Flatt, William P.	Sahagian, Benjamin M.
Frank, Oscar (C)	Sell, Jerry L.
Gaylor, James L.	Shah, Shantilal N.
Hintz, Harold F.	Shapiro, Irwin L.
Hoskins, Fred H.	Sheehy, Thomas W. (C)
Hoetzel, Dieter G.	Smith, James C., Jr.
Howard, Charles F., Jr.	Smith, Robert E.
Huber, Tyron E.	Stewart, Robert A.
Johnson, Herman L.	Suzuki, Minoru
Jones, James D.	Tews, Jean K.
Joslyn, Maynard A.	Thiele, Victoria F.
Kevany, John P. (C)	Tolbert, Bert M.
Khairallah, Edward A.	Underwood, Barbara A.
Kirksey, Avanelle	Van Campen, Darrell R.
Kokatnur, Mohan G.	Veen, Margaret J.
Krzywicki, Harry J.	Warnock, Laken G.
Kuo, Peter T. (C)	Wise, Milton B.
Lakshmanan, Florence L.	Wostmann, Bernard S.

* For institutional affiliations and addresses of new members, see the Federation Directory of Members, 1968.
(C) Also elected to membership in the Clinical Division at its Annual Meeting, May 4, 1968.

The following AIN members were accepted for membership in the Clinical Division: Mildred Adams, Conrado Asenjo, Robert Bradfield, Clinton Chichester, Jack Cooperman, Seymour Dayton, Stanley Gershoff, M. S. Read, John Schubert, and Herta Spencer.

HONORARY MEMBERS

The following distinguished scientists were elected to Honorary Membership in the AIN:

Professor Hans D. Cremer, *Professor of Human Nutrition, University of Giessen, Germany.*

His many important contributions to the advancement of the science of nutrition in both research and education have resulted in recognition of the importance of human nutrition among educators, government bodies and the populace as a whole. Professor Cremer developed an Institute of Nutritional Sciences within the Medical School of the Justus Liebig University in Giessen; and occupied the first Chair of Human Nutrition in Continental Western Europe.

Dr. Karl Guggenheim, *Professor of Nutrition, Hebrew University, Jerusalem, Israel.*

For his many contributions to nutritional research and to public health nutrition in Israel during the past 30 years. He has promoted the flour enrichment program in his country and, more recently, he developed a vegetable protein supplement appropriate for the needs of the Near East.

Dr. Juan S. Salcedo, Jr., *Chairman, National Science Development Board, Manila, Philippines.*

For his many contributions to research and training in nutritional science and public health nutrition in the Philippines. Through his leadership as past Secretary of Health and past Director of the Institute of Nutrition, Department of Health, he has brought the Food and Nutrition Research Center to its present state of eminence in the Philippines. He was director of the Bataan study which demonstrated that thiamine-enriched rice was a convenient and effective means for the eradication of beriberi.

VI. *President's Report*

VIIIth International Congress of Nutrition, August 28–September 5, 1969. AIN has filed application with the National Institutes of Health and the National Science Foundation for funds to help support the travel of U. S. scientists to the VIIIth International Congress of Nutrition in Prague. NIH has, at the moment, approved $15,000 of the $90,000 requested but actual funding is still open to question. The National Science Foundation has not responded to the AIN request as yet.

AIN Council has approved the use of $15,000 of the special Congress Fund to

be used for travel to the Prague Congress, and will explore additional sources of travel support.

Council Actions. Council accepted the recommendations of the AIN Committee on Nomenclature as the official nomenclature of the American Institute of Nutrition and has recommended to the editorial boards of the official journals that these recommendations be implemented as the nomenclature of the official journals of the AIN. (Report of the Nomenclature Committee appears later in these Proceedings.)

Dr. Ogden Johnson was named Editor, AIN Nutrition Notes, for a three-year term (retroactive to his original appointment in 1967). Dr. James Waddell was named Managing Editor.

In response to a proposal that the AIN encourage the study of the history of nutrition and take steps towards the preservation of historical materials of nutritional interest, the Council appointed Dr. E. N. Todhunter as AIN Archivist for a term of three years.

The completed manuscript on the history of the AIN, "The First Forty Years of the AIN," on which Dr. Agnes F. Morgan has been working for many years, was submitted to the Council. The Council gratefully accepted the manuscript. Ways and means are being explored for its publication and distribution.

Council approved the expenditure of up to $1000 for the development of a proposed brochure for use by teachers on science fairs and nutrition experiments. (Report of this Subcommittee of the Public Information Committee appears later in these Proceedings.)

VII. *Secretary's Report*

Dr. W. N. Pearson reported that the Council has added the following rules to those already adopted covering the submission of abstracts:

No member may sign an abstract of another member unless the signer is one of the authors.

Abstracts submitted to other societies and offered for transfer to the AIN program will be judged for eligibility within the framework of the AIN rules regardless of the rules of the society to which the abstract was originally submitted.

VIII. *Treasurer's Report*

The 1967 Financial Statements, reproduced here, were presented by Mr. John R. Rice, AIN Business Manager, and accepted and approved. The AIN Auditing Committee, Drs. G. V. Vahouny and A. E. Light, reported that they had examined the financial report and the records in the AIN business office and found them to be in order.

The recommendation of the Council that the AIN dues remain the same for the 1968–1969 year ($7.00) was approved by the membership.

IX. *Report from the Clinical Division*

All AIN and ASCN members have received the outline of the program and an invitation to attend the ASCN Annual Meeting to be held in Atlantic City, New Jersey on May 4. Formal presentation of the Fourth McCollum Award will be made to Dr. L. Emmett Holt, Jr. at the dinner that evening.

X. *Federation Affairs:* A. E. Schaefer

Mr. Robert H. Grant, Director of the new FASEB Office of Public Affairs, was introduced to the membership. The Office will establish and continue contact with the public and Government agencies and make available scientific guidance on matters of legislation.

Joint sponsorship of a proposed series of conferences on the History of Biology is being developed by the AIBS and FASEB.

The FASEB Finance Committee has approved an increase in the registration fee at the Annual Meeting for nonmembers, from $20 to $25, effective 1969. It was pointed out that this will not affect the registration fee paid by graduate students who register in advance.

Because of unforeseen commitments in Washington, The Honorable Hubert H. Humphrey, Vice President of the United States, was unable to speak at the General Session for the 1968 meeting as planned. Dr. William Stewart, Surgeon General, PHS, spoke on "Reorganization Within the Department of Health, Education and Welfare."

Financial Statement — December 31, 1967

ASSETS		EXHIBIT A
Cash		$ 25,938
Accounts Receivable		16,591
Investments		122,024
Furniture and Equipment	$4,173	
Less Accumulated Depreciation	(627)	3,546
Prepaid Expenses		382
Total Assets		$168,481
LIABILITIES AND FUND CAPITAL		
Accounts Payable		$ 28,498
Fund Capital		139,983
Total Liabilities and Fund Capital		$168,481

Statement of Revenue and Expense and Fund Capital
For the Year Ended December 31, 1967

Revenue:	TOTAL	GENERAL FUNDS	EXHIBIT B SPECIAL FUNDS
Membership Dues	$ 7,047	$ 7,047	$ —
Sustaining Associate Memberships	10,250	10,250	—
Annual Meeting Registration	5,326	5,326	—
Journal of Nutrition:			
Subscriptions	5,882	5,882	—
Editorial Allowance	9,700	9,700	—
Page Charges	23,285	—	23,285
Interest	7,573	3,075	4,498
Grants	500	—	500
Overhead	7,186	7,186	—
Miscellaneous	168	168	—
Total	$ 76,917	$ 48,634	$ 28,283
Expenses:			
Salaries, Payroll Taxes, and Fringe Benefits	$ 10,144	$ 8,927	$ 1,217
Communications, Postage and Travel	8,347	6,737	1,610
Supplies and Duplicating	1,279	1,045	234
Travel Awards	2,188	2,188	—
Journal of Nutrition Editor's Office	11,159	11,159	—
Rent Expense	2,276	1,763	513
Depreciation	336	328	8
Printing and Engraving	13,564	1,874	11,690
Miscellaneous Expenses	832	582	250
Total Direct Expenses	$ 50,125	$ 34,603	$ 15,522
FASEB Business Service Charge	931	764	167
Total	$ 51,056	$ 35,367	$ 15,689
Revenue Over Expenses	$ 25,861	$ 13,267	$ 12,594
Fund Capital:			
Balance, 12/31/66	114,122	53,525	60,597
Balance, 12/31/67 — To EXHIBIT A	$139,983	$ 66,792	$ 73,191

XI. *Editor's Report — Journal of Nutrition*

Dr. Richard H. Barnes submitted his report for the calendar year 1967. The report and proposed budget were approved and the report is summarized below.

Dr. Barnes gave recognition to the outgoing members of the Editorial Board and noted that the Board has been increased to 20 members, as approved by the AIN Council last April.

Editing and Publication Operations (Calendar year)

	1965	1966	1967
Volumes published	85,86,87	88,89,90	91,92,93
Pages published — total exclusive of supplements	(not recorded)	(not recorded)	1762
(Scientific papers)	(1313)	(1350)	(1606)
(Biographies, AIN business and letters)	(71)	(97)	(72)
(Index, contents, covers, etc.)	(not recorded)	(not recorded)	(84)
Papers published (including 3 biographies)	189	194	225
Papers submitted	303	316	326
Papers rejected during year regardless of when submitted	98	89	95
Supplements published	—	—	2
Letters to the Editor	2	—	1
Avg Monthly Operating Schedule			
Manuscripts with reviewers	0.72	0.76	0.74
Manuscripts out for revision	0.74	0.8	0.9
Manuscripts in office, in mail or in unavoidable delay	1.02	1.03	1.3
Time from receipt of manuscripts to mailing to press	2.5	2.6	2.9
Manuscripts with Wistar Press	3.0	3.9	3.9
Time from receipt of manuscripts to mailing by Wistar Press to subscribers	5.5	6.5	6.8

Journal of Nutrition, Editorial Office
Statement of Income and Expenditures
December 31, 1967

Balance on hand 1/1/67		$ 1,224.67
1967 Appropriation		10,000.00
Total Income Available		11,224.67
Expenditures:		
Communications	978.91	
General Office	768.66	
Payroll, Regular	8,419.96	
Travel Expense	266.24	
Other Expense	225.26	
Overhead to Cornell Univ.	500.00	
Total Expenditures		11,159.03
Balance on hand 12/31/67		$ 65.64

XII. *Reports of Committees and Representatives*

A. *Publications Management Committee:* W. J. Darby, Chairman.

Dr. Darby reported on the completion of negotiations with The Wistar Institute whereby the AIN now becomes the owner of the *Journal of Nutrition*. This transfer of ownership, effective January 1, 1968, was made in consideration of the payment by AIN of $62,500 over a five-year period and a contract with Wistar to continue as printer of the Journal over the same period. The Council expressed appreciation for the efforts of the Publications Management Committee in effecting this transfer of ownership, and in particular to Drs. Darby, Waddell, Kline and Mr. Rice.

Announcement was made that Dr. W. N. Pearson had been appointed Editor of the *Journal of Nutrition* to succeed Dr. R. H. Barnes, effective January 1, 1969. The Council took this action on the recommendation of the Publications Management Committee and also that of Editor Barnes and the Editorial Board.

The recommendation of the Council that the member subscription rate to the *Journal of Nutrition* be raised to $15 was approved by the membership at the business meeting. This action was necessary to comply with the second class postal regulations which require that members pay at least one-half of the regular subscription price ($30).

B. *Public Information Committee:* M. S. Read, Chairman.

During the 1966–1967 year, the members of the Public Information Committee included: Dr. C. J. Ackerman (Virginia Polytechnic Institute), Dr. Robert B. Bradfield (University of California, Berkeley), Dr. Robert S. Goodhart (New York Academy of Medicine), Dr. Frank L. Iber (Johns Hopkins University School of Medicine), Dr. Ruth L. Pike (Pennsylvania State University), Dr. Merrill S. Read (National Institute of Child Health and Human Development), Dr. John T. Sime (Ralston Purina Company), and Dr. Philip L. White (American Medical Association). All members participated actively in one or more Committee activities during the year.

The traditional responsibility of the Committee has been the review of abstracts of papers to be presented at the Annual Meeting for news value. As in the past, it was agreed that two members of the Committee would share this responsibility — the Chairman plus the member from the American Society for Clinical Nutrition, thereby assuring a broad interpretation of newsworthiness. Dr. Iber, who had volunteered to help, was out of the

country when the abstracts were received. Dr. G. Watson James III, Medical College of Virginia, willingly and capably helped in Dr. Iber's stead. Of the more than 400 abstracts reviewed, 44 were identified for special attention by the Federation Public Information Office. In addition, all AIN Symposia were recommended for individual press conferences during the FASEB meeting.

The Annual Banquet of the AIN honors the award winners and new Fellows of the Society. Each member of the Public Information Committee drafted one or more press releases in conjunction with these major recognitions for 1968. Special individualized plans for maximizing press pick-up of these news stories were developed in cooperation with the AIN Executive Secretary.

Continuing attention was devoted to exploring ways for increasing news coverage of scientific papers published in Society journals. Dr. Richard Barnes, Editor of the *Journal of Nutrition* has reported difficulty in developing methods for identifying potential interest of forthcoming articles. Recently the Editorial Board has helped in this task. Five papers have been selected and recommended to university information offices for special treatment. Dr. Milton Rubini reports he has not been Editor of the American Journal of Clinical Nutrition long enough to undertake a comparable program; this program will be developed in the coming year.

Dr. Ruth Pike has continued efforts to identify sources for objective, quotable evaluations of reputable and/or quack nutrition books to aid in answering inquiries in this area. Copies of the material obtained are in the AIN office.

Dr. C. Wendell Carlson, South Dakota State University, is Chairman of the Science Fair Subcommittee of the Public Information Committee. Drs. E. L. Wisman (Virginia Polytechnic Institute), Helen M. Dyer (Washington, D. C.), C. J. Ackerman, J. T. Sime, and M. S. Read are the other Committee members. A report of the extensive activities of this Committee follows.

Finally, the Chairman has also served as Chairman of the FASEB Public Information Committee and of the Closed-Circuit TV Committee. This has been both time consuming and rewarding.

Subcommittee on Science Fairs report. The response to the questionnaire sent to AIN members on the subject of science fairs was good, 475 responses being received. Of these 387 (81%) favored the proposal that AIN support programs in secondary schools which would advise and assist students in developing nutrition-oriented projects for use in science fairs; 88 (19%) felt that AIN should not become involved in such programs. Similarly, a majority (375) favored the development of a brochure which would help orient and encourage students in nutrition projects; a minority (99) did not agree. Specific projects were described by a majority of the respondents and many suggestions were offered for developing an appropriate brochure.

Many members noted that the AIN might become involved in controversy and, indeed, might invite legal action if it prepared student-directed educational material which strongly encouraged animal experimentation. The subcommittee, therefore, proposes the alternate approach of preparing teacher-directed materials designed to acquaint the latter with the opportunities and problems and increase his knowledge on nutrition subjects. Encouragement should also be given to the teacher to work with trained nutrition scientists in the community.

Based on these considerations, the subcommittee recommends that the AIN prepare a guide for use by teachers in incorporating nutrition activities into science programs. The objective would be to expose students to nutrition as a science, to demonstrate how nutrition affects everyday life, and to encourage them to view nutrition as a career opportunity. It is suggested that a booklet about 8 to 10 pages long with a text of 6,000–8,000 words, and amply illustrated, be developed for placement with science teachers upon request. A tentative outline of the content of such a booklet has been prepared.

The subcommittee looks forward to Council's decision concerning the above recommendation.

C. *Committee on Experimental Animal Nutrition:* G. F. Combs, Chairman.

The activities of the Committee included making arrangements for the 33rd Annual Poultry Nutrition Conference and the 9th Annual Ruminant Nutrition Conference held as a part of the annual meeting of AIN. Amino acid metabolism was emphasized at the Poultry Nutrition Conference while the Ruminant Conference dealt with unique aspects of metabolism in ruminants. Dr. A. I. Virtanen, Biochemical Research Institute, Helsinki, Finland, reported on his life-cycle studies in the bovine species using purified diets composed of cellulose, urea, minerals and vitamins.

Dr. D. C. Church, Oregon State University, has been appointed Chairman of a subcommittee to continue the work in developing a collection of slides depicting nutritional deficiency disease in the various species of experimental animals. At present this committee includes Drs. F. H. Kratzer, W. H. Pfander, R. W. Luecke, K. E. Harshbarger, and G. F. Combs. These committee members are responsible for collecting slides dealing with the turkey, adult ruminants, swine, the calf, and chickens, respectively. Two additional members will be appointed to deal with fur-bearing and other laboratory animals.

From over 1,000 slides collected by Dr. Church and other members of this committee, approximately 500 have been selected for further consideration. In addition, the slide collection of the late Dr. Follis is being made available by the Armed Forces Institute of Pathology for use in assembling this collection. The AIN Council has allocated up to $300 for reproduction of slides so that copies can be distributed to subcommittees for further screening and classification. It is

anticipated that funds may be obtained from a source outside of AIN for completing this activity.

The new members appointed to this committee by the AIN Council for three-year terms ending in 1971 are Drs. L. S. Jensen and Gennard Matrone.

D. *Committee on Nomenclature:* Stanley Ames, Chairman.

1. Organizational. The 1967–1968 Committee on Nomenclature was activated by President Briggs following the 1967 AIN meeting at Chicago, with Drs. E. W. Crampton, P. L. Harris, Q. R. Rogers and H. E. Sauberlich as members. Dr. C. G. King and S. R. Ames continue as members of the Commission on Nomenclature of IUNS. Liaison with IUPAC-IUB was continued through Dr. Waldo Cohn, Director, NAS-NRC Office of Biochemical Nomenclature. The committee met at the 1967 meeting at Chicago and on April 16, 1968 at Atlantic City.

Vitamin nomenclature. Proposed generic descriptors for the vitamins were discussed by this Committee at the 1967 AIN meeting. A final proposal was reviewed by the Committee at its annual meeting on April 16, 1968.

IUPAC-IUB Commission on Biochemical Nomenclature has amended rule M-7 of the "vitamin" rules (J. Biochem., *241:* 2987, 1966) to reject the name "pyridoxine" as a group name for all compounds with vitamin B_6 activity and recommends "vitamin B_6" for this purpose. While acceptable to nutritional scientists, this action is not compatible with CBN's previous position disclaiming any concern with generic descriptors of vitamins.

The report of Professor H. Dam and Dr. T. Moore (Int. Cong. Nutr., Hamburg, Germany, August 10, 1966) was submitted by Professor Dam as Chairman, Commission on Nomenclature, IUNS, to IUPAC-IUB with expressed concern on the IUPAC nomenclature of the fat-soluble vitamins.

2. Vitamin E. The system of nomenclature for the stereoisomers of the tocopherols and their esters (J. Nutr., *90:* 108–109, 1966) has been reviewed further by IUPAC-IUB Commission on Nomenclature. No decisions were reached and later the problem was referred to the IUPAC-Commission on Organic Nomenclature for further action.

3. Nutrition terminology. A "Guide to Nutrition Terminology" is currently being prepared by Dr. E. N. Todhunter under a contract from NIH. AIN is well represented on the Advisory Committee.

4. Chairman's comments. The procedures for implementing the recommendations of the Committee on Nomenclature, AIN, have not yet been established. Close liaison with IUNS has been accomplished. Since this Committee should not act independently of the IUNS Commission on Nomenclature, the actions of the AIN Committee on Nomenclature have of necessity been advisory in nature.

E. *Committee on Public Affairs:* O. L. Kline, Chairman.

Dr. Kline outlined the special activities, in the order of their priority, of the FASEB Office of Public Affairs. This Office works closely with the FASEB Committee on Public Affairs (consisting of representatives from each constituent Society). The activities include the 1968 Draft Regulations, animal care legislation, Fountain Committee report, grants versus contracts, national survey on nutrition and health, creation of target research centers, and a symposium on the ethics of animal and human experimentation. A FASEB newsletter with limited distribution will initially be established on an experimental basis.

The new Selective Service regulations eliminate deferments of most graduate students and this has caused concern among scientists who predict a hazardous effect in the nation's educational, health and research capability. Representing the Federated Societies, FASEB has endorsed a statement expressing concern and recommending revision of the regulations to allow a reasonable proportion of graduate students to complete their studies.

Individual members of AIN are encouraged to communicate with their representatives in Congress expressing approval or disapproval of pending legislation, and particularly their views relating to the need for a national nutrition and health survey.

F. *International Biological Program:* George K. Davis.

The biggest landmark in our efforts to have nutrition recognized as an integral part of the International Biological Program was the adoption by the U. S. National Committee for the IBP of a proposed integrated program in nutrition as a part of our overall program. In adopting this program the U. S. National Committee reaffirmed its previous action identifying the U. S. National Committee of the International Union of Nutritional Sciences as a coordinating group for the IBP Nutrition Program. In effect, this means that Dr. O. L. Kline and Dr. C. G. King will share primary responsibility for the nutrition integrated program.

It is also significant that the National Science Foundation has included in its line item budget to the Congress a line for the International Biological Programs. Actually, the National Science Foundation has been responsible for a very considerable support of IBP through its other programs and this is in effect recognition of a specific need for IBP. The other Federal agencies which make grants have been encouraged to include in their budgets line items for IBP.

The Special Committee for the International Biological Program of the International Council of Scientific Unions is meeting in Varna, April 3–10, 1968. One of the actions which will be taken there will be the formal recognition of nutrition as an interdisciplinary committee, hopefully with the International Union of Nutritional Sciences providing the coordinating leadership

and the sections of SCIBP in Use and Management and Human Adaptability coordinating their efforts with those of IUNS. Dr. C. G. King will be in Varna representing this aspect of our program.

It is only fair to point out that many of the integrated programs of the U. S. National Committee, such as those of migrant populations and circumpolar peoples, have a very important segment of nutrition included in them. Even the integrated project on Grassland Ecosystems includes an important area of nutrition, especially animal nutrition. The role of the integrated program in nutrition will be to develop its own program and at the same time keep up liaison with these other areas which are in large measure dependent on a good nutrition input.

G. *International Union of Nutritional Sciences:* O. L. Kline.

The International Biological Program (IBP), a five-year study patterned after the Geophysical Year, to examine ecological systems with emphasis on man, has nutrition application throughout the program. During the April 1968 meeting in Bulgaria, Dr. C. G. King (President, IUNS) was asked to assist in coordinating the international nutritional aspects of the IBP. Drs. King and O. L. Kline, acting as codirectors of the U. S. program, have solicited participation from AIN members with research projects involving international collaboration or application.

H. *AIN Office of Nutrition Science Services:* O. L. Kline, Director.

Dr. Kline reported on the status of the project providing nutritional services and information to the Agency for International Development. A roster of over 500 names of nutrition scientists with interest and experience in international nutrition programs has been developed as the result of a questionnaire survey. A list of over 2,700 names of foreign students trained in the United States has been collected. Under the program, sixteen libraries in foreign countries have been identified and are receiving complimentary subscriptions to nutrition journals. Food and nutrition library references have also been provided to the Food and Nutrition Research Center in the Philippines.

The AIN Council has approved AIN membership in the League for International Food Education (LIFE). This League consists of organizations to provide information on food problems in reply to requests for technical information, and include the Institute of Food Technologists, the Nutrition Foundation, Inc., VITA, the American Chemical Society, and the American Association of Cereal Chemists. Dr. Kline is currently serving on the Board of Governors for this consortium.

I. *Western Hemisphere Nutrition Congress II, San Juan, Puerto Rico, August 26–29, 1968.*

Dr. W. N. Pearson, Chairman of the Planning Committee, advised that $20,000 of the $30,000 requested from NIH for travel support to the meeting has been approved but funding is still open to question. Members of the pharmaceutical and food industries, however, have made contributions towards travel support in addition to support received from the sponsoring organizations.

Programs and registration forms have been sent to all AIN members.

J. *Representative to the AAAS:* Hartley W. Howard.

AAAS COUNCIL MEETING, 1967

The Council voted approval of a revised constitution and bylaws intended to simplify wording and provide editorial improvement. The only substantive change of importance was abolition of the Committee on Affiliation and transfer of its responsibilities to the Committee on Council Affairs.

The Committee on Council Affairs presented a report analyzing Council attendance in terms of scientific disciplines. Persons from the physical sciences attend in substantially lesser, and those representing biology in appreciably greater, proportion than the percentage of AAAS members in these fields.

The Council Study Committee on Population Explosion and Birth Control recommended that one session of the 1968 Council Meeting be devoted to a review of the activities of affiliated societies and other organizations as they relate to the population problem.

In summarizing AAAS' financial status the executive officer drew attention to a new Internal Revenue Service regulation under which nonprofit organizations might become liable for payment of income tax on advertising revenues derived from their periodical publications. This regulation has not yet been tested in the courts.

The Council, at the 1966 Annual Meeting, adopted a resolution relating to the use of biological and chemical agents which modify the environment (J. Nutr., *92:* 516–517, 1967). The Board of Directors has subsequently established a continuing Committee on Environmental Alteration to carry on a continuing evaluation of the implications of technological intrusions on environmental processes and the interrelations between environmental changes and population changes. As its first order of business the Committee has been requested to consider the problems of the use of chemical and biological agents in Vietnam (cf., Science, *159:* 751 (1968) for additional details).

K. *Representative to the Food and Agriculture Organization:* H. E. Sauberlich.

Attention should be drawn to several recent reports of FAO that are of particular interest to nutritionists:

1. Joint FAO/WHO Expert Committee on Nutrition, Seventh Report, Rome, 1967. (This report reviews the programs of FAO/WHO and summarizes exceedingly well the general food and nutrition situation and problems of the world. A "must" reading for all interested in nutrition.)

2. Requirements of Vitamin A, Thiamine, Riboflavin and Niacin. Joint FAO/WHO Report,

Rome, 1967. (The recommended intakes for these vitamins are detailed along with extensive pertinent background information and an outline of existing problems in this area.)

3. Nutrition Newsletter — published normally four times each year. Issues summarize activities of FAO and FAO members. The July-September 1967 issue gave a brief account and assessment of the applied nutrition programs in The Philippines, India, Ivory Coast, Colombia, Trinidad and Tobago. This issue also contained an article by Dr. G. D. Kapsiotis, entitled "Struggling to meet the protein needs." Dr. Kapsiotis is Chief, Food Industries Section, Food Science and Technology Branch, Nutrition Division, FAO.

4. "Nutrition and Infection in Africa," October 1967, by Dr. F. Lowenstein, WHO Secretary. Joint publication of FAO/WHO/OAU (STRC), Regional Food and Nutrition Commission for Africa.

5. CAJANUS: Newsletter of the Caribbean Food and Nutrition Institute. The first issue appeared in February 1968. The Institute is supported in part by PAHO/WHO and FAO. The initial issue contained 42 pages of short articles on nutrition activities in the Caribbean area.

XIII. *Resolutions*

A. A motion was made, seconded and passed unanimously directing that the President of the American Institute of Nutrition prepare a resolution in support of a national nutrition and health survey to determine and define the extent of nutritional deficiencies existing in different areas of the U. S. on which sound recommendations for their amelioration can be based. The following resolution was sent by incoming President Barnes to the Secretary of the Department of Health, Education and Welfare:

> WHEREAS there is increasing evidence and awareness of the existence of both undernutrition and malnutrition in important segments of the U. S. population which has caused wide concern, particularly as to the deleterious effects of these conditions in infants and young children; and
> WHEREAS methods for evaluating the nutritional status of large population groups have been developed and widely tested in foreign countries largely under the sponsorship and support of U. S. investigators; and
> WHEREAS these surveys have resulted in demonstrating the wide variety, incidence, and nature of nutritional deficiency in different areas of the world and in defining the nutritional programs needed to ameliorate the deficiencies; therefore,
> BE IT RESOLVED that the Secretary of the Department of Health, Education and Welfare and the Assistant Secretary for Health and Scientific Affairs be urged to proceed immediately with the comprehensive national nutrition and health survey to determine and define the extent of nutritional deficiencies existing in different areas of the U. S. on which sound recommendations for their amelioration can be based.

B. A motion was made, seconded and passed unanimously directing the AIN President to inform President Lyndon B. Johnson and the other members of the National Security Council that the American Institute of Nutrition endorses the resolution recently adopted by the National Research Council calling for reconsideration and modification of the new Selective Service regulations that eliminated deferments of most graduate students. After the meeting, incoming President Barnes sent the following resolution to President Johnson and the six other members of the National Security Council:

> WHEREAS the vitality and continued productivity of our economy is essential for the realization of our national and international goals, including the maintenance of our military strength; and
> WHEREAS the active participation in the operation of our economy of individuals who are highly trained in many fields of knowledge is an essential requirement for its vitality; and
> WHEREAS the challenges and problems facing the nation now and in the future — in human behavior, in retrieving the environment, and in meeting the problems of exploding populations and the universal urge for higher living standards obtainable only through new technology — will require even more highly trained personnel in many fields; and
> WHEREAS a careful analysis indicates that the effect of the Selective Service regulations presently set forth will be, within two years, *to reduce by more than half* the flow of such highly trained individuals into our industrial and economic complex; therefore
> BE IT RESOLVED that the American Institute of Nutrition directs its president to transmit to President Lyndon B. Johnson and to the members of the National Security Council its urgent request that these Selective Service regulations be reconsidered and modified in accordance with the principle of equitable liability for national service to provide a continuing balanced flow of the skills necessary for the growth and well-being of our nation.

XIV. *Votes of Thanks*

Sincere appreciation and thanks were expressed for the service of all outgoing Committee chairmen and members, and the outgoing Treasurer W. A. Krehl, and outgoing Councilor E. L. R. Stokstad. A standing ovation was given to outgoing President G. M. Briggs.

XV. *Future Annual Meetings*

1969: April 13–18, Atlantic City
1970: April 12–17, Atlantic City
1971: April 12–17, Chicago
1972: April 9–14, Atlantic City

ANNUAL DINNER AND PRESENTATION OF FELLOWS AND AWARDS

The annual banquet was held on Thursday, April 18, 1968 at the Traymore Hotel with 448 attending. Dr. Briggs presided.

Dr. Agnes F. Morgan introduced the newly appointed Fellows, whose citations follow:

HERMAN J. ALMQUIST

HERMAN J. ALMQUIST — for his outstanding contributions to experimental nutrition and for his determination of nutrient requirements, especially protein and amino acid requirements, of poultry and livestock; co-discoverer of vitamin K and the first to elucidate its chemical structure, its occurrence in several forms and its role in nutrition; his own research and his meticulous and continuing review of the work of others contributed quantitative figures on nutrient requirements of great value in the manufacture of animal feeds. For twenty years no voice has spoken with greater authority in this field.

PAUL H. PHILLIPS

PAUL H. PHILLIPS — for an outstanding career of research and teaching in the fields of nutrition, biochemistry, physiology and pathology; for his contributions in the areas of fluoride toxicity, dental caries, mineral and vitamin requirements; for the development of media for the preservation of spermatozoa; for his extraordinary talent as a teacher attested to by the number and quality of his students. His dedication, determination and productivity despite physical handicap have been an inspiration to all who know him.

W. HENRY SEBRELL, JR. — for a distinguished and highly productive career in nutrition research and important administrative responsibilities in the Public Health Service and in an academic department; for his studies of nutrition in relation to liver disease, nutritional anemias, and the identification of the symptoms of riboflavin deficiency; for his career as an officer of the Public Health Service and as Director of the National Institutes of Health; for his contributions as Director of the Institute of Nutrition Sciences and Professor of Nutrition at Columbia University. He has been the recipient of many honors and has served as President of the American Institute of Nutrition and also of the American Society for Clinical Nutrition.

AIN MEAD JOHNSON AWARD FOR RESEARCH IN NUTRITION

HECTOR F. DE LUCA

The 1968 Mead Johnson Award of $1,000 and a scroll was presented to Dr. Hector F. DeLuca, Steenbock Professor of Biochemistry, University of Wisconsin, Madison. The award was given for his clarification of the metabolic interrelationships among calcium, phosphate, vitamin D and parathyroid hormone; for his fundamental studies leading to new concepts concerning the mode of action of vitamin D; and for his studies of metabolites of vitamins A and D, particularly the demonstration of previously unrecognized active forms of the two vitamins.

BORDEN AWARD IN NUTRITION

C. L. COMAR

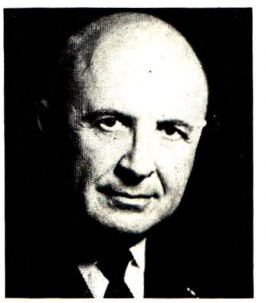
The American Institute of Nutrition's 1968 Borden Award of $1,000 and a gold medal was presented to Dr. C. L. Comar, Professor and Head of the Department of Physical Biology, and Director of the Laboratory of Radiation Biology, Cornell University. The award was given for his outstanding research contributions in mineral metabolism with special reference to the absorption, retention and secretion of calcium and other mineral elements in milk, studies on radionuclides in milk and other foods, and on the discriminatory factors for differential passage of mineral elements across physiologically active membranes especially the intestine, kidney, mammary gland and placenta. These studies have provided new insights in metabolism of minerals by a wide variety of animal species, techniques for nutritional studies with radioisotopes, and in the formulation of models most useful in predicting human exposure to radiation associated with the ingestion of radionuclide contaminants in foods.

OSBORNE AND MENDEL AWARD

CHARLES H. HILL

The 1968 Osborne and Mendel Award of $1,000 and a scroll was presented to Dr. Charles H. Hill, Professor of Poultry Science at North Carolina State University, Raleigh, North Carolina. The award was given in recognition of his outstanding studies on nutritional effects on disease resistance, for his fundamental contributions to our understanding of copper metabolism, and to metal-ion interactions in nutrition. His studies have provided new insight into vitamin levels and resistance of chicks to fowl typhoid, on the function of copper in the synthesis of elastin of the aorta, and on the relationship of the chemical parameters of metal ions and their antagonisms in nutrition.

THE CONRAD A. ELVEHJEM AWARD FOR PUBLIC SERVICE IN NUTRITION



W. HENRY SEBRELL, JR.

The 1968 Conrad A. Elvehjem Award of $1,000 and a scroll was presented to Dr. W. Henry Sebrell, Jr., R. R. Williams Professor of Public Health Nutrition, Director of the Institute of Nutrition Sciences, Columbia University, and Nutrition Consultant, St. Luke's Hospital Center in New York City. The award was made for outstanding research on nutrition in relation to liver disease, anemia and riboflavin deficiency; leadership in nutritional education for the public and within the professions; sound administrative guidance in the United States Public Health Service; leadership in professional societies; and advisory service to national and international organizations.

AMERICAN INSTITUTE OF NUTRITION

Founded September 27, 1928; Incorporated November 16, 1934; Member of Federation 1940

OFFICERS, 1968 — 1969

President: R. H. Barnes, Graduate School of Nutrition, Cornell University, Ithaca, New York 14850.

President-Elect: B. L. O'Dell, University of Missouri, Columbia, Missouri 65201.

Past President: G. M. Briggs, Department of Nutritional Sciences, University of California, Berkeley, California 94720.

Secretary: W. N. Pearson, Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee 37203 (1969).

Treasurer: H. W. Howard, Borden, Incorporated, 350 Madison Avenue, New York, New York (1971).

Councilors: A. E. Harper (1969), H. E. Sauberlich (1970), G. F. Combs (1971), A. B. Eisenstein (1969).

Executive Secretary: James Waddell, 9650 Rockville Pike, Bethesda, Maryland 20014.

COMMITTEES

Nominating Committee: H. P. Broquist, chairman; Hans Fisher, L. M. Henderson, Ruth L. Pike, R. E. Shank.

Membership Committee: Dena C. Cederquist (1969), chairman; C. H. Hill (1970), J. G. Bieri (1971), H. H. Sandstead (1972), F. W. Hill (1973).

Nominating Committee for Mead Johnson Award: L. M. Henderson (1969), chairman; H. E. Sauberlich (1970), H. P. Broquist (1971).

Nominating Committee for Borden Award: K. E. Harshbarger (1969), chairman; R. T. Holman (1970), R. G. Hansen (1971).

Nominating Committee for Osborne and Mendel Award: L. E. Holt, Jr. (1969), chairman; M. K. Horwitt (1970), E. L. R. Stokstad (1971).

Nominating Committee for Conrad A. Elvehjem Award: R. E. Shank (1969), chairman; Olaf Mickelsen (1970), O. C. Johnson (1971).

Fellows Committee: Agnes F. Morgan (1969), chairman; R. M. Forbes (1969), T. H. Jukes (1970), L. A. Maynard (1970), A. B. Morrison (1971).

Committee on Honorary Memberships: R. W. Engel (1969), chairman; L. C. Norris (1970), A. E. Schaefer (1971).

Finance Committee: H. W. Howard (1971), chairman; D. V. Frost (1969), C. H. Krieger (1969), E. E. Rice (1969), James Waddell (ex officio).

Auditing Committee: A. E. Light, chairman; D. A. Benton.

Committee on Nomenclature: S. R. Ames (1969), chairman; P. L. Harris (1969), H. E. Sauberlich (1969), E. W. Crampton (1970), Q. R. Rogers (1970).

Committee on Publications Management: W. J. Darby (1969), chairman; O. L. Kline (1970), J. F. Mueller (1970), F. L. Iber (1969), James Waddell (continuing).

Public and Professional Information Committee: M. S. Read (1970), chairman; C. J. Ackerman (1969), Ruth L. Pike (1969), R. B. Bradfield (1969), F. L. Iber (1969), J. T. Sime (1970), J. J. Vitale (1970), M. C. Nesheim (1970).

Science Projects Subcommittee: C. J. Ackerman, chairman; C. W. Carlson, E. L. Wisman.

Career Brochures Subcommittee: R. B. Bradfield, Chairman; G. J. Christakis, M. C. Nesheim, Ruth L. Pike.

Committee on Experimental Animal Nutrition: G. F. Combs (1969), chairman; R. W. Luecke (1969), W. H. Pfander (1970), J. K. Loosli (1970), L. S. Jensen (1971), Gennard Matrone (1971), B. L. O'Dell (1970) ex officio, R. L. Preston (1969) ex officio, R. H. Barnes (1969) ex officio.

Committee on Public Affairs: H. A. Schneider (1971), chairman; Leroy Voris (1969), O. L. Kline (1970).

Program Committee: W. N. Pearson (1969), chairman; J. G. Bieri, S. A. Miller, James Waddell (continuing).

Ad hoc AIN National Medal of Science Committee: W. N. Pearson, chairman; R. W. Engel, R. E. Shank.

Ad hoc Advisory Committee to AIN Office of Nutrition Science Services: D. B. Hand, chairman; G. F. Combs, A. L. Forbes, W. A. Gortner, W. N. Pearson, M. S. Read, M. L. Scott, L. J. Teply.

Symposia Committee Chairman: H. E. Sauberlich (1969).

Ad hoc Committee on Graduate Nutrition Training: A. E. Harper, chairman; W. D. Brown, Dena C. Cederquist, Hans Fisher, L. M. Henderson.

Ad hoc Committee on Undergraduate Nutrition Training: Olaf Mickelsen, chairman; Helen A. Hunscher, A. R. Kemmerer, Melvin Lee, Mary Ann Williams.

U. S. National Committee, IUNS: O. L. Kline (1969), chairman; W. A. Krehl (1970), A. L. Forbes (1971), G. F. Combs (1969), C. M. Young (1969), A. E. Schaefer (1970), G. F. Stewart (1970), R. O. Nesheim (1971), W. N. Pearson (1971). Ex officio (voting) R. H. Barnes, Grace Goldsmith; ex officio (nonvoting) C. G. King, A. G. Norman, R. K. Cannan, H. Brown, E. C. Rowan.

Archivist: E. Neige Todhunter (1971).

Historian: Agnes Fay Morgan.

REPRESENTATIVES

National Research Council Boards and Divisions: G. F. Combs (1969).

Food and Agriculture Organization: H. E. Sauberlich (1969).

National Society for Medical Research: P. H. Derse (1969).

American Association for the Advancement of Science: H. W. Howard (1969), H. C. Tidwell (1969).

U. S. National Committee for the International Biological Program: G. K. Davis.

Federation Public Information Committee: M. S. Read (1970).

Federation Proceedings Editorial Board: Olaf Mickelsen (1971).

Federation Public Affairs Committee: H. A. Schneider (1971).

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(A division of the American Institute of Nutrition)

President, J. F. Mueller; President-Elect, W. S. Hartroft; Past President, R. E. Shank; Secretary-Treasurer, A. B. Eisenstein, Director of Medicine, Cumberland Hospital Division, Brooklyn-Cumberland Medical Center, 39 Auburn Place, Brooklyn, New York 11205; Councilors: J. J. Will (1969), O. N. Miller (1970), C. S. Lieber (1971). M. E. Rubini (ex officio).

Editorial Board
American Journal of Clinical Nutrition

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Invitation for Nominations for 1969 American Institute of Nutrition Awards

Nominations are requested for the 1969 annual awards administered by the American Institute of Nutrition to be presented at the next annual meeting. Nominations may be made by anyone, including members of the Nominating Committees and non-members of the Institute.

The following information must be submitted: (1) Name of the award for which the candidate is proposed. (2) *A brief convincing statement setting forth the basis for the nomination and, where appropriate, a selected bibliography which supports the nomination. Seconding or supporting letters are not to be submitted.* (3) Five copies of the nominating letter must be sent to the chairman of the appropriate nominating committee *before October 1, 1968,* to be considered for the 1969 awards.

General regulations for A.I.N. awards. Membership in the American Institute of Nutrition is not a requirement for eligibility for an award and there is no limitation as to age except as specified for the Mead Johnson Award. An individual who has received one Institute award is ineligible to receive another Institute award unless it is for outstanding research subsequent to or not covered by the first award. A Jury of Award composed of A.I.N. members, which makes final selection and remains anonymous, may recommend that an award be omitted in any given year if in its opinion the work of the candidates nominated does not warrant the award. An award is usually given to one person, but, if circumstances and justice so dictate, a Jury of Award may recommend that any particular award be divided between two or more collaborators in a given research.

Presentation of awards will be made during the banquet at the annual meeting.

1969 Borden Award in Nutrition

The Borden Award in Nutrition, consisting of $1000 and a gold medal, is made available by the Borden Company Foundation, Inc. The award is given in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of milk or its components. The award will be made primarily for the publication of specific papers during the previous calendar year, but the Jury of Award may recommend that it be given for important contributions made over a more extended period of time not necessarily including the previous calendar year. Employees of the Borden Company are not eligible for this award nor are individuals who have received a Borden Award from another administering association unless the new award be for outstanding research on a different subject or for specific accomplishment subsequent to the first award.

Former recipients of this award are:

1944 – E. V. McCollum
1945 – H. H. Mitchell
1946 – P. C. Jeans and Genevieve Stearns
1947 – L. A. Maynard
1948 – C. A. Cary
1949 – H. J. Deuel, Jr.
1950 – H. C. Sherman
1951 – P. György
1952 – M. Kleiber
1953 – H. H. Williams
1954 – A. F. Morgan and A. H. Smith
1955 – A. G. Hogan
1956 – F. M. Strong
1957 – no award
1958 – L. D. Wright
1959 – H. Steenbock
1960 – R. G. Hansen
1961 – K. Schwarz
1962 – H. A. Barker
1963 – Arthur L. Black
1964 – G. K. Davis
1965 – A. E. Harper
1966 – R. T. Holman
1967 – R. H. Barnes
1968 – C. L. Comar

NOMINATING COMMITTEE:

K. E. HARSHBARGER, *Chairman*
R. T. HOLMAN
R. G. HANSEN

Send nominations to:

K. E. HARSHBARGER
Department of Dairy Science
University of Illinois
Urbana, Illinois 61801

1969 Osborne and Mendel Award

The Osborne and Mendel Award of $1000 and an inscribed scroll has been established by the Nutrition Foundation, Inc., for the recognition of outstanding recent basic research accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the

most significant published contribution in approximately the calendar year preceding the annual meeting of the Institute, or who has published recently a series of papers of outstanding significance. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration.

Former recipients of this award are:

1949 – W. C. Rose
1950 – C. A. Elvehjem
1951 – E. E. Snell
1952 – Icie Macy Hoobler
1953 – V. du Vigneaud
1954 – L. A. Maynard
1955 – E. V. McCollum
1956 – A. G. Hogan
1957 – G. R. Cowgill
1958 – P. György
1959 – Grace A. Goldsmith
1960 – N. S. Scrimshaw
1961 – Max K. Horwitt
1962 – William J. Darby
1963 – James B. Allison
1964 – L. Emmett Holt, Jr.
1965 – D. M. Hegsted
1966 – H. H. Mitchell
1967 – Samuel Lepkovsky
1968 – C. H. Hill

NOMINATING COMMITTEE:
L. E. HOLT, JR., *Chairman*
M. K. HORWITT
E. L. R. STOKSTAD

Send nominations to:

L. E. HOLT, JR.
Professor of Pediatrics
New York University School of Medicine
550 First Avenue
New York, N. Y. 10016

1969 Mead Johnson Award for Research in Nutrition

The Mead Johnson Award of $1000 and an inscribed scroll is made available by Mead Johnson and Company to an investigator who has not reached his 46th birthday during the calendar year in which the Award is given. Selection by the Jury of Award will be based primarily on a single outstanding piece of research in nutrition published in the year preceding the annual meeting or on a series of papers on the same subject published within not more than the three years preceding the annual meeting.

Former recipients of this award are:

1939 – C. A. Elvehjem
1940 – W. H. Sebrell, Jr.
J. C. Keresztesy
J. R. Stevens
S. A. Harris
E. T. Stiller
K. Folkers
1941 – R. J. Williams
1942 – G. R. Cowgill
1943 – V. du Vigneaud
1944 – A. G. Hogan
1945 – D. W. Woolley
1946 – E. E. Snell
1947 – W. J. Darby
P. L. Day
E. L. R. Stokstad
1948 – F. Lipmann
1949 – Mary S. Shorb
K. Folkers
1950 – W. B. Castle
1951 – no award
1952 – H. E. Sauberlich
1964 – J. S. Dinning
1965 – J. G. Bieri
1966 – M. Daniel Lane
1967 – W. N. Pearson
1968 – H. F. DeLuca

NOMINATING COMMITTEE:
L. M. HENDERSON, *Chairman*
H. E. SAUBERLICH
H. P. BROQUIST

Send nominations to:

L. M. HENDERSON
Department of Biochemistry
University of Minnesota
St. Paul, Minnesota 55101

1969 Conrad A. Elvehjem Award for Public Service in Nutrition

The Conrad A. Elvehjem Award for Public Service in Nutrition, consisting of $1000 and an inscribed scroll, is made available by the Wisconsin Alumni Research Foundation. The award is bestowed in recognition of distinguished service to the public through the science of nutrition. Such service, primarily, would be through distinctive activities in the public interest in governmental, industrial, private, or international institutions but would not exclude, necessarily, contributions of an investigative character.

Former recipients of this award are:

1966 – C. Glen King
1967 – J. B. Youmans
1968 – W. H. Sebrell, Jr.

NOMINATING COMMITTEE:
R. E. SHANK, *Chairman*
OLAF MICKELSEN
O. C. JOHNSON

Send nominations to:

R. E. SHANK
Department of Preventive Medicine
Washington University School of Medicine
4550 Scott Avenue
St. Louis, Missouri 63110

Invitation for Nominations for 1969 American Institute of Nutrition Fellows

The Fellows Committee of the American Institute of Nutrition invites nominations for Fellows in the Society. Eligible candidates are active or retired members of the Society who have passed their sixty-fifth birthday (by the time of the annual meeting) and who have had distinguished careers in nutrition. Up to three Fellows may be chosen each year.

Nominations may be made to the Chairman of the Fellows Committee by any member of the Society, including members of the Committee.

Nominations (in 5 copies) are due by October 1. A supporting statement giving the reason for the nomination is desirable.

Final selection will be made by the Fellows Committee and a suitable citation will be presented at the Annual Dinner in April.

Fellows Committee:

RICHARD M. FORBES, *Chairman*
T. H. JUKES
L. A. MAYNARD
A. E. MORRISON

Send nominations to:

RICHARD M. FORBES
124 Animal Sciences Laboratory
University of Illinois
Urbana, Illinois 61801

The following persons have been elected previously as Fellows of the Society:

Georgian Adams (1967)
Herman J. Almquist (1968)
J. B. Brown (1964)
Thorne M. Carpenter (1958)
George R. Cowgill (1958)
Earle W. Crampton (1967)
Henrik Dam (1964)
Eugene F. DuBois (1958)
R. Adams Dutcher (1961)
Ernest B. Forbes (1958)
Casimir Funk (1958)
Wendell H. Griffith (1963)
Paul György (1965)
Albert G. Hogan (1959)
L. Emmett Holt, Jr. (1967)
Icie Macy Hoobler (1960)
Paul E. Howe (1960)
J. S. Hughes (1962)
C. Glen King (1963)
Max Kleiber (1966)
Samuel Lepkovsky (1966)
Leonard A. Maynard (1960)
Elmer V. McCollum (1958)
Harold H. Mitchell (1958)
Agnes Fay Morgan (1959)
John R. Murlin (1958)
Leo C. Norris (1963)
Helen T. Parsons (1961)
Paul H. Phillips (1968)
Lydia J. Roberts (1962)
William C. Rose (1959)
W. D. Salmon (1962)
W. H. Sebrell, Jr. (1968)
Arthur H. Smith (1961)
Genevieve Stearns (1965)
Harry Steenbock (1958)
Hazel K. Stiebeling (1964)
Raymond W. Swift (1965)
Robert R. Williams (1958)
John B. Youmans (1966)

Invitation for Nominations for Honorary Membership in the American Institute of Nutrition

The Committee on Honorary Memberships of the American Institute of Nutrition invites nominations for Honorary Members.

Distinguished individuals of any country who are not members of the American Institute of Nutrition and who have contributed to the advance of the science of nutrition shall be eligible for proposal as Honorary Members of the Society.

Nominations may be made to the Chairman of the Committee on Honorary Memberships by two members of the Society.

Nominations (in three copies) are due by October 1. A supporting statement giving the reason for the nomination is desirable.

Final selection of nominees will be made by the Council of the American Institute of Nutrition and such nominations submitted to the Society at the spring meeting. Election requires a two-thirds majority of the ballots cast.

Honorary members pay no membership fees but are eligible to subscribe to the official journal(s) at member's rates.

Committee on Honorary Memberships:

R. W. ENGEL, *Chairman*
L. C. NORRIS
A. E. SCHAEFER

Send nominations to:

R. W. ENGEL
Associate Dean for Research
Virginia Polytechnic Institute
Blacksburg, Virginia 24061

The following persons have been elected previously as Honorary Members of the Society:

Kunitaro Arimoto
W. R. Aykroyd
Frank B. Berry
Edward Jean Bigwood
Frank G. Boudreau
Robert C. Burgess
Dame Harriette Chick
F. W. A. Clements
Hans D. Cremer
Sir David P. Cuthbertson
Herbert M. Evans
Karl Guggenheim
Joachim Kühnau
Joseph Masek
Toshio Oiso
H. A. P. C. Oomen
Lord John Boyd Orr
Conrado R. Pascual
V. N. Patwardhan
Sir Rudolph A. Peters
B. S. Platt
Juan Salcedo
Emile F. Terroine
Jean Tremolieres
Eric John Underwood
Artturi I. Virtanen

THE JOURNAL OF NUTRITION

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Guide for Authors

New policy concerning page charge, abstracts and summaries, and numerical citation of literature

The Journal of Nutrition welcomes concise reports of original research bearing on the nutrition of any organism. Preliminary notes, or papers already published or in press elsewhere are not acceptable. Consideration will be given to the publication of supplements at the author's expense.

Manuscripts are to be typewritten on bond paper (8½ × 11 inches). Double spacing should be used throughout, including that on title page, tables, legends, footnotes and references. A margin of about 1¼ inches (or 3 cm) is needed at each side of the sheets.

An abstract of 200 words or less, typed on a separate sheet and double-spaced, should be included. When published, this will appear ahead of the introductory section of the text in lieu of a summary at the end. The abstract should present the scope of the work and the principal observations.

An original and a carbon copy of the manuscript and abstract should be sent *flat* by registered mail to

RICHARD H. BARNES, EDITOR
THE JOURNAL OF NUTRITION
GRADUATE SCHOOL OF NUTRITION
CORNELL UNIVERSITY, SAVAGE HALL
ITHACA, NEW YORK

The following information should be listed on page 1:

1. Complete title of paper (in upper and lower case letters).
2. Name or names of authors.
3. Institution or laboratory, with city and state.
4. Shortened form of title (not more than 48 letters and spaces).
5. Address and name of author to whom proof is to be sent.

(*All footnotes*, including those pertaining to the title page, should be placed on a separate sheet, typed double-spaced.)

The text should begin on page 2. The manuscript should be prepared in complete and finished form. Number all pages consecutively in the following order: title page, text, literature cited, footnotes, abstract, tables and figure legends. *Never divide or hyphenate a word at the end of a line*, and do not staple together the pages of the manuscript.

Start each of the following sections on a new page, typed double-spaced:

1. The abstract.
2. Tables, with appropriate heading (a separate sheet for each table).
3. Explanation of figures, numbered and listed consecutively (do not affix illustrations to these sheets).
4. Footnotes — including those referring to title, author, institution and text.
5. Literature cited, arranged numerically in the order of first citation in the text, as "Jones and Smith (1) and others (2) have reported . . . ."

LITERATURE CITED

1. Jones, K. Z., and X. Y. Smith 1972 Growth of rats when fed raw soybean rations. J. Nutr., *95:* 102.
2. Brown, Q. R., V. A. Ham and I. V. Long 1971 Effects of dietary fat on cholesterol metabolism. J. Nutr., *94:* 625.

The metric system is used for all units, and temperature is expressed in the centigrade scale. The words "et al.," "per se" and "ad libitum" are not underscored. The repeated use of a unit in a sentence may be avoided by stating the common unit used, as "the diet consisted of the following: (in grams) sucrose, 50; casein, 130; . . ." Or, in other instances, as "8, 12 and 14%." The expression "*fed* a diet" is preferred to "*on* a diet." Certain other preferred usage is described in the *Style Manual for Biological Journals*.[1] The use of laboratory jargon is to be avoided, as well as such comments as "it can be seen that," " it is interesting that," and "it can be noted that." The word "quite" is often misused. Use of the active voice in all writing is preferred to the passive voice.

Registered trade names. Registered trade names are not used in text, tables or figures, except when necessary to identify certain equipment. A trivial or descriptive name should be used in text and tables, with a footnote giving the registered trade name, manufacturer and address (city and state).

Footnotes. In the text, footnotes should be numbered consecutively, including any indicated on the title page. For tables, footnotes should be typed *double-spaced* directly beneath the table, and numbered 1, 2, 3, etc. Superscripts in the table should appear consecutively, starting at the top of the table and reading from left to right across each line. Footnotes to tables are independent of the other footnote numbers in the text. Symbols are not used as superscripts and subscripts.

Acknowledgments. Financial support should be listed as a footnote to the title. Credit for materials should be listed as a footnote in the text. Technical assistance and advice may be cited in a section headed Acknowledgments, which will appear at the end of the text.

Literature Cited. Accuracy and adequacy of the references are the responsibility of the author. Literature cited should be checked carefully with the original publication. References to abstracts of verbal reports and to other unedited material (as the abstracts in Federation Proceedings, theses, and industrial technical bulletins) should be treated as text footnotes. Reference to a paper in press may be included in Literature Cited. If such a paper would be useful in the evaluation of the manuscript under consideration, it is advisable to make a copy of it available to the Editor. When a manuscript is one of a series of papers, the preceding paper should be included in Literature Cited. Personal communications and unpublished experiments should be treated as footnotes to the text.

Numbers. Use Arabic numerals throughout, including those in tables and figures. If possible, avoid beginning a sentence with a numeral; when necessary, numbers so used should be spelled out as well as any units immediately *following*.

Abbreviations and symbols. Letters in abbreviations such as DPN or IU are not spaced and periods are omitted except when the abbreviation might be read as another word. Following is a list of the more common abbreviations and symbols used in the *Journal*:

average	avg (*in tables*)
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

Other commonly accepted abbreviations may be found in the *Style Manual for Biological Journals*.[2] The isotope designation of a labeled compound should ordinarily appear *before* the name of the compound to which it applies. When following a symbol for a compound, it should be written as superscript (as, ^{14}C); when the name of the compound is spelled out, the isotope designation should be written on the same line (as, carbon-14).

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Original drawings, with two reproductions, to be sent to reviewers, or in the case of photographs, the original and two glossy prints, should accompany the manuscript. They should be marked on the back in ink with the author's name, complete address, and with the figure numbers. Such drawings and photographs must not exceed 8½ × 11 inches in size and must be at least 5¼ inches wide in order to fit the 2⅝-inch single column width when reduced by one-half. When a complicated figure requires more space for clarity, a proportionately larger illustration will be acceptable. But two copies of *prints* should be submitted on sheets of the same size as the text. Or if prints are smaller, they should be affixed to sheets of manuscript size. Oversize or undersize figures are difficult to handle in editing. Legends (including any keys to symbols or charts) should appear on a separate sheet. Drawings should be on white or blue-white paper or bristol board — not cream-white. They should be prepared for reproduction as line or halftone engravings. Letters and numbers should be uniform and large enough so that no character will be less than 2 mm high after reduction. A line 0.4 mm wide reproduces satisfactorily when reduced by one-half.

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Action to institute a page charge for publication in the Journal of Nutrition was taken at the April 1962 meeting of the AIN. Proceedings of this meeting are printed in

[2] See footnote 1.

the Journal of Nutrition, 78: 120–132, 1962.

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American Documentation Institute. To conserve space in the Journal, lengthy descriptions of experimental procedures, detailed data such as that on individual animals or subjects, extra figures and other material considered important supplementary information, may be deposited with the American Documentation Institute, Library of Congress, through the Editor's Office with a footnote reference.

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