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CHARLES FORD LANGWORTHY

1864 — 1932

C. F. Langworthy

Charles Ford Langworthy — A Biographical Sketch

(August 9, 1864 — March 3, 1932)

Scientific investigator of problems of fundamental importance in the life of American people, able translator of new knowledge in the printed form for the use of all, and catalytic influence in the sphere of higher education for women — so may be described the man who unmistakably left his imprint during the early decades of the century on basic and popular phases of the developing sciences of nutrition and home economics.

Charles Ford Langworthy was born in Middlebury, Vermont, on August 9, 1864. He was the son of Charles Parker and Ann Elizabeth (Ford) Langworthy. His father was a native of Middlebury; his mother was of English birth. The father was by occupation a clerk. Both parents were prominent in the church, civic, and school affairs of Middlebury — in Langworthy's time a small town of about 3000 inhabitants situated in a dairying area. As a boy, Langworthy attended the public schools of Middlebury and was graduated from its high school in 1883. He then entered Middlebury College, a college at that time admitting only men students. Founded in 1880, the institution had a faculty of eight professors and an enrollment of less than one hundred students. Langworthy was graduated along with eleven others in the class of 1887. During his college years, Langworthy's academic interests centered in chemistry, physics, and botany. After graduation his career followed the classic pattern of the time. He earned the A.M. degree from his alma mater in 1890, after which he studied chemistry in Germany for three years, receiving the degree of doctor of philosophy in 1893 from the Emperor Wilhelm University, Strasburg.

Immediately upon his return to the United States in 1893, Langworthy became associated with Dr. W. O. Atwater in the dual role of chemical assistant and instructor in chemistry at Wesleyan University in Middletown, Connecticut. His career from this point onward is surely the product of the time, the place, and the people.

Atwater, recognized today as the founder of the science of nutrition in the United States, had recently returned from a period of study in Voit's laboratory and had become acquainted with methods used by the Germans in the study of agriculture and nutrition. Stimulated to undertake such studies himself, Atwater was instrumental in the establishment of the first agricultural experiment station in the United States (1875), located initially at Wesleyan and transferred later to the Sheffield Scientific School at Yale University. He became its director.

Such experiment stations had been in operation in Europe for twenty-five years and had been remarkably successful in promoting the interests of agriculture by scientific investigation. The worth of the operation at Wesleyan was immediately discernible, and several states, within the next decade, followed the example of Connecticut and established state experiment stations.

Funds for their operation were scarce however, and Atwater and other agricultural leaders in the country campaigned for federal support for these stations and for the founding of similar agencies in other states. The needed support was provided in 1887 with the passage of the Hatch Act authorizing the promotion of agriculture by scientific investigation and experiment. The act provided for the establishment of a second station at Storrs, Connecticut. Atwater was made the director of this station also, with headquarters at Wesleyan University some thirty-five miles away. As a further consequence of the Act, an Office of Experiment Stations was

created in the United States Department of Agriculture to coordinate the programs in various parts of the country and to suggest lines of inquiry. Atwater was appointed as the first director of this federal agency with offices in Washington and at Wesleyan University.

Atwater recognized the close relationship of agricultural and nutritional investigation. He firmly believed that in studying the food of animals, workers had no right to neglect the food of man. But again, inadequate funds limited the expansion of programs in this direction. It was not until 1894, and then largely through Atwaters' efforts, that Congress appropriated a special fund to initiate the conduct of human nutrition investigations in the Department of Agriculture. Experiment stations throughout the country were expected to cooperate with the Secretary of Agriculture in carrying out these investigations. The new work was assigned to the Office of Experiment Stations, again with Dr. Atwater as special agent in charge. Headquarters were set up at Middletown University and at Storrs Experiment Station for investigations on the composition, nutritive value and cost of food materials, on the kinds of dietaries consumed by people, on the digestibilities of common foods, on the laws of nutrition of human beings, and on improvement of research methods together with the compilation of results of previous investigations and the publication of both technical and popular bulletins. Atwater also set up"centers of inquiry" in the state experiment stations for the study of nutrition problems in accord with the provisions of the 1894 legislation. By the end of 1894, eleven institutions had entered into such cooperation.

Thus, Langworthy upon his return from Germany in 1893 entered a laboratory dominated by a master mind and teeming with investigative excitement. The young man, with his training in science and an inherent concern for the welfare of man could not but respond to the influence of Atwater and his outstanding ability to organize and direct research. Research activity, too, soon reached a height, for Atwater with his gift of attracting promising young scientists assembled a group of laboratory workers destined to leadership in nutrition and related sciences. The stimulation of association with people like H. C. Sherman, F. G. Benedict, P. B. Hawk, J. F. Snell, and R. D. Milner can be imagined.

A promotion two years later made Langworthy an "expert in nutrition" in the Office of Experiment Stations. At this time, he also received an appointment as an associate editor of the Experiment Station Record, dividing his time for a short interval between Middletown and Washington. He held this position of associate editor until 1924, abstracting the literature first for the Animal Production Department, then Zootechny and Human Nutrition, and finally Foods and Human Nutrition.

In 1905, Atwater was forced to retire on account of illness, and Langworthy was made chief of the Nutrition Investigations. Many attributes probably were responsible for this appointment: his appreciation of the importance of the investigations, his wide knowledge of the nutrition movement gained through continuous contact with the literature over some years, and his scientific habit of mind.

The Nutrition Investigations were moved to the laboratories of the Department of Agriculture at Washington, D.C. in 1906, and were conducted under the auspices of the Office of Experiment Stations until 1915. In May of that year, the enactment of the Smith-Lever Extension Act brought about the reorganization of the Office of Experiment Stations into the States Relations Service so as to meet the needs of an enlarged extension program. A new agency, the Office of Home Economics, was created as a unit of this service and Dr. Langworthy was made its chief. The new office absorbed the activities of the Nutrition Investigations and initiated new lines of work related to Home Economics.

In 1923, the ranking of the home economics work in the Department of Agriculture was changed from an office to a bureau. At this time, the appointment of women to administrative offices in the government was much to the fore, and it was deemed appropriate to place a woman at the head of the new Bureau of Home Economics. Dr. Louise Stanley thus became its first chief. Dr. Langworthy, however, remained on the staff of the Bureau

as a "specialist in home economics" until 1929 when ill health necessitated his retirement. He died in Washington on March 3, 1932. Dr. Langworthy never married.

In 1912, Middlebury College honored its illustrious alumnus with the honorary degree of doctor of science. The citation read as follows: "Charles Ford Langworthy, chemist and expert in food and nutrition whose scientific investigations in problems of fundamental importance in the life of American people, have brought honor and gratitude to the National Department of Agriculture and reflected credit upon his alma mater."

Dr. Langworthy is cited in Who's Who in America, Who Was Who in America, and Rural Outlook Series. He was a Fellow in the American Association for the Advancement of Science and his wide range of scientific interest was reflected in membership in many societies such as the American Chemical Society, Society of Biological Chemists, Washington Academy of Sciences, Société Scientifique d'Hygiène Alimentaire Paris, American Forestry Association, American Home Economics Association, and American Dietetic Association. He also was a member of the Society of Colonial Wars.

Langworthy's contributions to nutrition, home economics, and to national life fall into a pattern which may be described in terms of his work in the Human Nutrition Investigations from its initial to final phases, his development of the program and activities of the Office of Home Economics, and his work as editor and author.

The human nutrition investigations

The spirit, objectives, and research methods of the Nutrition Investigations as envisioned by Atwater marked the continuance of the program under Langworthy's direction. A program as carefully thought out and executed as it was in its early phases could not be discarded, contributing as it did to the objectives of the Department of Agriculture and to the welfare of people generally. The way of the early investigations thus became the way of investigations in the Office of Experiment Stations and in the Office of Home Economics. Langworthy's contributions throughout the entire course of the studies fall into four general categories.

Collation of data and summarization of knowledge. In the early days of the investigations, knowledge was sparse and widely scattered. Atwater used every means possible to bring this information together for the use of workers in experiment stations and elsewhere. Langworthy's responsibilities in such collations served as a training ground for future responsibility. Early assignments involving access to Atwater's magnificent library and to the research data of the laboratory put him into direct contact with the budding science of nutrition and must have stimulated his interest and imagination. The first task to which Langworthy was assigned was the assembling of data for the famous bulletin no. 28 of the Office of Experiment Stations entitled "Chemical composition of American food materials." He also gave valuable aid in the preparation of bulletin no. 21 entitled "Methods and results of investigations on the chemistry and economy of food." Langworthy apparently was so skillful in this work that he was chosen to prepare later summaries of the status of nutritional knowledge about man and animals for publication in the Experiment Station Record.

Even as bulletin no. 21 was being prepared, Atwater and Langworthy began planning, as supplementary information, an ambitious "digest of metabolism experiments in which the balance of income and outgo was determined." The finished product proved to be a monumental work, more than 400 pages long and reporting some 3,600 experiments conducted with man and animals (Experiment Station Bulletin no. 45, 1897). Even today, this work constitutes one of the most complete repositories of information available about the era studied.

In other writings of this period, Langworthy seeks to show that metabolism or the chemical and physical changes which matter and energy undergo within the animal organism is basic to inquiries on nutrition of man or animals. The concepts expounded are very interesting in light of the newness of nutritional science. In "The value of experiments on the me-

tabolism of energy" (Experiment Station Record, *9:* 1001–1019, 1898), Langworthy writes that foods have a dual purpose: they must serve for the building and repair of body substance and they must supply energy for heat and work. These foods consist of the nutrients, protein, fat, carbohydrates and various mineral salts. Nitrogenous material is present in the cells of all tissues and organs. Hence, foods containing this element are an indispensable part of the diet. These concepts, in turn, depend upon certain fundamental facts which Langworthy designates as the "laws of nutrition." The more important of these follow: All nitrogen is derived by food, i.e., none from the atmosphere; all nitrogen is excreted in urine and feces, none as gaseous nitrogen; the animal adjusts itself to nitrogen intake and comes into nitrogen balance at which point intake and output are approximately equal; a certain amount of food material, i.e., protein, fat, and carbohydrate, is required for maintenance, mineral matter is also essential, but very little is known regarding the kind and amount necessary; a more abundant ration is required for muscular work, fattening, and milk production; food supplied in excess of all needs is stored, in part at least, as reserve material, principally as fat and glycogen; the body comes into nitrogen equilibrium at different levels of protein intake; body fat may be formed from fat and carbohydrate supplied in the food; as furnishers of energy, the different nutrients may replace each other in approximately the following ratios, protein: fat: carbohydrate as 1:2.5:1; if no food is supplied or if a diet containing no nitrogen is consumed, nitrogen derived from body tissues is excreted in the urine, the animal living on its own substance; and finally, nutrients of the food combine with oxygen within the body and undergo combustion, thus liberating energy for the body.

Basic investigations on the metabolism of man. Studies of what happens to food in metabolism began in the Wesleyan laboratory in 1897 with the building of a respiration calorimeter by Atwater and Rosa. With it, the amount of heat given off by the body could be measured directly, digestion studied, and nitrogen-carbon balances determined for the measurement of gain or loss of protein and fat.

The studies continued in Middletown until 1907, when at the death of Dr. Atwater, the calorimeter was moved to Washington and all activities transferred to the Office of Experiment Stations with Langworthy in charge. Money was appropriated by the Congress for the transportation of the calorimeter to Washington, but was then denied for active work until 1909 when funds again were authorized for the continuance of the investigations. This action brought some delay in the program, but Langworthy made extensive improvements in the calorimeter during its installation. Further modifications over the years made it an exceedingly accurate instrument for measuring heat changes and the gaseous exchange.

Langworthy, with F. G. Benedict and others, was an active participant in the experiments conducted with the use of the instrument during the time that it was in the Wesleyan laboratory. In general, the work had established the transfer of matter and energy that occurs in man's utilization of food for bodily activities, the demands of the body for nutriment, and the effect of muscular work on such demands, as well as providing information on the actual nutritive value of different kinds of food materials. When Langworthy assumed direction of the Nutrition Investigations, 500 metabolism experiments had been completed, 88 with the use of the calorimeter.

Langworthy thus had a sound base derived from personal experience, for the extension of metabolic studies when he became director of the Nutrition Investigations. In terms of general metabolism, the new studies moved toward determinations of the energy cost of work.

With the establishment of the Office of Home Economics in 1915, it was appropriate that studies turned to estimations of energy expenditures involved in the execution of household work. Previous studies had shown that the energy cost of any task varied with the nature of the task and the rate at which it was performed. It was hoped that the new experiments not only would provide information essential in estimating the energy require-

ments of an individual in the various circumstances of her daily life, but would give some rational estimates of what constituted the expenditures of energy involved in a reasonable day's work.

The investigations, comprehensive in scope and well-planned, controlled, and executed included not only measurements of the energy expended in the performance of different kinds of tasks then carried on in the home under different conditions, but also the amount of time involved in executing these tasks under varying conditions of personnel and routine. The relative energy demands of the various tasks studied were related directly to the hardness of the task and the activity involved, i.e., light, moderate, or heavy. H. G. Barott assisted in these investigations. The study represents one of the classic contributions of the Office of Home Economics. Today, as then, the question of the energy requirement occupies the attention of many nutritionists, and these initial investigations offer a base for comparison in the development of quicker and more telling methodology.

Langworthy also applied the methods of calorimetry to the problems of agricultural production. Cooperative work with the Bureau of Chemistry had demonstrated the applicability of the technique to studies of plant physiology. The respiratory processes that occur, for example, in the ripening of fruit, its holding and decay, as the result of enzyme action, were measurable in the calorimeter. The construction of a smaller calorimeter permitted the measurement of the gaseous exchange and heat elimination occurring in fruits and vegetables during their marketing, storage, and transportation with resulting improvement in the processes employed. This small calorimeter also was adaptable to problems relating to the incubation of eggs, the wintering of bees, and changes in the curing and storage of meats and cheeses but the demands of the war effort curtailed investigative activity along these lines.

Digestibility of foods. Since a considerable body of knowledge had accumulated in the early years of the nutrition investigations, studies of the proximate composition of foods were discontinued when the nutrition laboratories were established in Washington. Digestibility studies, however, remained an important phase of the continuing investigations, particularly those related to determinations of utilizable energy, for as Langworthy wrote in 1910, "no matter what its composition, food is of no use to the body unless it is digested."

Studies in addition embraced determinations of the energy cost of the digestion of food and the influence of various factors on the completeness of the digestive process. Data made possible estimations of the relative value of different agricultural products, both animal and vegetable, as sources of utilizable energy. Three different avenues of approach were used in pursuing these studies: artificial digestion studies for determinations of ease and rapidity of digestion, balance experiments involving analyses of nitrogen, carbon, and energy values of food and excretory products, and determinations of the gaseous and energy exchange with the use of the respiration calorimeter.

Results from one series of experiments relating to the digestibility of cheese serves to illustrate the kind of information sought. Making Cheddar cheese with different amounts of rennet and ripening it under different conditions did not affect the digestibility of the product. Different kinds of cheeses were equally well digested; and the energy costs involved in the digestion of cheese and meat were identical. The importance of cheese as a food, thus, was extended beyond its use as a condiment.

The digestibilities of a wide assortment of foods were determined in the interval from 1905–1924. Some were studied in view of the possibility of an extended agricultural production and use (nonsaccharine grain sorghums and millets), others because they represented new potentials in agricultural production (the dasheen), and still others because they represented less favored food (mutton). Certain other studies dealing with the digestibility of raw starches and carbohydrates provided important data with respect to many cereal products.

But perhaps more than any other factor, the need for feeding the civilian and mili-

tary populations of a nation at war dictated the nature of the studies made. Thus, we find reports of studies relating to the influence of milling on wheat, the digestibility of breads made from flours containing different proportions of the wheat kernel, wheat substitutes, and the respective digestibilities of unusual meats (seal, rabbit, horse, and dried powdered meats) which might find some place in the war program.

However, the most comprehensive experiments undertaken related to the digestibilities of various edible fats. An abundant supply of fat was considered of major importance to the nutrition not only of the individual but to that of a nation in war-time, and there was justification for efforts to find new sources of food fats and to make better use of those already available. Data had been limited on the energy supplied by fats originating from different sources and on the relation of melting point to thoroughness of digestion. In all, Langworthy and his co-workers (H. J. Deuel and A. D. Holmes) studied the digestibility of some 130 different fats. Various animal fats were studied as well as those derived from grains, legumes, fruits and vegetables, seeds and nuts, fish, poultry, cream, and butter fat. Hydrogenated oils, blends of hydrogenated oils, and oleomargarine also were included.

The investigations undoubtedly established the wholesomeness and usefulness of fats as a food and their place in the diet, a position enhanced, at about the time of these studies, by the fact that some fats may serve as carriers of the newly discovered vitamins.

Dietary surveys. The dietary surveys represented an important facet of study during the entire course of the Nutrition Investigations. Some 350 studies of the food intakes of people of varying occupation and of different economic and social strata, living in the rural areas, towns, and cities of the United States had been conducted under Atwater's guidance and general direction. Although emphasis on this phase of investigation was decreased when Langworthy assumed leadership, several studies contributing to understanding of the food patterns of people were completed. In one, concern was transferred from individuals and families to people living in public institutions, i.e., homes for children and for the aged. Another, an emergency dietary survey planned to gain information on the nature and adequacy of food consumed by the people of a nation at war, was comprehensive in scope involving about 2,000 dietary records of which 1,425 represented family records and 575 institutional studies.

Perhaps as important as the information provided on food intakes of people was the use Atwater and Langworthy made of the records in arriving at dietary "standards." Both workers believed that inasmuch as the diets recorded represented the food intakes of healthy and vigorous people, they could serve, when expressed in chemical terms, as a guide for evaluating adequacy of food practices. The chemical terms included, after corrections were made for waste and losses in digestion and metabolism, the protein, fat, and carbohydrate content of the food consumed together with an estimate of its total energy value.

Thus, we find Langworthy in 1908 expressing the dietary standard for man in full vigor at moderate muscular work as follows:

	Protein	Energy
Food as purchased	115 gm.	3,800 Cal.
Food eaten	105 gm.	3,500 Cal.
Food digested	95 gm.	3,200 Cal.

That these figures more or less express the food needs of man received confirmation in the data obtained in the 1918 war-emergency study. This survey disclosed the mean total energy value of food consumed per man per day as 3,225 Calories and the protein content of the diet, 96 grams. Protein provided about 12 per cent of the total calories; carbohydrates, 53 per cent; and fat, 33 per cent. Langworthy notes the differences in these figures and those proposed earlier by Atwater, and ascribes differences to the increase in the amount of accurate data available.

From data provided by the various dietary studies including those with children and with the aged, Langworthy gives some first estimates in concise form of the food needs of people of different sex and age. His concepts appear in a table published

in the U. S. Department of Agriculture Yearbook, 1907. In it the food requirements of persons of different age and occupation are compared with those of a man in full vigor at moderate work, the latter being assigned the value of 100.

Man, period of full vigor:	
At moderate work	100
At hard work	120
Sedentary occupation	80
Woman, period of full vigor:	
At moderate work	80
At hard work	100
Sedentary occupation	70
Man or woman:	
Old age	90
Extreme old age	70–80
Boy:	
15 to 16 years old	90
13 to 14 years old	80
12 years old	70
10 to 12 years old	60
Girl:	
15 to 16 years old	80
13 to 14 years old	70
10 to 12 years old	60
Child:	
6 to 9 years old	50
2 to 5 years old	40
Child under 2 years old	30

This compilation in relation to the recommended allowances of present-time food allowances is indeed interesting.

The development of Langworthy's nutritional concepts beyond the needs for energy and protein appears in his application of standards like those elucidated above to the problems of food selection. He now states that the quality of a diet may be evaluated on the basis of its energy value *providing* it supplies a variety of foods in reasonable quantities. A diet furnishing 3,000 Calories per man per day almost without question, he explains, furnishes the needed protein, ash, and other constituents if pains are taken to include in the diet a reasonable amount of *milk, green vegetables, and fruit.*

Langworthy goes on to say that acceptance of this conclusion as rational, permits nutritionists to go ahead without controversy until more abundant knowledge becomes available of the kinds and quantities of protein needed, the functions of the mineral elements; the best ways to meet body needs for them, and of the nature of vitamins and other regulatory substances.

Out of these concepts grew suggestions for food selection, which with the addition of knowledge over the years, gradually developed into the plan we know today for selection of food that will meet the requirements of an adequate diet (U. S. Department of Agriculture, ARS-62-4, 1956). Langworthy's plan was very simple. He arranged foods commonly appearing in the American diet into five groups. Each group contained foods particularly rich in one constituent, i.e., protein, starch and similar carbohydrates, fat, mineral substances and organic acids, and sugars. He then recommended that foods from each of these groups be included in each meal of the day if possible; if not, then at least once every day. Excessive use of foods from any one group day after day would lead to an unsatisfactory diet with distortions in the proportion of its protein and the fuel values.

Development of the program of the Office of Home Economics

The year 1915 saw the organization of the States Relations Service and the expansion of the Nutrition Investigations into the Office of Home Economics. The reorganization, involving as it did a larger staff, increased appropriations, and additional laboratory and office space, enabled Dr. Langworthy to enlarge his program and to increase its output. In developing the research activities of the office, Langworthy, although continuing the nutrition investigations as a legitimate part of the program, had the vision and foresight to see that other subjects relating to the "improvement of living conditions in the home, institutional household, and the community" were also significant and worthy of scientific effort. In the interval from 1915 to 1923, studies undertaken fell into three groups: food and nutrition; household labor, management, and equipment; and textiles and clothing.

In the food and nutrition area, Langworthy extended earlier work on food preparation and use to include problems pertaining to the chemistry and physics of cooking processes. A new experimental kitchen provided facilities for the stand-

ardization of cooking methods and the study of problems related to the influence of controlled variables (ingredients, manipulation, temperature, etc.) on the quality of food products. This expansion marked an important contribution to the scientific aspects of home economics, for out of its continuance in the laboratories of the Department of Agriculture and in those of the universities of the nation, the fields of academic and scientific endeavor developed, which we know today as food science and food technology.

Langworthy also promoted work in the area of household labor, management, and equipment, the aim of which "was to reduce the amount of physical labor required in housekeeping." Labor-saving devices which would lessen the amount of labor required to do the tasks of the household were experimented upon and improvements made. As already noted, the determination of the relative energy demands of different kinds of household work was undertaken in this period. The influence of the type of equipment used on the relative efficiency of the work performed was clearly demonstrated.

But very importantly, investigations in relation to household management and labor took a new turn when the Office realized "the importance of economic data in the consideration of home economics problems." Data on the standard of living in the farm home were secured in surveys conducted in the states of Michigan and New York. Items relating to income, expenditures for various purposes, sanitation, furnishings, time consumed in labor, the amount of leisure, social life and other socio-economic factors were studied.

In comparison with other activities, the Office gave relatively little time to work in the area of textiles and clothing, chiefly on account of the lack of research methods suitable for studying its problem. Such work as was done dealt chiefly with ways of prolonging wear and lengthening the period of usefulness of clothing and household textiles and fabrics.

Another important phase of the activities of the Office of Home Economics in this period must not be overlooked. World War I called attention "to the value of home economics and the need for further research as no other situation had ever done." Dr. Langworthy mobilized the work of his Office toward the war effort, enhancing its effectiveness by establishing cooperative relations with the United States Food Administration, the Council of National Defense, and the Department of the Treasury. Cooperation already active with various bureaus in the Department was continued and the Office found itself one of the leaders in the food conservation program.

War-time needs also called for reliable data about the rational and economical use of the food supply so as to provide a diet which conformed in so far as possible to dietary preferences and the standards of a satisfactory diet. The dietary-survey methods with individuals and families were applied to a nation-wide survey in a study already noted and cooperative with the Bureau of Markets. The survey provided per capita consumption data hitherto lacking as well as information essential to agricultural production intelligently adjusted to the people's needs. Very significantly, it suggested that about one-third of the families were eating diets supplying less than 3,000 Calories and 88 grams of protein per man per day, considered by some as the minimal allowance desirable. The importance of these studies cannot be over-estimated. In the years that have intervened since this first "national" study, Human Nutrition and Consumer Use Research, the counterpart of the Office of Home Economics in the Department of Agriculture today, has surveyed the American eating habits at intervals to gather and analyze data on food consumption patterns and nutrient levels of diets in the United States. In addition, the information produced has served as a guide in the formulation of food and national policies. Another such survey will be undertaken in 1965, and will include study of diets of individuals.

Much attention was devoted to the preparation of educational materials. "Ten lessons" summarizing information on food conservation topics were prepared as well as a series of food leaflets, all brief, concise, and non-technical. The total number of requests for these materials exceeded 21,575,000. In addition, publications in-

cluded circulars recommending use of substitutes to save wheat, meat, and fat, as well as the already well-known Farmers' Bulletins.

Thus, with limited funds and under difficult conditions, Dr. Langworthy laid the foundation for development and expansion of his office into a bureau from which grew the agency existing in the Department of Agriculture today dealing with research on human nutrition and consumer use and carrying on its far-flung work through its divisions of human nutrition research, consumer and food economics research, and clothing and housing research.

Editor and author

From 1895 to 1924, Dr. Langworthy served as associate editor of the Experiment Station Record abstracting the literature of physiological chemistry, food, and nutrition. His activities, however, became so broad in scope as time went on that his abstracting was increasingly overshadowed by other duties. Nevertheless, one is impressed in leafing through the pages of the Record by the breadth of the literature which he covered.

Langworthy also was a devoted member of the editorial board of the Journal of Home Economics for thirteen years. He often acted as an editor in periods of emergency. He seemed particularly responsive to its needs, remembering it in his wide reading and in personal contacts with leaders in key positions in related fields. During this period he was a consistent contributor to its pages.

Langworthy was a prolific writer. Many of his papers appeared in the publications sponsored by the Department of Agriculture. He also wrote frequently for scientific journals such as the Journal of Biological Chemistry, the Journal of Agricultural Research, the Journal of Industrial and Engineering Chemistry, the American Journal of Physiology, and the Journal of Home Economics. Often his articles were published in foreign journals. Papers based on original research as well as digests of developments in nutrition were included in the programs of the meetings of many learned societies, both here and abroad. He regularly participated in the Lake Placid Conferences. His work is also found in encyclopedias and popular magazines.

Special mention, however, should be made of Langworthy's contributions to the publications of the Department of Agriculture. These publications were the principal means by which data and information secured through the Nutrition Investigations were made available to the public. They included the bulletins of the Department of Agriculture, the annual reports of the Office of Experiment Stations, the bulletins of the Office of Experiment Stations, the summaries in the Experiment Station Record, the Farmers' Bulletins, documents, circulars, and leaflets. The course of publication of any material was fairly specific. Technical reports of interest to the student of nutrition first appeared as bulletins of the Office of Experiment Station or of the Department of Agriculture. These reports, then, very frequently were followed by summaries in the Experiment Station Record, the yearbooks of the Department, and the annual reports of the Office of Experiment Stations. These technical reports and digests, in the absence of text and reference books, were widely used in schools, colleges and medical schools. Also, they were quoted freely by writers of nutrition topics in this and other countries.

These publications, however, failed to meet the need of the general public, and so in turn they were condensed, rewritten in popular form, and issued as Farmers' Bulletins, circulars, and leaflets for the use of homemakers, teachers of home economics, and extension workers. These popular articles are for the most part, presentations of the nutritive value of nearly every common food appearing in the American dietary. Current information about each food is presented in a very comprehensive manner. In general, topics discussed deal with the history of the food, market conditions, quality, composition, methods of serving, place in diet, digestibility, effect of cooking on nutritive value, relative economy in terms of calories provided per unit cost, handling and marketing, and recipes for use.

Langworthy's interest in extending knowledge led to the development of popular methods for the presentation of scientific facts. He produced the first graphic material used in this country for the teaching of nutrition.

The number of people reached through these publications was tremendous. The technical articles were available either for limited free distribution or for sale at nominal price, and the Farmers' Bulletins and circulars could be obtained in unlimited numbers at no cost. Brief popular summaries supplied to the Office of Information further enhanced the spread of information.

Not to be omitted is mention of Langworthy's contribution to the building of nutrition subject matter through his many reports of the status of research activity in the field — general summaries as well as documented references to the literature. Even today they may serve the student of nutrition well.

It is doubtful whether a complete bibliography of Dr. Langworthy's writings ever has been prepared. Many of his contributions are lost as unsigned articles in the Experiment Station Record, the Journal of Home Economics, and in the annual reports, circulars, and leaflets of the Office of Experiment Stations. Others are hidden in less well known and more sporadic publications of the Department of Agriculture. Thus the bibliography prepared for the purposes of this biography and containing some 150 citations probably is far from complete. But it does give an appreciation of Langworthy's tireless efforts as an author and of his deep desire to reach as many people as possible with the findings and implications of the important new science of nutrition. The impacts of the information on the health and welfare of people and on the utilization of farm products for food cannot be measured.

Dr. Langworthy's role in the home economics movement

This biography would not be complete without reference to the role played by Dr. Langworthy in the home economics movement gaining momentum in the United States during the last decades of the nineteenth century. Indeed, in a personal communication, Dr. H. L. Knight, former editor of the Experiment Station Record and a colleague of Dr. Langworthy writes, "Personally I think his big contribution to society was his part in the formation of the American Home Economics Association." A recapitulation of the services he rendered so quietly and unostentatiously, may sharpen appreciation of the imprint he has left on the philosophy, standards, and activities of an organization now numbering nearly 24,000 members.

In Langworthy's day, higher education for women was still a matter of dispute in many quarters. The newly established Land-Grant institutions, however, had opened their doors to women. Dedicated to the philosophy of "education for all" and "knowledge for use," they believed that women had rights to education equal to those of their brothers. Furthermore, in light of this philosophy, a curriculum dealing with the application of science to the problems of the home and the welfare of the family gave promise of a field of study particularly suited for women. Thereupon, with attendant academic recognition, institutions of higher learning began introducing the new science, designated variously as domestic science, domestic economy, or household economy, into their regular college offerings. By 1880, three Land Grant colleges had established such departments; by 1905 the number had increased to thirty-six.

The new discipline, later to be known as home economics, probably dated its origin from Count Rumford's efforts in 1753 to apply the results of experiments in physics to the problems of the home. But it was in the interval from 1870 to 1905 that the need for training in the care and management of the home began to be felt acutely. Various forerunner activities had presaged the forthcoming movement, notably the Kitchen Garden Association, the New England Kitchen, the Rumford Kitchen, cooking schools, and others. But despite gains made in interpreting needs of the home to people in various circumstances and in obtaining academic recognition in institutions of higher learning, home economics at the turn of the century was struggling against public apathy. In the words of a distinguished scientist of the time,

The science of household economics is now in what chemists call a state of supersaturated solution which needs to crystallize out. Sometimes the point of a needle inserted will start such crystallization." The point of the needle proved to be Ellen H. Richards, the first woman graduate of the Massachusetts Institute of Technology, instructor in sanitary chemistry at the Institute, and scientist and humanitarian in her own right.

Mrs. Richards, a dynamic and far-seeing woman, had developed a keen interest in home economics by virtue of her scientific training and her interest in the welfare of society. The first led her to recognize the bearings of chemistry on practical life; the second gave her a perception of the role of the family as the structural unit in a healthy national life. At this time, she was particularly perturbed because she saw in the diversified trends within the home economics movement the need for a unification of its subject matter, and was exerting considerable influence on the thinking and philosophy of a little band of eleven men and women carrying the torch for the promotion of the new science. In 1899, Mrs. Richards called these people together to discuss the development of Home Economics in a conference held at the Lake Placid Club in New York at the invitation of Mr. and Mrs. Melvil Dewey, owners of the club. This conference proved so fruitful under Mrs. Richard's bracing leadership, that similar meetings were held annually thereafter for ten years, the number in the group growing from the original eleven to more than two hundred. The conferences elicited the interest of many broad-minded men and women; scientists, government workers, sociologists, economists, teachers, and college administrators. Among them were people of the stature of W. O. Atwater, C. F. Langworthy, T. D. Wood, W. A. Baldwin, Otto Folin, A. C. True, Benjamin R. Andrews, Lafayette B. Mendel, Mary Swartz (Rose), Amy Daniells, H. C. Sherman, J. H. Kellogg, and others.

Langworthy's activities in the developing home economics movement began in these Lake Placid conferences. A firm friendship existed between Mrs. Richards and Dr. Atwater, and Langworthy undoubtedly became acquainted with her through Dr. Atwater as well as through her involvement in certain phases of the Nutrition Investigations going forward under Atwater's leadership (U. S. Department of Agriculture Bulletin no. 21, 1895; U. S. Department of Agriculture Bulletin no. 129, 1903). Langworthy's active interest, however seems to stem from the time of a summer school, planned by Dr. Atwater and held at Wesleyan University in 1902, for the specific purpose of acquainting leaders in home economics with the work which the government was doing in the Nutrition Investigations. Especially challenging at this meeting was Langworthy's exposition of the need for more knowledge through research for the answering of questions and the building of subject matter needed by home economics in the area of foods and nutrition. As the result of this contact with the leaders in the field, Langworthy became a regular participant in the Lake Placid Conferences.

Over the ten years of their existence, the Lake Placid Conferences served as the rallying point for students, teachers, researchers, administrators, and professional workers in home economics. Langworthy engaged actively in all phases of its discussions, i.e., basic philosophical concepts relating to the education of women, the promotion of an education recognizing the home and the need of wholesome family living for all, the place of the new discipline of home economics in institutions of higher learning, the promotion of a literature for home economics, the synthesis of its subject matter, the organization of courses of study, the inadequacy of certain existing curricula, and the need for research in the university and by the government.

Then, when the Conference agreed that the time had come to dissolve and to reorganize as a national organization, it gave Langworthy a major role in effecting the change and in writing the constitution for the new organization to be called at his later suggestion, The American Home Economics Association. The reorganization was effected.

Dr. Langworthy gave of his time and energy to the American Home Economics Association for the rest of his professional life. He served as its vice president for six

years, its treasurer for three. He was instrumental in the creation of the Journal of Home Economics as an organ of the Association, editing the first two issues himself. He then served as a member of Journal's editorial board for thirteen years, often acting as editor in emergencies. He also contributed freely to its pages, his papers dealing not only with foods and nutrition but with other phases of home economics as well.

Special note should be made of the more important of his contributions in crystallizing the issues of the home economics movement. Of special interest was Langworthy's stand on the education of women. He is recorded as saying that education of women "has failed because until the home economics movement came about, we trained women in men's courses. We taught science with all the interesting material taken from the man's side of life. I want a woman to have as much education as a man but I do think that she is going to make a different use of her knowledge and we should give her the things she needs most. . . this movement is going to give to woman a scientific training in the way most useful."

Also important was Langworthy's stand on the role of graduate training and research in the sound development of the home economics discipline. Through his interest in the "graduate schools" established during the Lake Placid days, he made a vital contribution in establishing graduate training as a prerequisite to home economics. These graduate schools for home economics grew out of the summer school for advanced study held at Wesleyan University in 1902. Later graduate schools were organized in conjunction with the Graduate Schools of Agriculture with joint sessions at various colleges during the summers. The rosters of the faculties included in addition to personnel at each institution, many important leaders in the growing field of nutrition. In 1905, the two groups met jointly at the University of Illinois, convening again in 1908 at Cornell University, in 1910 at the Iowa State College of Agriculture (now Iowa State University), and in 1912 at Michigan State Agricultural College (now Michigan State University). Langworthy became intimately concerned with the work of the Home Economics Graduate Schools, participating in planning for them and taking a prominent part in their programs. We find him reporting on such topics as the literature on the physiology and chemistry of fatigue, the possibility of solving problems of fatigue with the use of respiration calorimeter, the scientific literature on domestic art, and the relation of physiological chemistry to animal nutrition.

Langworthy was indefatigable in his efforts to keep not only the Lake Placid Conferences but the American Home Economics Association abreast of developments in the field. Each year, beginning with 1905, he reported progress in food and nutrition for that year at the Lake Placid Conferences. In 1908 he published a comprehensive review of the publications of the United States government as sources of information for students of home economics in the Journal of Home Economics, and in 1910 in the same journal a comprehensive review of the contributions for 1908–09 dealing with all aspects of food and nutritional science. Langworthy's zeal in this direction was in all probability related to his desire to help in synthesizing a discipline of home economics. He saw the parallelism between the growth of agricultural science and of home economics. Agriculture was a side issue with other kinds of chemistry when it was first given a place in the curriculum. But its scientists, and Liebig in particular, began bringing together isolated facts and adding to them. Out of the grouping and classification of these facts, the subject of agriculture was produced. Langworthy credits Mrs. Richards with such a bringing together of facts for the creation of home economics and making its discipline worthy of serious study and practical application by both men and women. But Langworthy himself must be given proper credit for the part he played in this bringing together and arranging of information. He had the breadth of vision to catch the significance of expansions in home economics in relation to scientific developments of the times. This attribute, together with his ability to interpret home economics on a broad cultural basis, highlighted his contributions to a syllabus of

home economics published in 1913 as a memoir to Mrs. Richards. In it, home economics is grouped into areas relating to food, shelter, clothing, and household and institution management. Each topic is subdivided, and emphasis laid not only on theoretical, historical, and general aspects but on the respective relations of chemistry, physics, mathematics, biology, esthetics and engineering to each of the topics. An editorial, which, though unsigned, bears the stamp of Langworthy's writing (J. Home Econ., 5: 164, 1913) points out the wide scope of home economics and how it may lead a student along the paths of literature, science, social science, and general culture while providing instruction along general lines. The syllabus is very close indeed to present-day concepts of home economics which state that it is the field of knowledge and service primarily concerned with strengthening the family through educating the individual for family living, improving the services and goods used by families, conducting research to discover the changing needs of individuals and families, and furthering community, national, and world conditions favorable to family living.

Someone has written that the American Home Economics Association is the lengthened shadow of great personalities. Surely, Langworthy cannot be counted among the least of these.

Personal characteristics

One cannot help but be impressed at the extent and diversity of Dr. Langworthy's contributions to nutrition and home economics and the scope of his work as an editor and author. What were the personal characteristics of the man able to maintain these varied activities? Very little first-hand information about Dr. Langworthy as a person is available today and the best one can do is to piece together information gained from contacts with acquaintances still living and from notes in the literature.

It is said that Dr. Langworthy was a chubby man, almost cherubic, fastidious in appearance, and of sedentary habit. He apparently had no outdoor hobbies and took little time for recreation. A colleague recalls as his only diversion, the hiring of a horse and buggy for long rides into the country when he made his annual vists to Wesleyan University.

Dr. Langworthy had an unfailing friendliness. His talk was both stimulating and amusing. These qualities made his office a meeting point for colleagues and friends. Perhaps his greatest asset was his ability to interest influential people in relatively new fields. "Many of these," one colleague writes, "brought the meat to put in the pot but [Langworthy] kept it hot and well-stirred with his own distinctive flavor."

Dr. Langworthy was intensely loyal to his friends and to organizations with which he was connected. He had a genuine wish to be of service to the American Home Economics Association and was a generous financial backer in its early days when a small annual income made the balancing of its accounts a difficult achievement.

There seems little question but that this interest derived from inherent home-loving instincts. Although a bachelor, Dr. Langworthy had all the characteristics of a homebody, transforming his office into a cheerful study with oriental rugs, old china, and furniture. One of his favorite projects was the serving of impromptu luncheons to friends and visitors, the luncheons being made up of test foods brought in from the experimental kitchens. It is said that no visitor enjoying one of these luncheons could question Dr. Langworthy's culinary imagination. His tastes indeed were somewhat epicurean. The tomato soup and curry served at one of the luncheon occasions is etched in a visitor's memory. We find, too, in the Farmers' bulletins many suggestions for ways in which food can be made attractive and palatable: for instance, the transformation of mutton fat into a savory by the addition of a bit of onion, a vegetable or two, and some herbs. The writer once had a recipe in her possession for "Dr. Langworthy's Christmas fruit cake." In it were exotic ingredients too varied and numerous to mention, but thoughts of Christmas fruit cake seemed to tickle Langworthy's scientific thinking as well as his palate. An editorial in the Journal of Home Economics, unsigned but unmistakably of the Langworthy style, comes to the defense of the Christmas plum pudding. It is not "a deadly combina-

tion . . . but . . . a concentrated food containing protein, fat and carbohydrate in abundance!"

"It is easy to forget today," says the Experiment Station Record at the time of Dr. Langworthy's death, "how recent the developments of food and nutrition and home economics as sciences have been and how serious were the handicaps which confronted the pioneers in each cause. Dr. Langworthy began his work when reliable information was limited and objectives uncertain. He did much to aid in overcoming these obstacles, and thereby helped greatly to put each science on a high plane and a sound scientific basis. His services were important and timely and merit wide remembrance."

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Effect of Casein and Soy Protein Diets on the Growth of Ducklings [1]

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ABSTRACT The maximal growth rate of ducklings was obtained with a 30 to 35% soy protein diet (supplemented with methionine and glycine), but not with 35% casein or 25% casein plus 10% gelatin. Ducks responded like chicks and turkey poults in this respect, but unlike the latter, ducklings did not require a source of the fish-soluble factor for maximal growth. The better growth obtained with 35% soy protein as compared with 35% casein could not be attributed to differences in the amino acid composition of the 2 proteins, since a growth differential still existed when both diets were made equal in amino acid composition. The 25 to 35% casein diets supported the maximal growth rate when supplemented with 10% cottonseed meal.

The duckling grows rapidly and is, therefore, a convenient species for the rapid production of nutritional deficiencies (1). Hegsted and co-workers (2–4) used a purified 18% casein plus 10% gelatin diet and demonstrated that growth inhibitions with some vitamin-deficient diets could be obtained within a few days. Other studies on duck growth have used practical-type rations with unpurified food sources, and were concerned with the effects of protein (5, 6), unidentified growth factors (7), niacin levels (8) and antibiotics (9) in the diet. In previous studies from this laboratory (10, 11) a 35% soy protein diet (supplemented with methionine and glycine) supported growth better in chicks and turkey poults than did a 25% casein plus 10% gelatin diet; maximal growth rates were achieved when a 35% soy protein diet was supplemented with fish solubles. Chick "growth factors" were associated with both the soy protein and fish solubles.

The purpose of the present study was to determine whether the duckling also required the soy and fish-soluble factors, and also to help define a purified diet that would support maximal growth rate in ducks. The results have shown that the duckling, like the chick and poult, grew better with a soy protein diet than with a casein or casein-gelatin diet. As in the previous studies with chicks and poults, the better growth obtained with soy protein, as compared with casein, could not be attributed to differences in the amino acid compositions of the 2 proteins. Unlike the chick and poult, the duckling did not require any source of the fish soluble factor for maximal growth.

EXPERIMENTAL

Groups of 8 one-day-old Peking ducklings, weighing approximately 50 g, were housed in heated brooders with raised wire-mesh bottoms and were fed the diets [2] ad libitum for 14 days. Body weights were recorded twice weekly; all values reported are 14-day body weights. In addition to the kind and amount of protein listed with the results, and unless otherwise specified, all diets contained 10% purified cottonseed oil, 2% cod liver oil, 8.1% salt mixture, a vitamin mixture and enough starch to total 100%. The vitamins consisted of:

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[2] Sources of the ingredients were as follows: ADM C-1 Assay Soy Protein (Archer-Daniels-Midland Company, Minneapolis); vitamin-free casein and amino acids (Nutritional Biochemicals Corporation, Cleveland); gelatin powder (General Chemical Division, Allied Chemical, New York); Wesson Oil (the Wesson Sales Company, Fullerton, California); cod liver oil (Peder Devold Oil Company, New York); commercial duck pellets (Purina Duck Startena Checkers, Ralston Purina Company, St. Louis); cottonseed meal (Eufaula Cotton Oil Company, Eufaula, Alabama); fish solubles (Starkist Foods, Terminal Island, California). The ducklings were obtained from Thiel Brothers, Barker, New York and weighed 45 to 55 g.

(mg/100 g of diet) thiamine chloride, 0.5; riboflavin, 0.8; D-Ca pantothenate, 1.6; pyridoxine chloride, 0.4; niacin, 12; folic acid, 0.2; menadione, 0.05; biotin, 0.02; vitamin B_{12}, 0.005; mixed tocopherols, 7.2; *p*-aminobenzoic acid, 2; inositol, 100; and choline chloride, 200. The salt mixture was identical with that described by Reid et al. (12) except that the zinc content was increased to provide 50 ppm, anhydrous $CaHPO_4$ was used instead of the hydrated compound, and 0.2 mg of Na_2MoO_4 was added per 100 g of diet. All of the diets containing soy protein were fortified with 0.75% of DL-methionine and 0.4% of glycine. The casein-gelatin diets contained 10% gelatin plus the amounts of casein indicated in each experiment. All additions or changes in the diet were made in exchange for starch.

RESULTS

Figure 1 shows the relationship of the 14-day body weight of ducks to the amount and kind of protein in the diet.

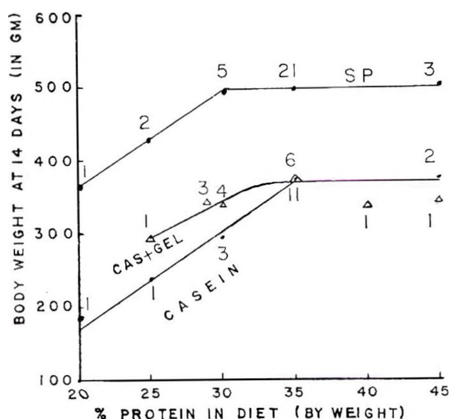

Fig. 1 The average growth of ducks (body weight at 14 days) in relation to the type and amount of protein in the diet (e.g., 35% protein refers to 35 g of casein or soy protein per 100 g of diet on the basis of weight of the source material). In addition to the proteins as illustrated, all diets contained 8.1% salt mixture, 2% cod liver oil, 10% cottonseed oil, 0.2% choline, 0.1% inositol, a vitamin mixture, and starch to make 100%; all casein-gelatin diets contained 10% gelatin and enough casein to total the amount illustrated; 0.75% DL-methionine + 0.4% glycine were added to all soy protein diets. The numbers given with each point refer to the number of experiments with 8 birds each, which were averaged to obtain the point.

The ducks grew better with soy protein (as supplemented with methionine and glycine) than with a casein or casein-gelatin diet. A minimal concentration of 30% soy protein (equivalent to a 24% protein content on the basis of nitrogen (N × 6.25))[3] was required to achieve maximal growth rate, and this appeared to be borderline. In respect to both the type and total amount of protein in the diet, the ducks behaved like chicks and turkey poults. Although casein-gelatin diets appeared to be slightly better than casein alone at concentrations below 35%, a 35% casein diet supported the growth of ducks to the same extent as a 25% casein plus 10% gelatin diet; both were significantly less than with 35% soy protein, and both resulted in poor feather development by comparison with soy protein. The 14-day body weight averages for 8 ducks fed the 35% soy protein diet varied from 450 to 550 g. Such variation is large when compared with the reproducibility of chick or turkey growth curves, but is not unusual in relation to a growth rate of 50 g/day for the 2-week-old duckling. Significant differences between test groups were established by repeating the same diets in several independent experiments. The reproducibility of such repeat experiments is illustrated by the following data. The average 14-day body weight of ducks fed the 35% soy protein diet was 500 ± a standard error of the mean of 6.6 (for 45 birds in the first 6 experiments); the corresponding figures for the 25% casein plus 10% gelatin diet and the 35% casein diet were 371 ± 13.4 (24 birds in the first 3 experiments) and 375 ± 6.8 (33 birds in the first 4 experiments), respectively. The over-all averages for these 3 diets were 495 (21 experiments), 376 (6 experiments) and 375 (11 experiments), respectively.

A 35% soy protein diet appeared to give the maximal growth rate which could be achieved with these ducks, and in comparative tests was appreciably better than the 18% casein-10% gelatin diet used

[3] The nitrogen content of the casein, soy protein and gelatin used in these experiments was 13.2, 12.8 and 15.4%, respectively. On the basis of a 16% N content, these sources of proteins represented 83, 80 and 96%, respectively, of actual protein. The cottonseed meal was reported to be 36% protein by the suppliers.

originally by Hegsted and Stare (1). It gave the same rate as a commercial duck pellet, and this rate was not increased further by adding 4% liver L-,[4] 10% egg yolk, 10% fermentation residue-,[5] 2, 10 or 20% fish solubles, 10% cottonseed meal or 1% arginine. A 25% soy protein diet contains a borderline or limiting amount of leucine and tryptophan for the growth of chicks, and was also deficient in these amino acids for the growth of ducks. When a 25% soy protein diet was further supplemented with 0.4% leucine and 0.1% tryptophan (in addition to the glycine and methionine added routinely), the maximal growth rate was obtained.

The following modifications of the 35% soy protein diet had little or no effect. Sucrose, as well as starch, supported growth. The salt mixture used by Hegsted (1) and by Fox and Briggs (13) gave the same results as the salts used routinely in these studies (12). Although the differences were too small to be statistically significant, the ducks appeared to grow slightly faster when the diet contained 10% as compared with 1.5% cottonseed oil (in addition to the 2% cod liver oil added routinely). A 35% lactalbumin, egg albumin or fibrin diet was no better than the casein or casein-gelatin diets. Little or no effect was obtained when a 35% casein diet was supplemented with 10% egg yolk, 10% fermentation residue or 1% creatine. Four per cent of liver L gave an intermediate response (440 g).

Amino acid supplementation. The better growth obtained with 35% soy protein (supplemented with methionine and glycine) as compared with 35% casein could not be attributed to differences in the amino acid composition of the 2 proteins. In 3 independent experiments, the appropriate amino acids were added to each diet so that the final amino acid composition of both diets was the same; i.e., the calculated amount of each amino acid was added to the appropriate diet to equal the larger amount of that amino acid provided by the other protein. The results of such experiments (table 1) show that: 1) the addition of the amino acids to the 35% soy protein diet had no effect on the growth rate; 2) the poorer growth obtained with a 35% casein diet was improved somewhat by the amino acid additions, but was not increased to the level achieved with soy protein. The same small growth response (415-g average of 3 experiments) was obtained when 1% arginine plus 1.4% glycine was added to 35% casein in place of the amino acid mixture listed in table 1.

Figure 2 illustrates growth curves for the ducklings receiving the soy protein or

[4] A solubilized dry concentrate containing the alcohol-insoluble fraction of liver obtained from Nutritional Biochemicals Corporation, Cleveland.
[5] Omafac, E. R. Squibb and Sons, New York.

TABLE 1

Effect on growth of balancing the amino acid composition of a 35% soy protein diet and a 35% casein diet to the same amino acid composition for both

Diet [1]	14-day Body weights			
	Exp. no. 1	Exp. no. 2	Exp. no. 3	Average
	g	g	g	g
35% Soy protein (+ 0.75 methionine + 0.4 glycine	519 ± 11.2 [2]	468 ± 12.1	475 ± 15.7	487 ± 7.6
35% Soy protein + 5.28% AA(SP) [3,4]	493 ± 15.7	480 ± 15.7	460 ± 15.9	478 ± 8.7
35% Casein	390 ± 11.6	376 ± 6.1	331 ± 17.2	366 ± 7.6
35% Casein + 3.08% AA(C) [3,5]	488 ± 21.4	420 ± 17.0	345 ± 19.4	418 ± 10.8

[1] In addition to the protein, the diet contained 10% cottonseed oil, 2% cod liver oil, 8.1% salts, 0.2% choline, 0.1% inositol, a vitamin mix, and starch to 100%.
[2] Body weights ± SE; from 6 to 8 ducks per group for each experiment; the average of 3 experiments represented 21 to 25 ducks.
[3] All amino acids were calculated to the same 16% N content for each protein (using: casein = 13.2% N; soy protein = 12.8% N).
[4] Amino acid mixture (SP) in g/kg diet: L-aspartic acid, 0.7; L-glutamic acid, 8.4; L-histidine, 0.1; L-leucine, 7.4; L-isoleucine, 0.5; L-lysine, 3.6; L-phenylalanine, 1.0; L-proline, 16.5; L-tryptophan, 0.9; L-tyrosine, 8.8; L-threonine, 0.3; and L-valine, 4.6.
[5] Amino acid mixture (C) in g/kg diet: L-alanine, 1.9; L-arginine, 11.4; L-cystine, 0.6; glycine, 14.1; DL-methionine, 0.6; and L-serine, 2.2.

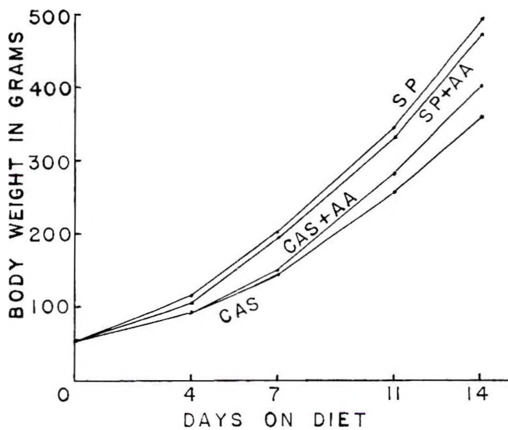

Fig. 2 The growth of ducks fed a 35% soy protein (supplemented with 0.75% methionine and 0.4% glycine) or a 35% casein diet with and without the addition to each diet of an amino acid mixture that would give each diet the same amino acid composition (see table 1 for amino acid mixtures used).

casein diets with and without the amino acid supplementation. The major point to be noted is that the relatively small growth stimulation produced by adding the amino acids to the casein diet became evident only after the diet had been fed for 7 days. The differences between the soy protein and casein diets was evident within 4 days, and this early difference between the 2 proteins was not influenced by the amino acids at all.

Cottonseed meal. Twenty-five to 35% casein diets supported the maximal growth rate in ducklings when they were supplemented with 10% cottonseed meal (table 2). A further addition of 0.5% glycine, 1% arginine or 0.6% methionine (in various combinations) did not increase or decrease this response to cottonseed meal. Fifteen per cent cottonseed meal (added to 25% casein) gave the same response as 10% cottonseed meal, whereas 4 to 7% cottonseed meal (added to 35% casein) was somewhat less effective (465 to 485 g). Because the cottonseed meal was 36% protein, a casein-cottonseed meal diet containing 25% total protein on a nitrogen basis gave as good growth as the 30 or 35% soy protein diet.

A number of additional studies were conducted in an attempt to duplicate the cottonseed meal response with pure amino acids. The results (table 2) tended to be erratic in replications, and the conclusions are somewhat ambiguous. The addition of 1% arginine to 20 to 35% casein diets gave a growth stimulation which would suggest an arginine deficiency in the casein diets. However, the addition of 0.5% methionine plus 0.5% glycine to these diets gave a comparable response and the addition of all 3 together to 25 to 30% casein diets supported good but not maximal growth; the effect was not sufficient to account for the full response to cottonseed meal.

DISCUSSION

Ducks, like chicks and turkey poults, grew better with soy protein than with casein diets. An unusually high requirement for arginine, when chicks are fed a casein diet (14, 15), has been attributed to an amino acid imbalance in the casein by Anderson and Dobson (16). In support

TABLE 2

Effect of cottonseed meal or selected amino acids on the growth of ducklings fed casein diets

Casein in diet	14-day Body weights				
	No additions	+ 10% Cottonseed meal	+ 1% Arginine	+ 0.5% Methionine + 0.5% glycine	+ 0.5% Methionine + 0.5% glycine + 1% arginine
%	g	g	g	g	g
35	375 (11) [1]	505 (3)	440 (3)	—	405 (1) [2]
30	295 (3)	500 (5)	375 (2)	400 (1)	460 (5)
25	240 (1)	495 (5)	360 (1)	395 (1)	475 (2) [3]
20	190 (1)	455 (6)	310 (1)	300 (1)	395 (2)

[1] Values in parentheses show the number of experiments with 8 birds each that were averaged.
[2] Additions consisted of 0.7% methionine, 1.4% glycine and 1.5% arginine instead of the amounts indicated in this column.
[3] Additions consisted of 0.6% methionine, 1.0% glycine and 1.5% arginine.

of this concept, Klain et al. (17) obtained a growth response in chicks when additional arginine was added to an amino acid mixture which simulated 30% casein. A growth response to arginine when a casein diet is fed has also been observed in ducks in this study and in chicks in numerous laboratories, and much of the growth-stimulating effect of cottonseed meal in chicks has been attributed to its arginine content (18). Lysine toxicity was readily induced in chicks fed a casein but not a soy protein diet, and this was counteracted by additional arginine (19).

All of these studies indicate clearly the limiting role of arginine in casein diets for birds. However, if differences in the amino acid composition of casein and soy protein were the only reason for the differences in growth, then the growth rates of the ducks should have been the same when the appropriate amino acids were added to make the final amino acid composition of both diets the same. This did not occur in the present studies, and Westerfeld and Hermans (10) could not achieve equal growth in chicks and poults when the amino acid composition of the 2 proteins was equalized by appropriate additions.

Casein diets for birds are unusual with respect to methionine (10) as well as arginine. Either the actual requirement for each of these amino acids is higher with a casein than with a soy protein diet, or these amino acids are not readily available to birds when they are supplied as casein. If birds had difficulty digesting an "unnatural" protein such as casein, the higher requirement for arginine and methionine with a casein diet would merely reflect a partial availability of these amino acids from this source. However, this possibility is not supported by the studies of O'Dell et al. (20) in chicks, nor by the current studies in which casein was an adequate protein when supplemented with only an additional 3.6% protein in the cottonseed meal.

The explanation for the difference between casein and soy protein diets for birds is not clear. The growth obtained by supplementing casein diets with amino acids was better than that obtained with casein alone, but was not the maximal rate achieved with soy protein or with casein plus cottonseed meal.

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Certain Factors Including Fluoride which Affect Magnesium Calcinosis in the Dog and Rat [1]

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ABSTRACT The studies herein reported confirm in part observations previously made and indicate further that certain interrelationships between Mg, Ca, P and F^- were involved in the low Mg induced calcinosis. A balance of these elements was essential for its prevention and control. Dietary F^- greatly reduces, or largely prevents calcinosis in the dog, whereas the rat is less likely to show a reduction of excessive calcification of low Mg-induced calcinosis. The evidence at this time suggests a species differences. Minimal prevention level in the dog was 25 ppm F^-. This effect was independent of feed intake. From the results in the response of calcinosis in dogs by fluoride treatment during magnesium depletion and 25-day repletion, it appeared that fluoride prevented the initiation of the calcification of soft tissues but it had no effect on the preformed calcified lesions during the course of 25-day repletion period.

The effects of dietary magnesium upon the calcification of tissues and structures of the animal body have been widely recorded and accepted. Dietary calcium, phosphorus, level of fat, age of test animals and fluoride supplements have been shown to influence the response of the test animal fed low magnesium diets (1–9). Tufts and Greenberg (10) reported an increase in the severity of the calcinosis syndrome with increased levels of calcium and phosphorus. Vitale et al. (11) reported that the requirements of the growing rat quadrupled when fed an atherogenic diet. Bunce et al. (8) observed increased severity in the calcinosis in dogs when the calcium or phosphorus of the ration, or both, was raised from 0.6 to 0.90% and 0.4 to 0.9%, respectively, with the effect of phosphorus being more critical. Reduction of the phosphorus content of the diet to 0.22% had an alleviating affect but the reduction of the calcium to 0.3% had none. Fluoride supplementation adversely affected the growth rate and feed intake of young dogs and prevented the appearance of magnesium calcinosis without the alteration of other magnesium deficiency symptoms (8, 9). No protective effect of fluoride on soft tissue calcification was observed in rats fed a low magnesium diet (9).

Data on the recovery of the calcified lesions induced by magnesium deficiency is very limited. Recently Forbes (12) showed that it may be slow or irreversible in rats.

A number of factors and interrelationships exist and information regarding their physiological effects needs to be further explored. The purpose of the present studies was to ascertain the effect of fluoride on magnesium calcinosis by the use of pair-fed dogs; to establish the level of fluoride required for this purpose; to study species differences between magnesium-deficient dogs and rats in responding to fluoride treatment; to investigate the reversibility of the calcinosis in dogs and to determine the effect of fluoride on recovery therefrom; and to make a preliminary test on the effect of hormones, cortisone and estradiol on magnesium calcinosis.

EXPERIMENTAL

The composition of the basal diet, the forms of magnesium and fluoride supplements and the husbandry practices were the same as those described previously (9). Blood samples were obtained at 2-week intervals and the serum calcium, magnesium and inorganic phosphorus were analyzed. The magnesium depletion period was 6 weeks except in the fifth experiment when it was terminated at the end of the fifth week. A magnesium repletion period of 25 days was used in all recovery experiments. At the termination of the experiment aortas, hearts, and kidneys were examined grossly and analyzed for calcium,

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[1] The authors are indebted to Wilson and Company, Chicago, for partial support of this project.

magnesium and phosphorus. Femurs were analyzed for ash, magnesium and in some experiments for fluoride. In the case of rats, blood samples, kidney, heart and femurs were analyzed at the end of the 4-week experimental period. Analytical methods were the same as those described previously (9) except for the calcium determination of the ashed samples where strontium chloride was added at 1% concentration to eliminate phosphate interference. The atomic absorption spectrophotometer was equipped with a slotted burner.

A low Mg calcinosis of the soft tissues of the dog has been demonstrated (13) and appears to involve alterations of the ground substance. Cortisone and estradiol have been shown to reduce the synthesis and turnover of mucopolysaccharides and to influence collagen metabolism (14–17). A preliminary study of the affects of these substances upon low Mg calcinosis was made with cortisone acetate and estradiol 17B fed at 160 and 8 ppm respectively. The results did not warrant further effort in this respect.

RESULTS AND DISCUSSION

Experiment 1. Deficiency symptoms of inadequate Mg were observed in animals restricted to the basal diet. Their average cumulative body weight gains were 2.62, 2.77, 2.06 and 2.07 kg for lots 1 to 4, respectively. No evidence of gross calcinosis was observed when the ration was supplemented with either F^-, or 200 ppm of Mg. There was calcification in the animals in lots 1 and 4 which was observed in the aortas, heart valves and kidneys but not in the heart (table 1). Bone ash Mg was normal with 569 mg/100 g ash. All others were reduced to less than 263 mg/100 g ash.

In the previous work reported by Chiemchaisri et al. (9) no information was obtained to indicate the role of reduced feed intake which accompanies feeding of F^- at high levels. Low Mg diets also affect feed intake adversely. A study was made with young dogs pair-fed in lot 4 comparable with those fed 200 ppm F^- in lot 3 (table 1). The low Mg ration without and with adequate Mg, lots 1 and 2, respectively, were those used in experiment 2 and since they met our requirements for age, type and size, these rations served as controls for both experiments. Low magnesium calcinosis was not the direct result of the reduction of feed ingestion or the reduction of weight gain caused by fluoride.

Experiment 2. The second study was made to determine the amount of F^- needed to inhibit low Mg-induced soft tissue calcification. The F^- supplements were fed at 4 levels, 25, 50, 100 and 200 ppm of F^- (lots 3, 4, 5 and 6). The basal ration was the low Mg diet containing 30 ppm Mg. This diet was supplemented by incorporating 200 ppm Mg, an adequate level, and dogs so fed constituted the positive control animals for the study.

Magnesium deficiency symptoms were observed in the dogs of lot 1 (low Mg), and were not observed in those animals fed Mg or NaF. The data in table 2 show a reduction in cumulative weight gain when more than 50 ppm of F were incor-

TABLE 1

Calcium content of tissues of dogs pair-fed low magnesium diets

	Lot 1 (3) [1]	Lot 2 (3)	Lot 3 (4)	Lot 4 (2)
	30 ppm Mg ad libitum	200 ppm Mg ad libitum	30 ppm Mg + 200 ppm F	30 ppm Mg pair fed L-3
Calcium, mg/100 g dried tissue				
Aorta	301.7	45.8	38.8	739.0
Valve	739.2	35.7	36.5	915.2
Heart	20.6	19.8	21.6	31.1
Kidney	204.0	35.6	31.4	55.5
Bone magnesium, mg/100 g of ash	232	569	263	225

[1] Numbers in parentheses indicate number of dogs per lot.

TABLE 2

Tissue of dogs fed low magnesium diets

Lot no.	1	2	3	4	5	6
Magnesium, ppm	30	200	30	30	30	30
Fluoride, ppm	—	—	25	50	100	200
No. of dogs/lot	3	3	3	3	3	4
Calcium, mg/100 g dried tissue						
Aorta	301.7	45.8	47.2	50.9	55.2	38.8
Valve	739.2	35.7	50.4	73.6	35.9	36.5
Heart	20.6	19.8	18.8	202.3	16.5	21.6
Kidney	204.0	35.6	32.8	34.6	39.4	31.4
Bone magnesium, mg/100 g of ash	232	569	233	222	225	263
Bone fluoride, ppm of ash	89	101	960	1751	3480	6251
Cumulative weight gain, 6 weeks, kg	2.62	2.77	2.29	2.23	1.82	1.72

TABLE 3

Effect of 400 ppm of fluoride on growth and calcium content of hearts and kidneys of rats fed at various levels of magnesium

Lot no.	Dietary treatment		Cumulative wt gain at end of 4th week	Calcium content	
	Mg	F		Kidney	Heart
	ppm	*ppm*	*g*	*mg/100 g dried tissue*	
1	30	—	50	46.8	47.1
2	30	400	37	365.4	39.4
3	200	—	114	32.0	19.8
4	200	400	94	46.0	20.8
5	400	—	100	33.7	19.2
6	400	400	96	49.8	19.2

porated in the diet. Bone Mg was reduced approximately 50% except in lot 2 which contained 569 mg/100 g ash. Bone F^- retention was evident and measurable in all groups fed NaF supplements. The results indicated that the low Mg diet caused marked calcinosis of the aorta, heart valve region and kidney, and variable results if any in the heart. The effective level of dietary F^- which protected the dog from low Mg calcinosis was 25 ppm.

Gross lesions occurred in the aorta, heart valve area and kidneys of the Mg-deficient dog, whereas the positive control dogs fed 200 ppm Mg showed little if any evidence of calcinosis. The data obtained from gross inspection and mineral analyses of selected tissues support the interpretation that dietary F^- of 25 ppm or more reduced the severity of low Mg-induced calcification.

Experiment 3 (rats). A third experiment was set up to obtain further data for comparative reaction between species to low Mg calcinosis diets, with and without F^-. Holtzman male rats were allotted 8 per lot and fed supplements as indicated in table 3.

Cumulative weight gains, calcium content of kidneys and hearts of rats fed diets containing 30, 200 and 400 ppm of Mg with or without F^- are summarized in table 3. The low Mg rats and those fed F^-, lots 1 and 2, were retarded in weight gains. Animals fed the basal diet only and those in lot 2 fed F^- showed an increased calcium content in the kidney and heart, when compared with the control lot 3. The Ca content of the heart was unaffected by Mg increased to 400 ppm with or without added F^-. In view of the demonstrated preventive action of F^-

against calcinosis, it was expected that the heart Ca concentration would be normal as for lots 4, 5 and 6.

Kidney Ca content was somewhat variable but was increased by added F^- at 400 ppm, lots 2, 4 and 6. The high value obtained for the kidney, lot 2, cannot be explained aside from its stimulatory affect upon Ca elimination via the kidney. It may be due to kidney damage since kidney Ca of the rats fed adequate Mg 200 or 400 ppm Mg was also increased by feeding F^- (compare values reported for lot 3 vs. lot 4, and lot 5 vs. lot 6). Thus it appears that high dietary F^- intake caused an increased concentration of kidney Ca.

Experiment 4. A study of recovery from low Mg calcinoses was made in experiments 4 and 5. In the fourth experiment, 8 growing puppies were fed the low Mg basal ration for a 6-week depletion period. Two dogs were used as representatives of the depletion period and tissue analyses made at the end of that time for comparison with values following recovery, or the repletion period of 25 days. The treatments during the repletion period were: lot 1, low Mg diet + 170 ppm Mg (total Mg 200 ppm); lot 2, low Mg diet + 370 ppm Mg (total Mg 400 ppm); lot 3, low Mg diet + 25 mg Mg as $MgSO_4$ daily subcutaneously; and lot 4, depletion group terminated before repletion period.

Mg deficiency symptoms were observed during the Mg depletion period including spasmatic seizures which subsided by the fourth day of repletion. The dogs sustained continued growth and activity and appeared normal by the second week of repletion. In this experiment 200 ppm of Mg supplied adequate dietary Mg and the repletion was accomplished by subcutaneous injection as well as by supplementation of the diet. Gross inspection at necropsy revealed that calcinosis lesions had increased in all dimensions during the repletion period and had invaded all layers of the aortic wall. The low Mg-induced calcinosis lesions did not disappear during the 25-day repletion period.

Analytical results confirm the gross observations recorded above (table 4).

The calcium content of the aorta, heart valve area and kidney was increased markedly. It had no consistent effect on the heart. Two hundred parts per million of Mg appeared to meet the requirements under these conditions. The bone Mg was reduced in lot 4 and in the depleted group fed for 25 days in comparison with that of the other groups.

Serum data are summarized in table 5. Low Mg-induced calcinosis caused a steady reduction of serum Ca content during depletion with a rapid return to normal during the first week of repletion. In the depletion period phosphorus concentration remained constant or became slightly higher as the calcium was decreased; for example, the level of Ca in dogs of lot 1 was 7.97 at 6 weeks and P in lot 1 for the same time interval was 11.33 Mg/100 ml.

Serum Mg values for the dog with adequate Mg lie within the range from 1.7 to 2.5 with high intake increasing the level

TABLE 4

Effect of depletion and repletion of magnesium on bone magnesium and tissue calcium content of dogs

Lot no.	1	2	3	4
Depletion diet, ppm Mg	30	30	30	30
Repletion diet, ppm Mg	200	400	30 [1]	—
No. of dogs/lot	2	2	2	2
Calcium, mg/100 g dried tissue				
Aorta	1309.5	1538.8	818.6	426.0
Valve	1611.3	3725.6	4108.4	1379.4
Heart	27.1	73.1	29.2	24.0
Kidney	71.2	189.8	99.7	61.9
Bone magnesium, mg/100 g of ash	449	573	451	280

[1] Injection, 25 mg of Mg/day.

to 3.0 mg/100 ml serum. Mg deficiency was evident when the serum Mg values were one or less milligrams per milliliter. Injection of magnesium sulfate with 25 mg daily was equivalent to about one-half the daily ingestion of the dog fed a ration containing 200 ppm of Mg, which accounts for the slower response to Mg repletion. The data further indicate that normal serum values were re-established much more quickly in the case of Ca and P than was Mg.

Experiment 5. It became evident from early data obtained from experiment 4 that more information was required on the relationship of F^- to Mg in the problem under study. A 6-week depletion period followed by a 25-day repletion period was used with 6 lots of 3 to 5 dogs per lot (table 6).

Fluoride at the level of 200 ppm slightly decreased the rate of growth of the dogs in both the depletion and repletion periods. Calcification of aortas and heart valve area were observed in all animals fed the low Mg diet (lots 1 and 5, table 6). The dogs in lot 6 were exposed to 200 ppm of F^- until the end of the depletion period and results obtained at that time indicated that F^- prevented low Mg-induced calcinosis of the soft tissues studied. In contrast, those fed the same depletion diet and repleted as indicated for lot 2 showed very slight aortic lesions in three of five dogs.

The production of low Mg-induced calcinosis by dietary means has been established for the dog. It was prevented by adequate dietary Mg, with 140+ ppm of Mg as the minimal level. This series of studies shows that F^- in amounts as small as 25 ppm reduce or prevent its occurrence and 100 to 200 ppm F^- have been used with excellent results. The data may be summarized as follows. The residual or retained calcium was higher in the aorta, heart valves, heart and kidney tissues in Mg-deficient animals repleted for 25 days with 200 ppm Mg. To a lesser degree the repletion period showed a similar pattern when Mg and F^- both were fed at 200 ppm

TABLE 5

Effect of depletion and repletion of magnesium on serum calcium, phosphorus and magnesium (dog)

Lot no.	Treatment	Weeks on experiment					
		0	2	4	6	7	9
		mg/100 ml serum					
		Calcium					
1	30 ppm Mg diet, 6 weeks 200 ppm Mg diet, 25 days	11.33	9.88	8.51	7.59	11.63	10.80
2	30 ppm Mg diet, 6 weeks 400 ppm Mg diet, 25 days	11.33	9.21	8.38	7.38	11.33	11.35
3	30 ppm Mg diet, 6 weeks 25 mg Mg/day injection	11.46	9.79	8.51	8.23	9.55	10.56
4	30 ppm Mg diet, 6 weeks, control	10.94	10.03	8.41	7.83	—	—
		Phosphorus					
1	30 ppm Mg diet, 6 weeks 200 ppm Mg diet, 25 days	10.77	9.95	10.77	11.33	9.55	8.22
2	30 ppm Mg diet, 6 weeks 400 ppm Mg diet, 25 days	11.27	10.73	11.05	10.51	9.31	10.64
3	30 ppm Mg diet, 6 weeks 25 mg Mg/day injection	8.27	10.12	11.33	9.68	10.06	10.53
4	30 ppm Mg diet, 6 weeks, control	11.10	11.70	9.75	10.28	—	—
		Magnesium					
1	30 ppm Mg diet, 6 weeks 200 ppm Mg diet, 25 days	1.85	0.50	0.37	0.31	0.82	1.11
2	30 ppm Mg diet, 6 weeks 400 ppm Mg diet, 25 days	1.87	0.41	0.32	0.27	1.27	1.56
3	30 ppm Mg diet, 6 weeks 25 mg Mg/day injection	1.80	0.42	0.36	0.44	0.65	0.79
4	30 ppm Mg diet, 6 weeks, control	1.91	0.59	0.40	0.32	—	—

TABLE 6

Bone magnesium and tissue calcium at the end of 25-day recovery period

Lot no.	1	2	3	4	5	6
Diet, first 6 weeks, ppm Mg	30	30	200	200	30	30
Diet, first 6 weeks, ppm F	—	200	—	200	—	200
Diet, last 25 days, ppm Mg	200	200	200	200	200	—
Diet, last 25 days, ppm F	—	—	—	—	200	—
No. of dogs/lot	4	5	5	4	3	3
Calcium, mg/100 g dried tissue						
Aorta	648.1	43.4	36.4	45.4	454.4	40.0
Valve	588.8	168.3	51.1	50.2	2948.9	44.5
Heart	49.5	39.7	20.1	20.8	68.1	20.5
Kidney	206.7	42.3	39.8	53.5	82.6	37.2
Bone magnesium, mg/100 g bone ash	463	424	567	591	458	214

each, but the analyses were less consistent. The inconsistent results for the calcium concentrations suggest that these most often follow the use of a combination of both Mg and F^- (200 ppm of each). Results for lots 3, 6 and 4 provide data which are consistently regular and that fall into the normal range for the species. In view of the knowledge concerning the effects of F^- on the excretion of Ca from the kidney it is not unexpected to have increased Ca in kidney tissue. The bone ash Mg content at the end of the repletion period was 214 mg which is less than 50% of normal. The control lot 3 averaged 567 mg/100 g bone ash.

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The Utilization of Ethanol

III. LIVER CHANGES INDUCED BY ALCOHOL [1]

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ABSTRACT Fatty livers frequently develop subsequent to chronic consumption of ethanol. To evaluate the possible role of alcohol dehydrogenase (ADH), the rate-limiting enzyme of ethanol metabolism, in the production of fatty infiltration, weaning rats were used. The animals were fed an adequate purified diet until the end of the experiment. A group of rats was given 20% ethanol as the only drinking fluid, and another group was given water and served as a control. Periodically some of the animals were killed and the alcohol dehydrogenase levels and total hepatic lipids determined. Liver tissue sections were made simultaneously for microscopic study. The results indicated that as the level of ADH increased concomitant to alcohol treatment the amount of total hepatic lipids also increased. Likewise, when ADH activity decreased, the quantity of total lipids decreased. Similarly, the degree of liver change measured in terms of cytoplasmic vacuolization followed a pattern similar to that of the ADH level. When alcohol was withdrawn the liver cells tended to revert to near normal. These observations were interpreted to mean that a relationship between ADH level and hepatic lipid synthesis may exist and that ADH may, to a certain extent, be linked to the process of fatty infiltration usually observed in the alcoholic.

Several explanations to liver fatty infiltration during alcohol consumption have been suggested (1–10). Whereas these vary with respect to the mechanism by which alcohol contributes to the development of fatty livers, they appear to agree that ethanol is involved directly or indirectly in certain liver changes. This is particularly true in the case of fatty livers and liver cirrhosis (7, 10–12).

Earlier studies (13–16) have shown that prolonged ingestion of ethanol induced several enzymatic alterations in the liver of rats which could contribute to the usually observed increase in hepatic lipid level under such circumstances. The present paper is an extention of the previously published study (16) and reports certain possible relationships between fatty infiltration and the level of alcohol dehydrogenase (ADH), which is considered the rate-limiting factor in the course of alcohol utilization. The role of other enzymes of alcohol oxidation in the process of fatty infiltration is also discussed. The paper further presents data on the effect of withdrawal of alcohol on ADH activity as well as on the degree of fatty infiltration.

EXPERIMENTAL

Maintenance of animals and assay of alcohol dehydrogenase. The maintenance of animals was essentially the same as that previously described (16) except that in the present experiment the equilibration period with the purified diet was extended to 5 weeks instead of four and that the number of control animals was increased to thirty and the test rats to seventy. The preparation and assay of ADH followed the procedure outlined in the earlier communication (29). However, beginning with the twelfth week after feeding the 20% ethanol, 4 alcohol-treated and 2 control rats were killed every 2 weeks for each determination instead of the 1 or 2 rats killed every 4 weeks as was proposed in the procedure cited above. Seventeen weeks later, the alcohol was withdrawn from 22 rats and replaced by water until the termination of the experiment. The remaining 12 rats continued

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to receive the alcohol and were killed periodically, 4 animals each time.

Histological studies. From the same livers used for the assay of ADH a wedge-shaped piece weighing approximately 0.5 to 0.75 g was cut from the large left lobe of the liver and another small segment from the median lobe was also taken for microscopic studies. The tissue preparations were all stained with hematoxylin and eosin and some with periodic acid-Schiff stains.

Total lipid determination. In a second series of experiments 10 control and 20 alcohol-treated rats were maintained as described in the first series to determine total liver lipids simultaneously with ADH activity. Again on the twelfth week after the introduction of the 20% v/v ethanol and subsequently at 2-week intervals, one control and 2 alcohol-treated rats were killed for these studies. One-half of the livers was used for the assay of ADH and the other half for lipid determination. Total hepatic lipids were analyzed by the method of Floch et al. (17).

RESULTS

The dietary effect on growth and liver weights is recorded in table 1. Statistical evaluation shows that although there were slight differences in weight between the alcohol-treated and control rats, these differences were not striking ($P = 0.063$). This held true before withdrawal of ethanol. After reverting to water, however, the weight difference became definitely non-significant ($P < 0.40$). On the other hand, the weights of the livers per 100 g of total body weight showed highly significant differences before ($P < 0.001$) but not after removal of alcohol ($P < 0.20$).

The results of the ADH determinations (fig. 1) show that the activity of the enzyme followed a course similar to that reported earlier (16). Figure 1 further represents the effect of removing alcohol and substituting water for it, which was effected in this investigation on the 29th week, i.e., after the activity of ADH had attained peak value. This is in contrast with the earlier study (16) where withdrawal of ethanol was made in the 20th

TABLE 1

Average body weights of rats and of their livers

No. weeks on experiment	Avg wt/rat		Avg wt of liver/ 100 g body wt	
	Alcohol-treated [1]	Normal [2]	Alcohol-treated	Normal
	g	g	g	g
12	408.5	417.0	3.55	3.02
14	391.5	435.0	3.03	2.78
16	436.5	481.0	3.34	1.95
18	390.3	403.0	3.09	2.65
20	430.8	437.0	3.29	3.20
22	443.8	455.0	2.96	2.22
24	378.0	376.0	3.04	2.89
26	404.0	416.0	3.40	2.49
28	427.5	467.5	3.45	3.32
		Alcohol withdrawn		
30	426.0 (425.0) [3]	458.0	3.28 (3.20)	2.98
32	439.8	470.5	3.13	3.02
34	495.5	531.5	3.28	3.19
36	499.0		3.68	
38	(527.3)	540.7	(3.19)	3.02
40	607.5	551.0	3.45	3.46
42	587.0		3.27	
44	590.5 (585.5)	578.0	3.33 (3.25)	3.28

[1] Average weight of 4 rats.
[2] Average weight of 2 rats.
[3] Values in parentheses indicate rats continuing to receive alcohol after the 29th week of experiment.

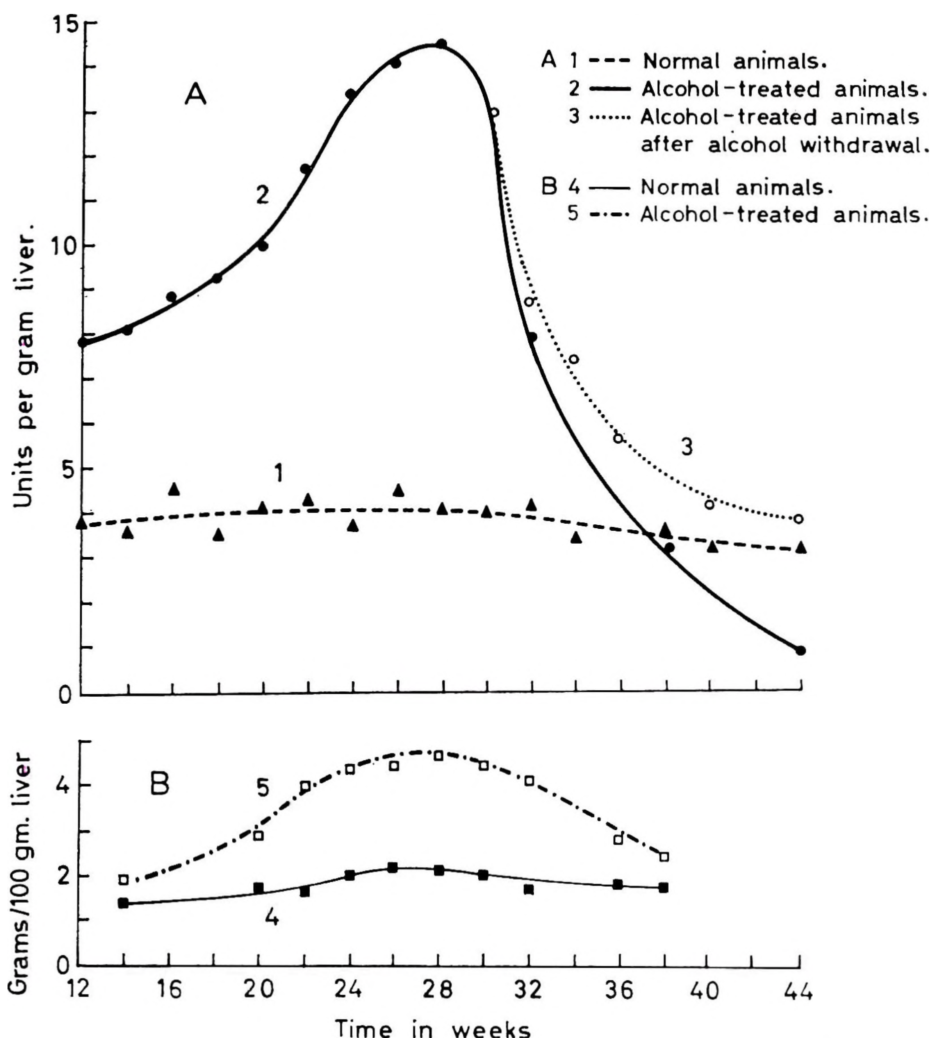

Fig. 1(A) Alcohol dehydrogenase levels in livers of normal and alcohol-treated rats. (B) Total lipids in livers of normal and alcohol-treated rats.

week, a time when maximal activity of the enzyme had not yet been reached. The data indicate that in both studies the level of ADH approached nearly normal values at the end of the experiment. The implications of these observations are, however, different for the 2 studies as will be explained later.

In addition to weight differences, the gross appearance of the livers of the alcohol-treated rats was larger than that of the controls with a friable texture and occasional yellowish discoloration which were suggestive of fatty infiltration. Clear cellular changes other than intracellular vacuolization and fatty infiltration were not observed in the histological study. The degree of cytoplasmic vacuolization observed was used to devise an arbitrary grading system for comparison of the cytological effects of ethanol. Cell alterations were interpreted on a scale ranging from ± (slight vacuolization observed in few cells) to 3+ (about one-half of the lobule showing markedly distended cytoplasm with large coalescent vacuoles). A zero was assigned to non-vacuolated cells having no fat, whereas 1+ represented the

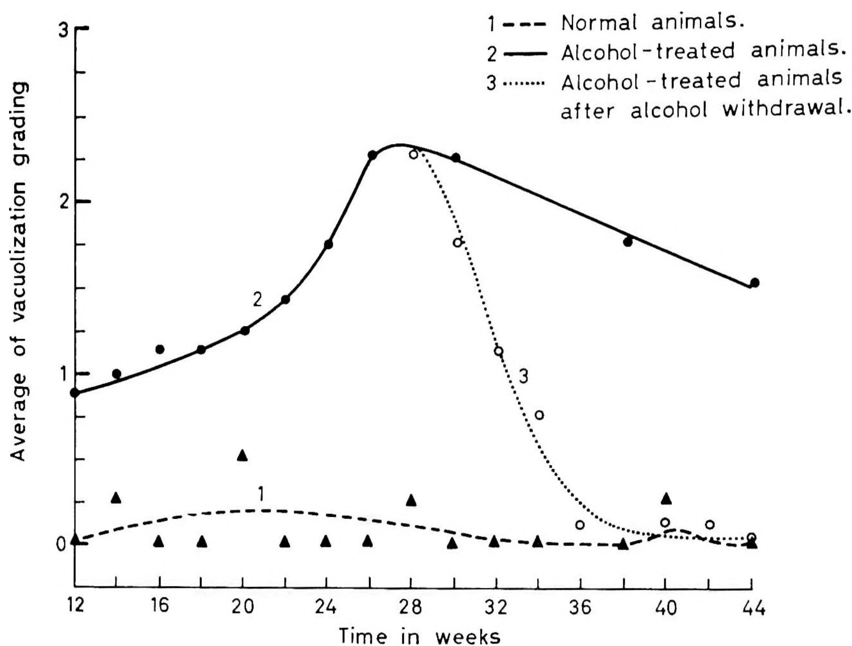

Fig. 2 Average degree of cytoplasmic vacuolization in livers of normal and alcohol-treated rats.

least degree of unequivocal vacuolization and fatty infiltration. It was evident from the microscopic examination that continuous ingestion of ethanol induced a gradual increase in cell vacuolization. Toward the end of the study and after removal of ethanol all livers tended to exhibit a nearly normal picture with respect to size, color, vacuolization and fatty infiltration. However, the shape of cells and distribution of cytoplasmic content appeared to differ slightly from those of the controls.

When averages of the vacuolization gradings for each 4 alcohol-treated rats and for 2 control rats were plotted against the number of weeks (time of killing the animals), the data shown in figure 2 were obtained. The shape of the curve for the alcohol-treated rats, as well as that for the control rats, appears to correspond closely with the respective curves in figure 1(A). This observation suggests a possible relationship between the activity of ADH and the extent of vacuolization and fatty infiltration under the conditions of the experiment. The probable existence of such a relationship will be discussed.

Since the grading system mentioned above is not considered completely quantitative, the actual determination of total hepatic lipids was considered necessary before any attempt was made to correlate its level with ADH activity. Hence in another series of experiments an assay for ADH was conducted simultaneously with lipid determination on livers of the same rats. The results are shown in figure 1(B).

DISCUSSION

Until recently, the part played by ethanol in liver cell injury had not been conclusively elucidated. At present many investigators are of the opinion that alcohol, a principal source of calories, may cause liver damage by producing nutritional deficiency in proteins and essential lipotropic agents (1, 10, 12). Others, however, believe that the effect of ethanol is a more direct one than a mere interference in the nutrition of the subject (9).

Based on unpublished data referred to in the earlier investigation (16) the diet fed to rats had proved to be quite adequate

for growth and maintenance. Despite the use of the same diet in the present study, fatty infiltration was generally produced in the alcohol-treated rats but not in the controls (fig. 1(B)). Other laboratories had reported similar observations (18, 19).

Also, whereas the food consumption of the alcohol-treated rats was somewhat less than that of the normal animals (16), the differences in body weights (table 1) were not significant ($P < 0.05$) for the 2 groups. This would be expected if the decrease in dietary calories was compensated for by the ethanol ingested. On the other hand, relative liver weights (liver weight/100 g of body weight) were significantly greater ($P < 0.001$) in the alcohol-treated group as compared with those for the corresponding control rats (table 1). This observation is consistant with previous reports that relative liver weight is increased by the continued ingestion of ethanol (10, 12, 15).

From the foregoing observations, it might be postulated that alcohol exerts an effect which permits accumulation of fat within the liver cells and that this effect operates separately from that of an extrinsic deficiency of lipotropic or other dietary factors. In support of the above mentioned postulate several mechanisms have been proposed (2–4, 8). They are not entirely convincing, however, and sometimes present opposing views (20, 21). Whereas there is evidence favoring each of the proposed mechanisms, one should not overlook the probability that some may be an effect of liver damage rather than a cause.

In the present work it was generally noted that during the first 28 weeks of the experiment the livers of the alcohol-treated rats accumulated increasing amounts of lipids (figs. 1(B) and 2). This increase was concomitant with a corresponding increase in the level of ADH. Moreover, the gradual increase in vacuolization (fig. 2), observed mainly in the liver cells of the alcohol-treated group which also reflects the degree of fatty infiltration, likewise appears to follow a course similar to that of ADH. Such liver changes were not noted in the control rats except in scattered cases even though intensive search for advance liver injuries, aside from vacuolization, was made in the tissue slides. No sign of the presence of cirrhosis, fibrosis, focal necrosis, cellular infiltration or alcohol hyaline was evident. It is possible, however, that cirrhosis would have developed eventually if the experiment had been continued longer. On the other hand, glycogen deposition was more marked in the tissue preparations obtained from the alcohol-treated livers as compared with those of the controls.

These observations suggest that a relationship, not yet clearly defined, may exist between the amounts of hepatic lipids and ADH activity. It is also possible that another hepatic aberration not apparent at present is causing part or all of the changes observed in this study. Definitive answers to these points must await the results of enzymatic studies in liver slices and other suitable systems for an actual correlation between ADH levels and fat synthesis. Whereas it is conceivable that the amounts of total lipids may decrease as the ADH level decreases, it is not clear, however, why vacuolization, which is thought to be a morphological change, should also decrease simultaneously and then ultimately disappear after alcohol withdrawal (fig. 2). It may be that certain regenerative processes in the cytoplasm were responsible for this phenomenon.

The concept that alcohol induces hepatic fatty acid synthesis gained support from the independent observations of Forsander and Raiha (5) and Lieber and his associates (4). These investigators related this effect to an increase in the amounts of reduced diphosphopyridine nucleotide (DPNH) generated in the liver in the early stages of ethanol metabolism. The role of DPNH as an essential co-factor in certain aspects of fatty acid synthesis has been well documented (22, 23). Furthermore, it has been reported by Lieber et al. ('65) that the incorporation of labeled acetate into fatty acids in rats given ethanol was much greater in the liver than in adipose tissue, suggesting that ethanol stimulates hepatic fatty acid synthesis,

and that the incorporation of labeled acetate was dependent on the abundance of ADH in the liver. Thus, a more active ADH system, as is evident from the present investigation, would be expected to induce more generation of DPNH at the expense of DPN. This increase would be accentuated by the greater activity of the acetaldehyde dehydrogenase (ACDH) system operating under similar experimental conditions (16). The shift in the amounts of DPN and DPNH thus produced causes a change in the relative disposition of acetyl-CoA in such a way that more acetate is incorporated into fatty acids, and less is oxidized via the citric acid cycle (4, 6). Diminished citric acid cycle activity, during alcohol utilization or by increasing the amounts of extrinsic DPNH in vitro experiments, has been reported by several laboratories (4, 15, 18). Moreover, a reduction in the level of DPN during ethanol utilization by ADH and ACDH would impair fatty acid oxidation, since DPN is known to be important in these reactions (43). As a result of this a corresponding reduction of fat removal from the liver would occur. Coupled with these observations is the fact that part of the acetyl-CoA pool from which the fatty acids are derived is provided by ethanol (15, 26). All of the reactions mentioned above would favor, undoubtedly, a greater hepatic synthesis and less utilization of fatty acids, a combination which is conducive to fatty livers.

The effect of ethanol withdrawal on ADH activity and fatty infiltration before reaching the maximal level has been reported (16), and that after attaining maximal levels is represented in figures 1(A) and 2. In both cases the substitution of alcohol by water caused a progressive decrease in the activity of ADH as well as of hepatic total lipids, which finally reached near-normal values. As a partial explanation it was suggested in the previous communication that a decrease in ADH activity in the latter stages of alcohol treatment is probably due to an irreversible cellular damage, which in turn reduced the ability of the liver to form ADH. Diminished ADH activity was also observed recently in liver preparations obtained by biopsy from human alcoholics with cirrhosis (14). A reduction in this enzyme similar to that reported previously (16) was, likewise, observed in the present study. Actually, the activity of ADH and the amounts of total lipids decreased to subnormal levels. In contrast, the ultimate levels reached in the earlier experiment in which alcohol was withdrawn before ADH had attained maximal values were generally a little above normal. No definitive or complete explanation can be offered which would account for the occurrence of these differences. Possibly they may be explained, at least in part, by the premise that alcohol, which would otherwise alter the integrity of the liver cells, leaves the liver uninjured when withdrawn before ADH has reached peak levels. Thus the higher levels of ADH which are usually required to handle the continually ingested ethanol become unnecessary when alcohol, the normal substrate of the enzyme, is not administered further. In this way, the adaptive mechanism, which might be developed by alcohol for the production of excessive amounts of the ADH, would cease to function. This would ultimately lead to a progressive decrease in the formation of the enzyme until it reaches normal values but apparently never below normal. On the other hand, the integrity of the hepatic cells is affected when alcohol withdrawal is delayed, as in the present investigation, thus leading to their injury. Beside cessation of the adaptive mechanism referred to above, certain unknown functional alterations in the livers could also have occurred which caused spontaneous diminishing in the enzyme activity as well as of total lipids until subnormal levels were reached. If these alterations were actually present they would not be of advance nature, however, as the results of the present work appear to indicate. This is clear from the fact that the microscopic appearance of the hepatic cells taken from the alcohol-treated animals reverted to near normalcy. It must be assumed that the discontinuation of ethanol in this study was not started at such a late stage as to prevent cytoplasmic regeneration. This assumption finds support in

the observation that withdrawal of alcohol from cirrhotic subjects often leads to marked improvements in the clinical, functional and histologic state of their livers (27). That a true and complete reversal to normal condition of the liver could be effected by withdrawal of ethanol at the proper time is still speculative and warrants detailed investigation.

CONCLUSION

The evidence presented in this paper demonstrates that during prolonged alcohol treatment liver fatty infiltration could occur despite adequate food consumption. Moreover, hepatic lipid synthesis appears to be very much influenced by the metabolic reactions which ethanol undergoes in the liver. The increased levels of both ADH and ACDH and the decreased activity of the citric acid cycle during chronic ethanol ingestion appear to play a role which is not clear at present, probably a direct one, in the process of fatty infiltration. Furthermore, withdrawal of ethanol before the occurrence of advance liver damage appears to cause reversion of liver cells to near normalcy.

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Use of Free Amino Acid Concentrations in Blood Plasma in Evaluating the Amino Acid Adequacy of Intact Proteins for Chick Growth [1]

I. FREE AMINO ACID PATTERNS OF BLOOD PLASMA OF CHICKS FED UNHEATED AND HEATED FISHMEAL PROTEINS

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ABSTRACT Concentrations of free amino acids in the plasma of chicks were studied to develop a method which will enable the prediction of the amino acid adequacy of intact proteins for chick growth. Plasma free amino acid patterns of chicks fed a standard crystalline amino acid diet were compared with those resulting from feeding the same diet in which the crystalline amino acid component was replaced by an equivalent amount of protein (N $\times$ 6.25). Various fishmeals, some overheated, were used as the source of intact protein. Feeding procedures were used that assured an equal amount and rate of intake of all nutrients among experimental groups prior to time of blood sampling. A comparison of the plasma amino acid levels of the chicks fed fishmeal with those fed the control diet showed that, in general, the concentrations of the essential amino acids were lower in the fishmeal group, with the exception of lysine. This amino acid occurs in relatively high concentrations in good quality fishmeal. The amino acids which appeared to be most deficient in the fishmeals were histidine, valine, methionine and threonine. Chick growth studies, however, have established that these fishmeals respond first to methionine then to single additions of histidine, phenylalanine and possibly threonine. When the intact fishmeals were mildly overheated prior to feeding, lower plasma concentrations of lysine and threonine resulted indicating a loss of availability of these amino acids. More drastic heating resulted in a decrease of all amino acids. The technique described offers a method of comparing the relative availability of amino acids in proteins subjected to various processing alterations, but appears to be ineffective in its present form for predicting the order and degree of amino acid limitation in the fishmeals studied.

Within the past decade an increasing number of investigators have attempted to relate dietary amino acid levels to concentrations of free amino acids in blood plasma. A relationship between the concentrations of plasma free amino acids and the level of the same amino acids in the diet has been demonstrated (1–3). Inconsistencies may arise, however, in the definition of protein quality when the commonly accepted technique is used (3). This technique generally takes the form of a comparison of the plasma patterns of animals having ingested the test protein with those having ingested a standard diet. It appears that factors contributing to the discrepancies which arise are related to the feeding practices used by the various researchers and the use of a standard diet which is comprised of intact proteins consisting of amino acids whose availabilities are not known.

The factors primarily overlooked with respect to feeding practice are time of blood sampling in relation to time of ingestion of the test meal, and total intake of the test diet. Apparently several conditions are essential for a valid comparison of a test protein with a reference diet using plasma amino acid titres. First, an equal and concomitant feed intake must be established for the 2 diets. Also, to insure that the amino acids of dietary origin are reflected in the plasma pattern, maximal feed intake must be achieved, preferably on a voluntary basis. Also, to circumvent the possibility of a lack of uniform absorption of the amino acids the diets should be fed as often as possible, to establish a

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[1] This investigation was conducted by the senior author while on educational leave from the Canada Department of Agriculture.

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"steady state" with respect to amino acid uptake into the blood stream.

Dean [3] has developed a method which satisfies the feeding stipulations outlined above by feeding 800 mg of feed every 30 minutes over a 6-hour period to 2-week-old experimental chicks. During this period, chicks that have been fasted 4 hours just prior to being offered the experimental diets, will voluntarily consume twice as much diet as non-fasted individuals. Under these conditions known amino acid deficiencies in a crystalline amino acid diet were studied by comparing the resultant plasma amino acid concentrations after feeding the deficient diet, with those obtained on feeding a complete amino acid diet to a similar group of chicks. Without exception, a marked reduction in the plasma amino acid titre was noted when chicks were fed amino acid-deficient diets.

The experiments reported here were initiated to extend this concept to the point where the plasma amino acid patterns of chicks fed a standard reference diet (4), containing crystalline amino acids, could be compared with those resulting from the feeding of an intact protein. It was then proposed to use this information to predict the amino acid inadequacies of the protein on the basis of differences in the amino acid patterns. Information on the effects of heat treatment upon protein quality was also examined by comparing the plasma patterns that resulted from feeding heated and unheated proteins.

METHODS

New Hampshire × Columbian crossbred males were fed a practical ration of the corn-soybean oil meal type for the first 7 days of life. Approximately twice the number of chicks needed for an experiment were then selected from this population and were placed 2 to a cage in individual (rat type) electrically heated wire cages. These chicks were then fed on an ad libitum basis the purified diet (isolated soybean protein-glucose) described in table 1 for the next 4 days. On the eleventh day feed was removed for 4 hours, replaced for 2 hours and then both feed and water were removed for an overnight fast. The following morning the chicks were weighed to the nearest gram and the actual number of chicks to be used in an experiment (8/treatment plus 2 extra) were selected from the most uniform chicks in this smaller population. Each of the selected chicks was placed in a single cage and fed the standard reference diet containing crystalline amino acids (table 2) ad libitum until the fourteenth day. On

TABLE 1

Composition of the isolated soybean protein pretest diet

	%
Glucose [1]	48.93
Isolated soybean protein [2]	35.30
Corn oil	10.00
Salts 59A [3]	5.27
DL-Methionine	0.30
Choline chloride	0.20
Vitamins (2 g/kg) [4]	—
Penicillin (11 mg/kg)	—
Antioxidant [5]	—
Total	100.00

[1] Cerelose, Corn Products Refining Co., New York.
[2] ADM C-1 Assay Protein, Archer-Daniels-Midland Company, Cleveland.
[3] Salt mixture as a per cent of the total diet: $CaCO_3$, 2.166; KH_2PO_4, 1.05; $CaHPO_4 \cdot 2H_2O$, 0.94; NaCl, 0.8; $MgSO_4$, 0.25; $FeSO_4 \cdot 7H_2O$, 0.03; $MnSO_4 \cdot H_2O$, 0.02; $ZnCO_3$, 0.01; $CuSO_4 \cdot 5H_2O$, 0.002; KI, 0.001; $Na_2MoO_4 \cdot 2H_2O$, 0.001. Total, 5.27.
[4] Klain et al. (11).
[5] Santoquin (125 mg/kg diet), Monsanto Chemical Company, St. Louis.

TABLE 2

Composition of the standard reference diet

	%
Cornstarch	50.15
Amino acid mixture [1]	24.28
Corn oil	15.00
Salt mixture [2]	5.37
Cellulose [3]	3.00
Antacid [4]	1.00
$NaHCO_3$	1.00
Choline chloride	0.20
Vitamins (2 g/kg) [5]	+
Total	100.00

[1] As a % of the diet: L-arginine·HCl, 1.33; L-histidine·HCl·H_2O, 0.62; L-lysine·HCl, 1.40; L-tyrosine, 0.63; L-tryptophan, 0.225; L-phenylalanine, 0.68; DL-methionine, 0.55; L-cystine, 0.35; L-threonine, 0.85; L-leucine, 1.20; L-isoleucine, 0.80; L-valine, 1.04; glycine, 1.60; L-glutamic acid, 12.00; L-proline, 1.00. Total, 24.275%.
[2] Klain et al. (11). ($ZnCO_3$ substituted for $ZnCl_2$.)
[3] Solka Floc, Brown Company, Chicago 3, Illinois.
[4] Antacid adsorbent (an aluminum hydroxide-magnesium trisilicate preparation), Warner-Chilcott Laboratories, Morris Plains, New Jersey.
[5] See footnote 4, table 1.

[3] Dean, W. F. 1963 The development of a crystalline amino acid reference diet for chicks with special reference to its use in studying the effect of suboptimal and superoptimal dietary concentrations of amino acids on the free amino acid content of blood plasma. Doctoral Thesis, University of Illinois, Urbana, Illinois.

this day feed was removed for 4 hours, at the end of which time each chick was fed 0.8 g of the standard reference diet once every 30 minutes over a 6-hour period. Chicks that would not voluntarily consume the full amount of feed offered within the allotted time were discarded and their place taken by the spare chickens. At the end of 6 hours all chicks were returned to the standard reference diet ad libitum. This training procedure not only exposed the chicks to an interrupted feeding program but also insured a similar amino acid intake across all groups prior to the actual feeding of the test diets. On the morning of the fifteenth day all feed was removed for 4 hours. Following this fast the groups were fed the experimental diets at the rate of 0.8 g/chick every 30 minutes for 12 feedings. Thirty minutes after the final feeding blood samples were taken.

Approximately 3 ml of blood were withdrawn by heart puncture from each chick, heparinized and centrifuged. One and one-half milliliter of plasma from each of the 8 chicks in each treatment group were pooled and centrifuged for an additional 10 minutes. A 10-ml aliquot was deproteinized with picric acid (5). Two milliliters of deproteinized plasma (equivalent to 1.67 ml of the original plasma sample) were added to the columns of an automatic amino acid analyzer [4] and the amino acid content of the sample determined (6, 7).

EXPERIMENTAL

Experiment 1. Prior to the initiation of studies on plasma amino acids, it was considered essential to determine whether the growth response of chicks fed the amino acid mixture of the standard reference diet could be equated to that of the intact proteins when the latter were fed at an equivalent level of protein in the same high energy diet. In addition it was necessary to see whether the fishmeal proteins to be studied could be improved by the addition of their known first limiting amino acid under the conditions of the relatively high level of protein and high energy diets to be used in the plasma assays.

In experiment 1 the standard reference diet served as treatment 1 (table 1). Diets for treatments 2, 3 and 4 were made by removing the amino acid mixture from this diet and substituting in turn the equivalent of 18.34% crude protein from fishmeal L, from fishmeal L which had been steam autoclaved 2 hours at 121°, and from fishmeal L which had been steam autoclaved 12 hours at 121°. The diets were fed ad libitum for 6 days (8 to 14 day) to 3 replicates of 3 chicks. Body gain and feed efficiency were used as the criteria of comparison.

Experiment 2. The response of growing chicks to methionine supplementation of fishmeal when fed as the sole source of protein at 10% crude protein (8) prompted the study of the effect of methionine supplementation of fishmeal L when fed at the higher level of crude protein (18.34%). The experimental conditions were identical to those of the foregoing experiment with the treatments used being fishmeal L and fishmeal L, autoclaved 2 hours at 121°, fed at 18.34% crude protein with and without 0.1 and 0.2% supplementary DL-methionine.

Experiment 3. The first plasma amino acid assay involved a comparison of 4 treatments. Treatment 1 consisted of the standard reference diet (table 2) in which the amino mixture provided the equivalent of 18.34% crude protein and 3 diets in which fishmeal L was substituted for the amino acid mixture to provide the equivalent amount of protein. The fishmeals studied were unheated fishmeal L, and fishmeal L autoclaved at 121° for 2 and 12 hours, respectively. The feeding program, sample preparation, and analysis were conducted as described under Methods. Unfortunately, the blood sample collected from the 12-hour heat-treated group was lost during the final preparative stages. As a result only the first 3 treatment comparisons could be made.

The tables associated with the results of these experiments contain a summary of the average amino acid concentration (μg/ml of plasma) of each amino acid in the pooled plasma of 8 chicks fed the standard reference diet and various fishmeal diets under the appropriate headings. These headings are also described with letters, as

[4] Beckman, Model 120B, automatic amino acid analyzer, Palo Alto, California.

A, B, C, for purposes of easily identifying the percentage change calculations which also appear. This value was calculated as follows:

$$\frac{\text{Test protein plasma conc} - \text{reference plasma conc} \times 100}{\text{reference plasma conc}}$$

The reference plasma concentration for most comparisons will be that level of amino acid noted in the plasma, through the feeding of the standard reference diet, although in desired comparisons of heat treatment effects the unheated protein plasma levels have been used as a reference concentration. The magnitude of this percentage change is considered to indicate the relative adequacy of an amino acid to that of the reference diet, where inadequacies or amino acid deficiencies are indicated by increasing negative values. The plasma levels of 17 amino acids or their products have been tabulated. The discussion will consider primarily the first 11 amino acids because the requirement for these amino acids has been accurately determined for the standard reference diet, and certain of the nonessential amino acids can and do undergo biological alteration (e.g., transamination, destruction, etc.) which render an exact quantitation impossible (9).

Experiment 4. To broaden the scope of these investigations another source of fishmeal was studied. Fishmeal M (an eel meal) had responded to methionine supplementation both before and after 12 hours of heating and at the same time had indicated a loss of threonine on heat treatment (8, 10). In an attempt to verify this order of limitation, plasma amino acid levels arising from the feeding of fishmeal M before and after 12 hours of autoclaving at 121° were compared with those obtained from feeding the standard reference diet on an 18.34% crude protein equivalent basis. In this trial, however, the valine content of the standard reference diet was lowered from 1.04% of the diet to 0.86% as a result of evidence which showed that a level of 0.82% L-valine would support optimal chick growth.[5]

RESULTS AND DISCUSSION

Experiment 1. The standard reference diet and the diet containing fishmeal L as the sole source of protein supported approximately equal growth (table 3). Autoclaving the fishmeal for 2 and 12 hours resulted in the proportional decrease in growth and feed efficiency witnessed in earlier experiments at lower levels of protein (8).

Experiment 2. The growth response of the chicks fed the unheated and the 2-hour heated fishmeal L were found to be highly significantly different indicating

[5] Unpublished data, Dean, W. F., and H. M. Scott, 1963.

TABLE 3

Comparative growth responses when fishmeal L and heated fishmeal L with and without methionine supplementation are incorporated as the sole source of protein into the standard reference diet

Exp. no.	Diet no.	Supplement to standard reference diet [1]	Gain/chick/day	Gain/Feed
			g	
1	1	Amino acid mixture [1]	14.3	0.77
	2	Fishmeal L [2]	14.9	0.76
	3	Heated fishmeal L [3]	13.0	0.64
	4	Heated fishmeal L [4]	9.2	0.47
2 [5]	1	Fishmeal L [2]	13.9	0.70
	2	As 1 + 0.10% DL-methionine	14.9	0.78
	3	As 1 + 0.20% DL-methionine	14.9	0.75
	4	Heated fishmeal L [3]	13.1	0.64
	5	As 4 + 0.10% DL-methionine	13.5	0.67
	6	As 4 + 0.20% DL-methionine	13.6	0.68

[1] Table 2.
[2] Fishmeal L to provide the equivalent of 18.34% crude protein.
[3] Fishmeal L (autoclaved 2 hours at 121°) to furnish 18.34% crude protein.
[4] Fishmeal L (autoclaved 12 hours at 121°) to furnish 18.34% crude protein.
[5] Mean daily gain of chicks fed fishmeal L (diets 1, 2, 3) = 14.6 g. Mean daily gain of chicks fed heated fishmeal L (diets 4, 5, 6) = 13.4 g. SE = ± 0.19.

that the alteration of the protein quality after only 2 hours of heat treatment is of measurable consequence (table 3). Although the growth and feed efficiency data indicate a slight response to methionine supplementation under either treatment the differences were not statistically significant. This indicates that the beneficial response to supplementary methionine noted in chicks fed diets containing 10% protein might not be noted when these fishmeals are incorporated into the diet at higher levels. This lack of response to methionine in terms of a 6-day growth assay, however, does not mean that a methionine deficiency in the fishmeal may not be indicated by the 6-hour plasma assay to be used in the subsequent experiments.

Experiment 3. Having established that the intact protein (fishmeal) and the crystalline amino acid mixture resulted in comparable gains and that supplementation of the fishmeal with methionine was not necessary for maximal gain, the plasma experiments were undertaken. Table 4 lists the plasma amino acid concentrations obtained from the chicks fed the standard reference diet, fishmeal L and heated fishmeal L. In comparing the amino acid levels of the chicks fed fishmeal L with those fed the control diet, the concentrations of the first 11 amino acids are lower in the fishmeal group except for lysine, arginine and phenylalanine. The relatively high lysine content of good quality fishmeals would account for the positive value for lysine whereas arginine and phenylalanine appear to be unchanged. The maximal negative percentage changes between the plasma concentrations of chicks fed the reference standard versus the fishmeal diet appear to have occurred in histidine, valine and methionine, indicating a possible deficiency of these amino acids in the fishmeal in that order. Growth studies have shown, however, that fishmeal L responds first to methionine, then to single additions of histidine, phenylalanine and possibly threonine (8).

When the 2-hour heated fishmeal is fed the maximal amino acid deviations from the control group occur in histidine, valine and methionine, but, in addition, losses in lysine and possibly threonine are indicated. The final column of table 4 shows the comparison of the plasma amino

TABLE 4

Free amino acid levels in chick blood plasma resulting from the feeding of a crystalline amino acid reference diet and diets containing fishmeal L, before and after heat treatment

		Standard reference diet [1] (A)	Fishmeal L [2] (B)	Heated fishmeal L [3] (C)	$\frac{B-A}{A} \times 100$	$\frac{C-A}{A} \times 100$	$\frac{C-B}{B} \times 100$
		µg/ml plasma	*µg/ml plasma*	*µg/ml plasma*	%	%	%
1	Threonine	212.9	169.6	149.0	−20	−30	−12
2	Valine	58.9	24.1	24.1	−59	−59	0
3	Cystine	13.0	10.7	10.6	−18	−18	− 1
4	Methionine [4]	34.6	20.8	16.2	−40	−53	−22
5	Isoleucine	16.3	13.4	13.8	−18	−15	3
6	Leucine	20.4	18.8	19.9	− 8	− 2	6
7	Tyrosine	19.5	16.9	16.7	−13	−14	− 1
8	Phenylalanine	14.7	14.6	16.0	− 1	9	10
9	Lysine	70.8	77.5	47.4	9	−33	−39
10	Histidine	27.1	9.3	7.4	−66	−73	−20
11	Arginine	43.7	43.3	41.1	− 1	− 6	− 5
12	Serine	79.3	108.1	99.8	36	26	− 8
13	Asparagine and glutamine	163.0	125.2	111.5	−23	−32	−11
14	Proline	68.9	39.9	31.7	−42	−54	−21
15	Glutamic acid	119.0	25.8	25.1	−78	−79	− 3
16	Glycine	105.9	89.4	78.1	−16	−26	−13
17	Alanine	317.9	99.3	70.7	−69	−78	−29

[1] Table 2, diet contains the equivalent of 18.34% crude protein.
[2] Standard reference diet (table 2 minus amino acid mixture) + fishmeal L to provide the equivalent of 18.34% crude protein.
[3] Standard reference diet (table 2 minus amino acid mixture) + fishmeal L (autoclaved 2 hours at 121°) to provide the equivalent of 18.34% crude protein.
[4] Corrected for methionine sulfoxides.

acid levels of the chicks fed the 2-hour heated and the unheated fishmeal and, as a result, offers an indication of the effect of heating upon the protein. The largest relative change occurred in the lysine levels of the plasma, supporting the evidence offered previously that lysine availability is adversely affected by heat treatment (10). In addition, methionine, histidine and threonine appear to be adversely affected, but to a lesser degree. Although the studies just mentioned (10) showed that threonine might be rendered less available on heating, only a slight indication of a reduction of histidine was observed and no adverse effect of heating upon methionine availability was shown. Such discrepancies, where histidine and valine, rather than methionine, appear as first limiting in the fishmeal, plus the fact that the 12-hour heated sample was lost during the analysis, prompted a second trial in which fishmeal L, autoclaved for 12 hours, was incorporated in the design.

Table 5 lists the results of the plasma amino acid comparisons for this repeated experiment. Calculations involving the percentage change in amino acid concentration have been restricted to those comparing various fishmeal L treatments. However, if the net plasma amino acid concentrations of the chicks fed fishmeal are compared with the amino acid concentrations in the standard group, lysine again is the only amino acid to exceed the control diet plasma levels; hence valine, threonine, methionine and histidine are once again the amino acids which appear to be most limiting in the unheated fishmeal.

A comparison between groups fed unheated fishmeal and 2-hour heated fishmeal shows histidine and lysine were adversely affected by 2 hours of heat treatment. This was also demonstrated above. However, the relative magnitude of change, and, more important, the order of limitation between lysine and histidine appear to have been altered. No measure of the magnitude of change necessary for statistical significance is possible since there was no replication. Nevertheless, the very close agreement between the plasma amino acid concentrations of the 2 experiments would

TABLE 5

Free amino acid levels in chick blood plasma resulting from the feeding of a crystalline amino acid reference diet and diets containing fishmeal L, before and after heat treatment

		Standard reference diet [1] (A)	Fishmeal L [2] (B)	Heated fishmeal L [3] (C)	(D)	(C − B)/B × 100	(D − B)/B × 100	(D − C)/C × 100
		μg/ml plasma	μg/ml plasma	μg/ml plasma	μg/ml plasma	%	%	%
1	Threonine	261.3	151.7	156.0	100.4	3	−34	−36
2	Valine	55.3	24.1	28.5	21.4	18	−11	−25
3	Cystine	14.2	15.6	17.6	9.2	13	−41	−48
4	Methionine [4]	31.6	19.9	25.6	16.8	29	−16	−34
5	Isoleucine	14.8	13.2	16.5	10.7	25	−19	−35
6	Leucine	19.3	17.8	22.7	16.3	28	−8	−28
7	Tyrosine	18.4	14.2	21.3	18.1	50	27	−15
8	Phenylalanine	12.9	13.7	19.1	15.1	39	10	−21
9	Lysine	68.7	82.7	67.4	23.1	−19	−72	−66
10	Histidine	27.7	17.3	13.4	6.8	−23	−61	−49
11	Arginine	40.0	48.7	63.8	34.7	31	−29	−46
12	Serine	83.0	96.7	103.7	65.1	7	−33	−37
13	Asparagine and glutamine	176.2	107.7	135.1	107.9	25	0	−20
14	Proline	66.9	36.8	39.6	24.9	8	−32	−37
15	Glutamic acid	96.7	23.3	26.6	18.3	14	−21	−31
16	Glycine	97.9	90.6	91.9	52.4	1	−42	−43
17	Alanine	313.7	82.7	84.0	60.2	2	−27	−28

[1] Table 2, diet contains the equivalent of 18.34% crude protein.
[2] Standard reference diet (table 2 minus amino acid mixture) + fishmeal L to provide the equivalent of 18.34% crude protein.
[3] Standard reference diet (table 2 minus amino acid mixture) + fishmeal L (autoclaved 2 hours and 12 hours, C and D respectively, at 121°) to provide the equivalent of 18.34% crude protein.
[4] Corrected for methionine sulfoxides.

indicate that it may take only very small changes to be meaningful. These data tend to substantiate those of the biological assays for the availability of amino acids (10), particularly with respect to the loss of the diamino acids and threonine on heating. Heating the fishmeal for 12 hours appears to have reduced all plasma amino acid concentrations to a large degree, especially lysine, histidine, cystine, threonine and arginine. This reduction in the plasma concentration for all amino acids after 12 hours of heating the protein may indicate that a significant loss of digestibility per se occurs on severe heating.

Experiment 4. The results of this experiment are presented in table 6. The effect of reducing the valine content of the standard reference diet is readily observed in the plasma data. In experiment 3 where the 1.04% level of valine was fed, plasma levels of 58.9 and 55.3 μg/ml were observed. However, in this experiment the plasma level in the reference group was lowered to 28.6 μg/ml. Not only does this observation indicate the sensitivity of the assay to detect dietary amino acid alterations but, equally important, valine now does not appear to be limiting in fishmeal M. Furthermore, it can be speculated that it would no longer be limiting in fishmeal L, thus accounting for the discrepancy noted previously between the plasma assay and previous growth assays (8) which did not show this amino acid to be deficient in the fishmeals.

With respect to the results of the unheated fishmeal M assay, histidine, methionine and threonine appear to be most limiting in that order, although growth studies (8), at the 10% level of protein, indicate a significant response primarily to methionine with the slight possibility of a histidine response and even a detrimental effect due to threonine addition. Comparison of the heated fishmeal with the control shows histidine, methionine and threonine to be most limiting in that order. A comparison of the unheated with the 12-hour heated meals reveals that histidine and lysine were the most adversely affected, although with the severe heat treatment used, all amino acid concentrations in the plasma, except for tyrosine and phenylalanine, were reduced markedly.

TABLE 6

Free amino acid levels in chick blood plasma resulting from the feeding of a crystalline amino acid reference diet and diets containing fishmeal M, before and after heat treatment

		Standard reference diet [1] (A)	Fishmeal M [2] (B)	Heated fishmeal M [3] (C)	$\frac{B-A}{A} \times 100$	$\frac{C-A}{A} \times 100$	$\frac{C-B}{B} \times 100$
		μg/ml plasma	*μg/ml plasma*	*μg/ml plasma*	%	%	%
1	Threonine	246.2	153.0	94.3	−38	−62	−38
2	Valine	28.6	26.6	18.7	−7	−35	−30
3	Cystine	12.4	14.1	9.8	14	−21	−30
4	Methionine [4]	33.7	18.1	11.5	−46	−66	−36
5	Isoleucine	14.6	12.6	7.9	−14	−46	−37
6	Leucine	20.9	17.2	13.8	−18	−34	−20
7	Tyrosine	18.7	13.3	13.1	−29	−30	−2
8	Phenylalanine	14.1	13.4	13.2	5	−6	−1
9	Lysine	65.8	67.0	27.3	2	−59	−59
10	Histidine	29.5	11.5	4.2	−61	−86	−63
11	Arginine	50.5	56.3	40.1	11	−21	−29
12	Serine	79.7	126.1	79.1	58	−1	−37
13	Asparagine and glutamine	174.6	146.0	119.6	−16	−32	−18
14	Proline	73.0	53.2	28.8	−27	−61	−46
15	Glutamic acid	83.2	26.0	19.1	−69	−77	−27
16	Glycine	131.3	131.6	73.1	0	−44	−44
17	Alanine	346.9	120.1	60.3	−65	−83	−50

[1] Table 2, diet contains the equivalent of 18.34% crude protein.
[2] Standard reference diet (table 2 minus amino acid mixture) + fishmeal M to provide the equivalent of 18.34% crude protein.
[3] Standard reference diet (table 2 minus amino acid mixture) + fishmeal M (autoclaved 12 hours at 121°) to provide the equivalent of 18.34% crude protein.
[4] Corrected for methionine sulfoxides.

The foregoing experiments indicate that the plasma amino acid technique as described may prove useful in comparing the availability of amino acids among various protein sources. However, because no agreement between the first limiting amino acid, as predicted by the plasma studies, and those predicted by growth and amino acid supplementation studies can be found when the plasma aminogram of chicks fed the standard reference diet is used as the criterion of adequacy, it appears that the technique has not yet reached the point where it can be used to anticipate the order of amino acid limitations in various proteins for chick growth. The implication that the standard reference diet, when used as a comparative base, might contain excess levels of certain amino acids and hence introduce inflated requirements, resulting in "apparent" deficiencies as reflected by the plasma pattern, merits further attention. This and related factors will be considered in a subsequent communication.

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Use of Free Amino Acid Concentrations in Blood Plasma in Evaluating the Amino Acid Adequacy of Intact Proteins for Chick Growth [1]

II. FREE AMINO ACID PATTERNS OF BLOOD PLASMA OF CHICKS FED SESAME AND RAW, HEATED AND OVERHEATED SOYBEAN MEALS

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ABSTRACT Plasma free amino acid patterns of chicks fed a standard crystalline amino acid diet were compared with those resulting from feeding (a) sesame meal with and without lysine supplementation, and (b) raw, heated and overheated soybean meal, with and without methionine supplementation. A low level of lysine in the plasma of sesame-fed chicks showed that lysine is the first limiting amino acid in this protein. Similarly, methionine was observed to be the first limiting amino acid in all the soybean meals studied. Upon supplementation of these meals with their respective limiting amino acids, deficiencies of histidine, threonine and methionine were still indicated. Comparisons of meals with and without supplementation of their first limiting amino acid showed an increased utilization of all amino acids for protein synthesis. This was demonstrated by a marked reduction of all other essential amino acids in the plasma. An overall apparent loss of digestibility results from severe heat treatment of proteins. In addition, the amino acids of raw soybean meal were shown to be less available to the chick than those of properly heated soybean meal. It appears that the plasma amino acid assay described can be of considerable value when a qualitative comparison of the amino acid availability of similar proteins is desired. The use of the technique to predict the amino acid adequacy of intact proteins for chick growth, however, was shown to be dependent upon the extent to which the standard reference diet provides the amino acid requirements for chick growth. Such a reference diet must contain neither a deficient nor an excess quantity of any of these amino acids.

Sesame meal, when fed as the sole source of protein in a chick diet, has been shown to be severely deficient in lysine. Soybean similarly exhibits a sulfur amino acid deficiency even when fed at a level of 18% crude protein (1). However, no clear-cut second limiting amino acid has been demonstrated for either of these proteins for chick growth, particularly at high levels of incorporation in the diet. For this reason sesame and soybean proteins were selected for these studies, since by feeding sesame and soybean meals it was felt that the plasma amino acid patterns should clearly exhibit a lysine and a methionine deficiency, respectively, but no marked second limiting amino acids should appear. Such a result would add support to the validity of the technique described in the preceding report and any deviations from these expectations would outline clearly its shortcomings.

Due to the widespread search for the biochemical and biological reasons accounting for the poor growth exhibited by animals fed raw soybean meal it was decided to include raw, properly heat-treated, and overheated soybean meals in this experiment. It was also considered desirable to include these test proteins with and without their known first limiting amino acids, to observe the effects of these supplementations upon the resultant plasma patterns.

EXPERIMENTAL

The feeding, blood sampling, plasma preparation and analyses used in the present experiment were the same as those of

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[1] This investigation was conducted by the senior author while on educational leave from the Canada Department of Agriculture.

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the preceding report (2), with the exception that the standard reference diet was modified to provide 0.82% rather than 0.86% L-valine. The experiment consisted of 8 treatments as follows: 1) the standard reference diet (table 2, (2) with valine at 0.82%) to provide 18.34% crude protein; 2) basal (treatment 1 minus the amino acid mixture) plus sesame meal to provide 18.34% crude protein; 3) as treatment 2 plus 0.55% L-lysine, shown to adequately supplement sesame for maximal chick growth at this protein level;[3] 4) basal (treatment 1 minus the amino acid mixture) plus raw soybean meal to provide 18.34% crude protein; 5) as treatment 4 plus 0.24% DL-methionine, also shown to adequately supplement soybean meal at this level of protein;[4] 6) basal (treatment 1 minus the amino acid mixture) plus properly heated (autoclaved 30 minutes at 110°) soybean meal to provide 18.34% crude protein; 7) as treatment 6 plus 0.24% DL-methionine; and 8) basal (treatment 1 minus the amino acid mixture) plus overheated (autoclaved 4 hours at 110°) soybean meal to provide 18.34% crude protein.

RESULTS

The effect of decreasing the valine content of the standard reference diet can be readily seen. In table 6 of the preceding report (2) the plasma level of valine was 28.6 μg/ml when the diet contained 0.86% L-valine. In the present experiment the valine content was 20.4 μg/ml with the diet containing 0.82% L-valine (table 1). This observation further substantiates the sensitivity of this method.

A comparison of the plasma amino acid levels of chicks fed the standard reference diet with those fed the sesame diet shows a large difference in their relative lysine content. The lower level of lysine in the plasma of chicks fed sesame shows that lysine is the first limiting amino acid in this protein. The relatively high arginine content of sesame meal is also demonstrated by the large positive change observed in this amino acid in the plasma. In addition, however, "apparent" deficiencies of methionine, histidine and threonine are also indicated despite evidence from growth assays that the addition of any of

[3] Unpublished data, H. M. Scott, W. F. Dean, R. E. Smith and R. A. Zimmerman, 1962.

[4] Unpublished data, A. Aguilera and H. M. Scott, 1962.

TABLE 1

Free amino acid levels in chick blood plasma resulting from the feeding of a crystalline amino acid reference diet and diets containing sesame with and without adequate lysine supplementation

		Standard reference diet [1] (A)	Sesame [2] (B)	Sesame + lysine [3] (C)	(B − A)/A × 100	(C − A)/A × 100	(C − B)/B × 100
		μg/ml plasma	*μg/ml plasma*	*μg/ml plasma*	%	%	%
1	Threonine	216.1	137.4	110.7	−36	−49	−19
2	Valine	20.4	37.9	31.4	86	54	−17
3	Cystine	13.0	15.0	13.0	15	0	−13
4	Methionine [4]	32.3	15.1	18.5	−53	−43	23
5	Isoleucine	13.5	16.5	13.2	22	−2	−20
6	Leucine	20.5	25.3	18.3	23	−11	−28
7	Tyrosine	19.7	31.3	29.0	59	47	−7
8	Phenylalanine	13.8	18.2	18.6	32	35	2
9	Lysine	69.6	14.1	50.0	−80	−28	255
10	Histidine	26.7	16.4	18.2	−39	−32	11
11	Arginine	41.4	111.1	133.9	168	223	21
12	Serine	79.2	55.1	55.5	−30	−30	1
13	Asparagine and glutamine	180.5	100.1	121.1	−45	−33	21
14	Proline	68.1	24.1	23.3	−65	−66	−3
15	Glutamic acid	96.9	19.6	19.1	−80	−80	−3
16	Glycine	127.7	37.0	35.2	−71	−72	−5
17	Alanine	323.4	53.8	63.6	−83	−80	18

[1] Table 2, Smith and Scott (2).

[2] Standard reference diet (table 2 minus amino acid mixture) plus sesame meal to provide 18.34% crude protein.

[3] As footnote 2 plus 0.55% L-lysine.

[4] Corrected for methionine sulfoxides.

these amino acids to a sesame meal diet does not improve chick growth.[3]

Supplementing the sesame meal diet with lysine to the requirement level increased the concentration of this amino acid in the plasma by 255% (table 1). With this supplementation threonine, methionine and histidine now appear more limiting than any other amino acid. The supplementation of sesame meal with the first limiting amino acid, however, has also reduced the net plasma level of most of the other amino acids, a possible reflection of improved amino acid utilization, by improved amino acid balance.

The difference between the plasma levels of methionine of chicks fed the standard reference diet and those fed the raw soybean diet demonstrates the limitation of this amino acid in the raw soybean meal (table 2). However, rather severe limitations of threonine and histidine are also indicated. Supplementation of the diet with a level of methionine shown to be adequate for growth increased the plasma level of this amino acid but did not bring it up to that of the standard diet. All other amino acids with the exception of arginine and phenylalanine appear to remain slightly lower than those of the standard reference fed group under the conditions of adequate methionine supplementation. This could be a reflection of a change in the rate of amino acid absorption or perhaps a more rapid disposal of the absorbed amino acids.

A comparison of the plasma levels of amino acids from chicks fed the properly heated soybean with those of the standard reference group also reflects the extreme deficiency of methionine in this meal (table 2). Once again there are indications of "apparent" deficiencies of threonine, histidine and lysine, but all the other amino acids appear to be adequate when compared with the standard reference diet. Supplementation of this properly heated meal with methionine increases the methionine in the plasma but not up to the level exhibited by the reference diet. Threonine,

TABLE 2

Free amino acid levels in chick blood plasma resulting from the feeding of a crystalline amino acid reference diet and diets containing raw and heated soybean meal with and without supplemental methionine

		Standard reference diet [1] (A)	Raw soybean [2] (B)	Raw soybean + methionine [3] (C)	Heated soybean [4] (D)	Heated soybean + methionine [5] (E)	Over-heated soybean [6] (F)
		µg/ml plasma	*µg/ml plasma*	*µg/ml plasma*	*µg/ml plasma*	*µg/ml plasma*	*µg/ml plasma*
1	Threonine	216.1	102.6	120.6	113.1	114.9	137.4
2	Valine	20.4	16.2	12.8	29.6	20.9	27.1
3	Cystine	13.0	7.2	11.0	9.1	10.7	9.8
4	Methionine [7]	32.3	2.7	14.7	3.1	17.0	2.8
5	Isoleucine	13.5	11.9	11.6	18.8	16.1	18.6
6	Leucine	20.5	16.1	14.2	23.7	18.8	23.2
7	Tyrosine	19.7	15.1	17.4	19.6	30.1	28.5
8	Phenylalanine	13.8	15.6	15.3	18.5	19.7	20.6
9	Lysine	69.6	61.5	48.9	59.5	45.7	28.2
10	Histidine	26.7	14.2	15.6	15.3	20.7	12.9
11	Arginine	41.4	47.5	48.4	66.1	59.5	45.1
12	Serine	79.2	68.6	59.7	67.3	56.9	76.0
13	Asparagine and glutamine	180.5	128.3	124.0	129.7	122.7	133.3
14	Proline	68.1	25.4	21.6	32.4	27.0	30.3
15	Glutamic acid	96.9	17.4	16.7	20.2	17.1	20.0
16	Glycine	127.7	32.3	28.3	40.8	29.4	39.1
17	Alanine	323.4	56.5	50.3	64.6	63.9	60.4

[1] Table 2, Smith and Scott (2).
[2] As footnote 1 (minus amino acid mixture) plus raw soybean to provide 18.34% crude protein.
[3] As footnote 2 plus 0.24% DL-methionine.
[4] As footnote 1 (minus amino acid mixture) plus heated soybean meal (autoclaved 30 minutes at 110°) to provide 18.34% crude protein.
[5] As footnote 4 plus 0.24% DL-methionine.
[6] As footnote 1 (minus amino acid mixture) plus overheated soybean meal (autoclaved 4 hours at 121°) to provide 18.34% crude protein.
[7] Corrected for methionine sulfoxides.

lysine and histidine still appear to be limiting after methionine supplementation.

Chicks fed the overheated soybean meal also demonstrated a severely reduced methionine level in their plasma. In comparison with chicks fed the properly heated soybean meal plasma lysine, histidine and arginine were reduced. This observation tends to confirm the reduction in availability of these amino acids shown to occur upon heating fishmeals (3).

Supplementation of the raw soybean meal with methionine increased plasma levels of this amino acid more than 400% (tables 2 and 3). Threonine, cystine, tyrosine and histidine also increased while valine, leucine and lysine decreased; the remaining amino acids (isoleucine, phenylalanine, arginine) were not appreciably altered by methionine supplementation. Methionine supplementation of the properly heat-treated soybean meal resulted in a change in the plasma pattern not unlike that noted for the raw meal when it was supplemented with methionine. Proper heat treatment of the raw soybean meal increased the plasma concentration of all amino acids except lysine. As compared with properly heated soybean meal, overheating the meal decreased the concentration of many amino acids in the plasma, particularly lysine and arginine (tables 2 and 3).

DISCUSSION

The experiment in this report was designed so that comparisons of the plasma amino acids resulting from the feeding of several proteins could be made with those of chicks fed a standard reference diet, while at the same time comparisons within protein sources were also possible under the specified conditions of amino acid supplementation or heat treatment.

By using the technique which compares the plasma pattern of amino acids from chicks fed the test protein with those fed a crystalline amino acid reference standard diet, it has been demonstrated that acute amino acid deficiencies in proteins can be identified readily. However, in addition to the reduction in the plasma concentration of the limiting amino acid there has been a continual "apparent" deficiency of histidine, threonine and methi-

TABLE 3

Selected comparisons of the data from table 2 expressed as a percentage change in free amino acid levels of the chick blood plasma

		The effect of methionine supplementation		The effect of heat treatment	
		$\frac{C - B}{B} \times 100$ [1]	$\frac{E - D}{D} \times 100$ [2]	$\frac{D - B}{B} \times 100$ [3]	$\frac{F - D}{D} \times 100$ [4]
		%	%	%	%
1	Threonine	18	2	10	21
2	Valine	−21	−29	83	− 8
3	Cystine	53	18	26	8
4	Methionine [5]	444	448	15	−10
5	Isoleucine	− 3	−14	58	− 1
6	Leucine	−12	−21	47	− 2
7	Tyrosine	15	54	30	45
8	Phenylalanine	− 2	6	19	11
9	Lysine	−20	−23	− 3	−53
10	Histidine	10	35	8	−16
11	Arginine	2	−10	39	−32
12	Serine	−13	−15	− 2	13
13	Asparagine and glutamine	− 3	− 5	1	3
14	Proline	−15	−17	28	− 6
15	Glutamic acid	− 4	−15	16	− 1
16	Glycine	−12	−28	26	− 4
17	Alanine	−11	− 1	14	− 7

[1] Raw soybean meal with (C) and without (B) methionine supplement.
[2] Heated soybean meal with (E) and without (D) methionine supplement.
[3] Properly heated soybean (D) vs. raw soybean (B).
[4] Overheated soybean (F) vs. properly heated soybean (D).
[5] Corrected for methionine sulfoxides.

onine in proteins where no deficiency can be demonstrated by amino acid supplementation and growth trials.

In an effort to explain this discrepancy, the information derived from the effects of excess valine in the standard reference diet led to the belief that perhaps other amino acids were also present in excess in this diet, the primary suspects of course being these 3 amino acids. As a direct consequence of the results in this plasma work, chick growth assays were subsequently carried out to reestablish the amino acid requirements of chicks fed the crystalline amino acid standard reference diet. Experiments directed toward this end have shown conclusively that the levels of valine, threonine, histidine, lysine and methionine in the standard reference diet were all in excess of the chick's requirement. The standard reference diet, as revised, now contains valine 0.82% (from 1.04%), threonine 0.65% (from 0.85%), histidine 0.30% (from 0.50%), lysine 0.95% (from 1.12%) and methionine 0.35% (from 0.55%).[5] The fact that the standard reference diet contained excess amounts of these amino acids should serve to clear up most of the discrepancies noted in the prior experiments with fishmeal. Since the reduction in the level of dietary valine in the reference standard removed it from appearing as limiting in the proteins so would the reduction of threonine, histidine, methionine and lysine. In addition, it is now obvious that where adequate supplementation of the sesame meal with lysine, and soybean meal with methionine, failed to raise the plasma levels to those of the standard, it was because the plasma levels with the standard reference diet were in excess of those required for optimal growth.

The ability of the technique described, to predict the amino acid adequacy of intact proteins for chick growth, depends upon the extent to which the standard reference diet provides the amino acid requirements for chick growth. Such a reference diet must contain neither deficiencies nor excesses. Although Dean and Scott (4) were able to define their amino acid diet, which supports good chick growth, by determining in turn the level of each amino acid required to maximize gain, little attention was paid to amino acid levels in excess of requirement unless obvious growth depressions resulted. It is now clear that if this diet is to be used for more precise work (i.e., as a standard for plasma comparisons) amino acids present in excess of the minimal requirement must be excluded. By inference it may be pointed out that the selection of an intact protein or some other amino acid mixture as a control diet merely because it promotes good growth of the animal can lead to serious misinterpretation when used to measure the amino acid adequacy of various other proteins by this technique.

The plasma amino acid assay as carried out in these experiments can be of considerable value in direct comparisons between similar protein sources. The evidence of a detrimental effect of mild heat treatment of fishmeals upon the di-amino acids, especially lysine, and upon threonine using this technique has been supported by previous studies (2, 3). The overall apparent loss of digestibility through severe heat treatment as indicated in these assays would tend to explain the inability of investigators to regain the growth supported by unheated proteins by the addition of one or only a few amino acids.

Supplementing intact proteins (sesame meal and soybean oil meal) with their first limiting amino acid tended to reduce the concentration of other amino acids in the plasma. This effect probably reflects an improved utilization of all plasma amino acids for protein synthesis.

In comparing the raw, properly heated, and overheated soybean meal treatments, this technique has indicated that all amino acids of the raw soybean meal are less available to the chick than are those of the properly heated meal. Borchers (5) has shown that supplementation of raw soybean meal with 4 amino acids will enable the rat to grow equally as well as they do with a properly heated soybean meal. However, Saxena et al. (6) and Aguilera and Scott[4] have been unsuccessful in their attempts to overcome the growth inhibition of chicks fed raw soybean oil meal by supplementing the diet with individual amino acids. The data reported herein

[5] Unpublished data, H. M. Scott, W. F. Dean, R. E. Smith and R. A. Zimmerman, 1963.

tend to support the view that an overall reduction in amino acid availability is characteristic of raw soybean meal rather than an interference in the availability or metabolism of one or only a few amino acids.

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Chromium, Cadmium and Lead in Rats:

EFFECTS ON LIFE SPAN, TUMORS AND TISSUE LEVELS [1]

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ABSTRACT Groups of 50 or more Long-Evans rats in a low metal environment and fed a diet devoid of cadmium and low in many trace metals were given 5 ppm chromium (III), cadmium or lead in drinking water from weaning until death. Life span was shortened in those fed lead and cadmium; tissue concentrations were within human ranges. Longevity of the last 10% was increased in those fed chromium; tissue concentrations were within ranges of young human beings, and females resisted an epidemic of pneumonia. Rats fed lead had fewer tumors than controls or other groups. Arteriolar sclerosis in kidneys and ventricular hypertrophy occurred largely in cadmium-fed animals; cirrhosis of the liver in all groups. Organs of controls were cadmium-free; the metal occurred in animals from another laboratory. Cadmium did not accumulate in kidneys at older ages. Older rats fed lead showed less in organs than younger ones. Chromium did not accumulate in tissues. Extension of life span by restriction of food was reproduced by restriction of lead and cadmium and feeding of chromium. Results indicate that lead and cadmium at human tissue concentrations are toxic to rats in terms of life span and longevity, whereas chromium (III) is not.

Life-term studies on mice exposed to abnormal trace metals with concentrations in tissues within human ranges have been reported (1). Males exhibited excessive mortality from low doses of cadmium and lead, whereas survival was increased by those given chromium. To confirm and extend these observations, rats were maintained on identical regimens; up to 23 months of age mortality of both sexes was significantly higher in those fed cadmium and lead than in those fed chromium or in the controls (2). Furthermore, trivalent chromium appeared to act as an essential trace element, improving growth rates of mice and rats in comparison with those on a regimen containing little of this metal (2, 3). The present report concerns these same rats in respect to life span and longevity, response to infection, causes of death and accumulated tissue metals, concentrations of which in 5 organs approximated those noted in American human beings (4).

METHODS

The regimen, low metal diet and the specially designed laboratory have been described in detail (3), as have the methods used in handling tissues (1). In brief, a diet of rye, dried skim milk and corn oil, to which necessary vitamins and iron were added, was given to 237 male and 224 female rats of the Long-Evans strain. The diet was deficient or low in the metals under study [3] and was fed ad libitum. To doubly deionized spring water were added simple salts of the essential elements manganese, cobalt, copper, zinc and molybdenum in amounts similar to those in commercial diets. From the time of weaning until death this water, to which was added 5 ppm chromium (III), cadmium or lead as acetates, was given as the sole source of fluid to groups of 50 or more rats of each sex; the controls received none of the metals. Water consumed was weighed throughout life. Extensive precautions were taken to avoid extraneous metallic contamination in a laboratory situated on a remote hilltop.

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[3] The diet contained the following trace metals by analysis: (μg/g, wet weight) Zn, 2.98; Cd, 0.0; Pb, 0.05–0.22; Cr, 0.07–0.17; Ti, 0.0–0.06; Ni, 0.4; V, 3.2; Sn, 0.28; Nb, 3.61; As, 0.02; and Ge, 0.24.

Cannibalism and autolysis made a number of carcasses unfit for analysis. At necropsy, heart, lung, spleen, kidney and liver were placed in polyethylene bottles and frozen until needed, precautions being taken to avoid metallic contamination. They were thawed, blotted dry with paper towels having low content of the metals to be analyzed, weighed, dried at 110° and ashed at 450° for 24 to 48 hours in silica crucibles in a muffle furnace lined with silica. Analyses were made on 1 to 6 samples of each tissue pooled according to age. Cadmium and chromium were measured by the methods of Saltzman (5, 6); zinc and lead by those of Sandell (7). Sensitivities in wet tissue were: Cd < 0.02 µg/g; Pb < 0.05 µg/g; Cr < 0.006 µg/g; Zn < 0.05 µg/g. When 0.0 is reported in the tables it indicates less than these concentrations. All values are given in terms of wet tissue. Data were analyzed by chi-square or Student's t where applicable and standard errors of the means are shown. Sections of fresh tissues numbering 524 were fixed in Bouin's solution, stained with hematoxylin-eosin and examined under a light microscope. Urine was assayed semi-quantitatively with reagent strips[4] for pH, protein and glucose.

RESULTS

Survival. Under the conditions of the experiment only one early death occurred in the control and chromium-fed groups. There was considerable mortality before 3 months of age, however, in animals fed lead and cadmium, 36 out of 160 having died (22.5%, $P < 0.0005$). For this reason 45 animals were added to bring each group to 52 or more; 9 died before 3 months of age (20%). These differences in early mortality were significant ($P < 0.0005$). Mean age at death of the second animals in the control and chromium

[4] Combistix, Ames Company, Elkhart, Indiana.

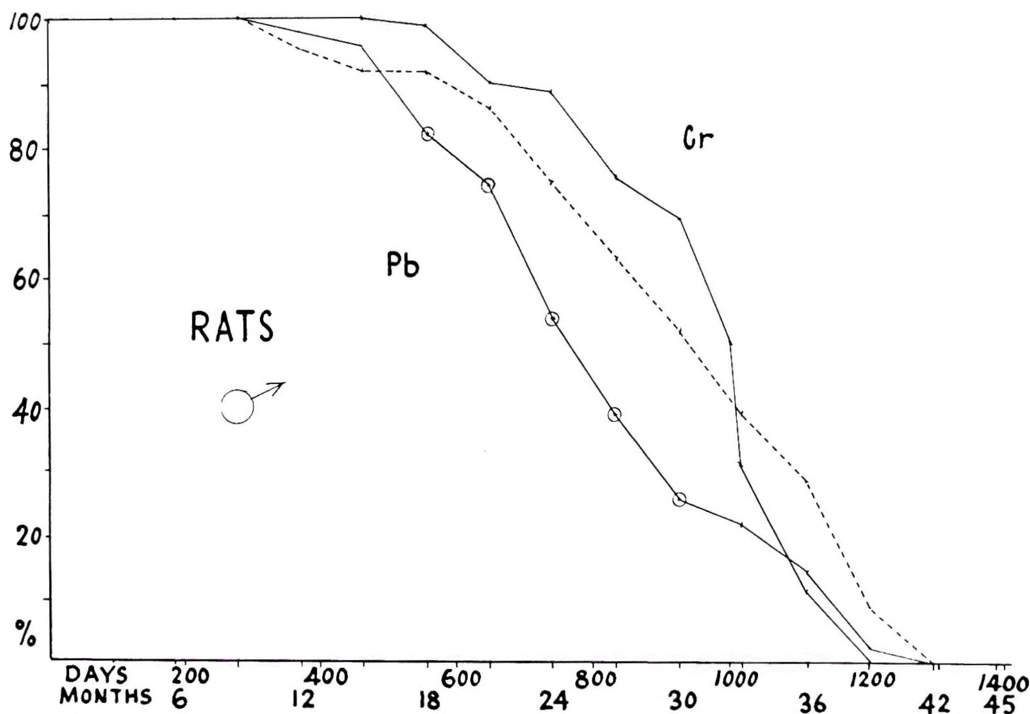

Fig. 1 Survival curves of male rats, 42 controls (solid line), 46 fed chromium (hatched line) and 47 fed lead (solid line), corrected as discussed in text. The circles on the lead curve represent significant differences from the controls ($P < 0.05$ at 18 months, increasing to $P < 0.0005$ at 30 months). Differences also occurred from chromium-fed rats, from 21 to 30 months ($P < 0.05$–0.001).

groups was 510 ± 58.2 days and in the lead and cadmium groups, 31 ± 1.39 days ($P < 0.0001$).

Survival curves are shown in figures 1 to 3, which exclude deaths at less than 3 months of age. Because the supplier of this strain [5] was considering closing his business, in March, 1963, we obtained 25 new breeder animals which started an epidemic of caseous pneumonia; 22.7% of the survivors died within a month's time and all groups were affected. The curves were corrected by subtracting from each group the number of animals dying during the next month, April, which exceeded the mean number of deaths in each of the preceding and following 3 months, and reducing the total accordingly.

Not only did lead and cadmium in the doses given affect survival in young animals but they also influenced it adversely more or less throughout life. At each 3-month interval, mortality was increased significantly in cadmium-fed males from 6 to 30 months of age and in females 30 and 33 months of age. Mortality of lead-fed males was greater from 18 to 30 months of age and of females from 9 to 33 months of age than that of their controls. Chromium exerted no such effect, curves being similar to those of the controls, except at 3 years of age when there were more animals of both sexes alive than in the control ($P \sim 0.025$), and lead ($P < 0.001$) groups.

Longevity. The term longevity is used here to indicate the mean age of the last surviving 10% of the population. The longevity of each of the chromium groups was greater than that of both lead, male control and female cadmium groups (table 1). Longevity of the male cadmium group exceeded that of the controls, whereas in females the opposite situation occurred. Males fed cadmium lived longer than males fed lead.

[5] Rockland Farms, New City, New York.

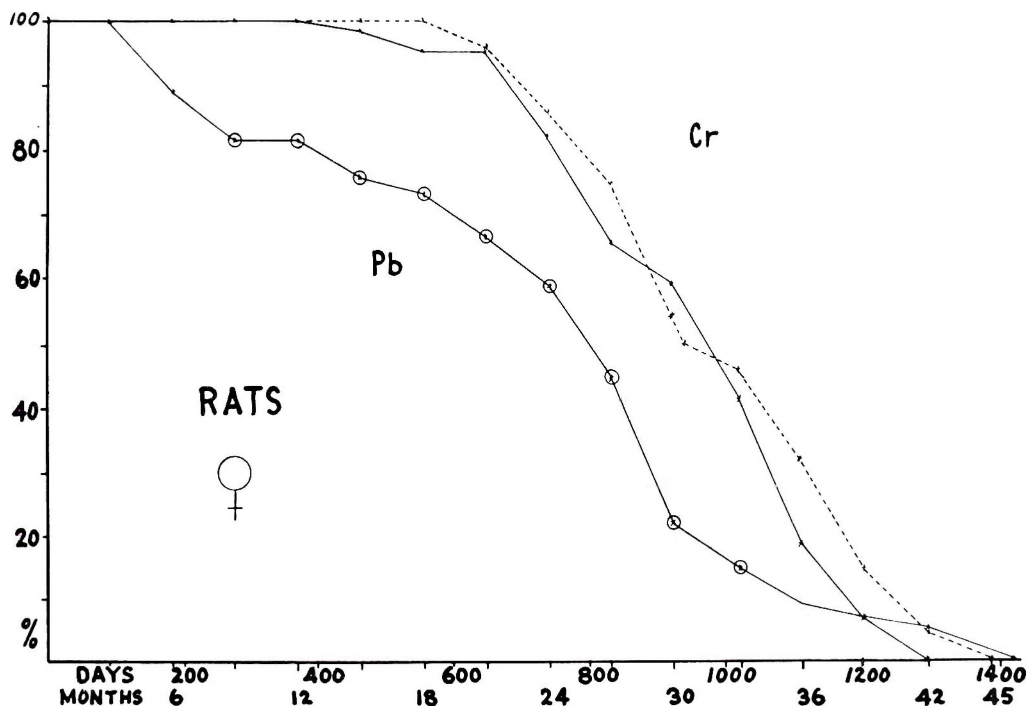

Fig. 2 Survival curves of female rats, 44 controls (solid line), 50 fed chromium (hatched line) and 46 fed lead (solid line) corrected as discussed in text. The circles on the lead curve represent significant differences from the controls ($P < 0.05$ at 24 months, < 0.01–< 0.001 at the remainder). Differences for chromium-fed rats were similar.

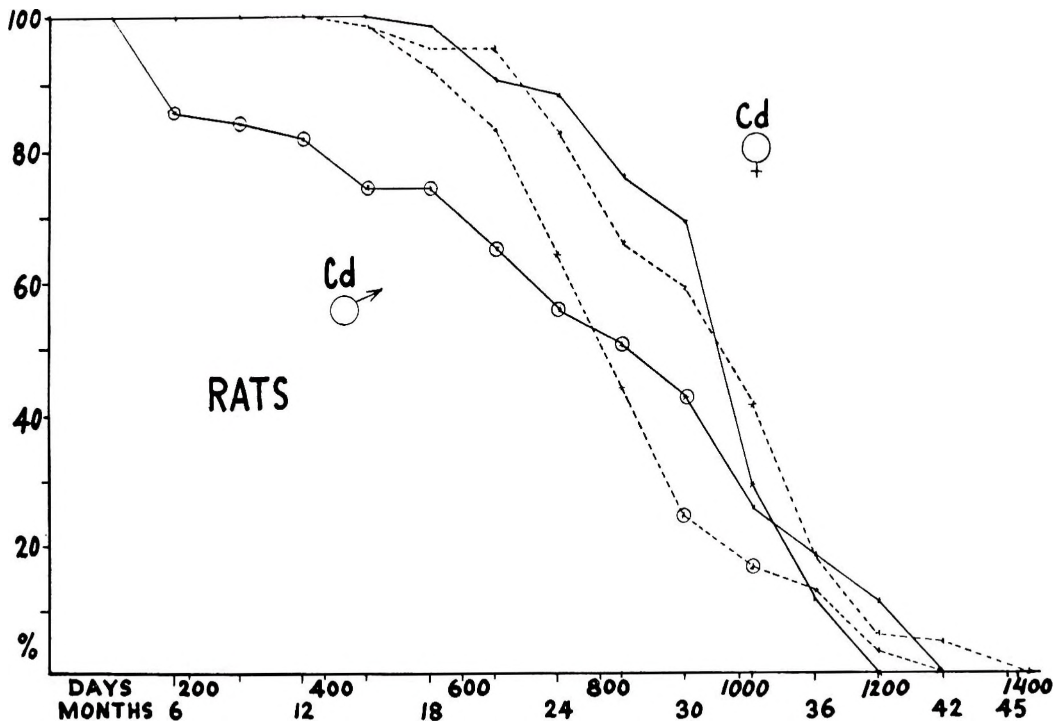

Fig. 3 Survival curves of 42 male (solid line) and 44 female control (hatched line), and 50 male (solid line) and 46 female rats (hatched line) fed cadmium, corrected as discussed in text. Significant differences from the controls are shown in circles (males, $P < 0.05$ at 6 months, < 0.025 at 9 and 27 months, < 0.01 at the remainder; females, $P < 0.005$ at 30 months and < 0.025 at 33 months). Curves of cadmium-fed animals are on the left.

TABLE 1

Life span and longevity of rats given chromium, cadmium and lead; median age at death and ages when 75 and 90% were dead; mean ages of last surviving 10% and ages of last survivors

	No. of rats [1]		No. dead	Median age at death	75% dead	90% dead	Mean age of last 10% surviving	Maximal [2] age
	Gross	Corrected	< 3 months					
				days	*days*	*days*	*days*	*days*
Males								
Control	52	42	0	978	1016	1083	1141 ± 13.2 [3]	1185
Chromium	54	46	0	922	1099	1143	1249 ± 33.2	1320
Cadmium	69	50	12 [4]	822 [4]	996	1134	1221 ± 20.2	1267
Lead	62	47	10 [4]	729 [5]	903 [4]	1069	1123 ± 24.2 [3]	1197
Females								
Control	52	44	0	945	1036	1156	1245 ± 36.0	1400
Chromium	54	50	1	950	1099	1189	1288 ± 13.9	1321
Cadmium	58	46	8	805 [4]	902 [4]	1018	1146 ± 19.4 [6]	1213
Lead	60	46	10 [4]	727 [5]	925 [4]	1069	1162 ± 33.5 [6]	1278

[1] Total number beginning experiment as weanlings, corrected for mortality at less than 3 months of age and during epidemic (see text).
[2] The following lived more than 1200 days: male, 4 chromium, 4 cadmium; females, 3 control, 6 chromium, 1 cadmium, 2 lead.
[3] Significance of difference from chromium group: $P < 0.01$.
[4] Significance of difference from control group: $P < 0.005$; the control groups differed from comparable cadmium groups ($P < 0.025$); the cadmium and lead males also differed ($P < 0.025$).
[5] Significance of difference from control group: $P < 0.0005$; the control groups differed from comparable cadmium groups ($P < 0.025$); the cadmium and lead males also differed ($P < 0.025$).
[6] Significance of difference from chromium group: $P < 0.005$.

Life span as measured by median age at death and when 75% were dead was less in the lead groups by 249 and 113 days for males and 218 and 111 days for females, respectively, compared with controls. These differences were significant ($P < 0.005$–0.0005). Median ages at death were adversely affected by cadmium, differing from the controls by 156 days for males and 140 days for females ($P < 0.005$). Chromium-fed animals approximated the controls in these respects, although tending to live to older ages. The 7 oldest animals, 2 controls, 3 chromium-fed females and 2 chromium-fed males, lived 1300 to 1400 days. Life spans of males were similar to those of females.

Response to exposure to infection. Mortality in the several groups, that resulted from the epidemic of pneumonia, is shown in table 2. The lowest was in chromium-fed females and the next in females fed lead. Other groups differed little. There were fewer exposed survivors in the lead and cadmium groups owing to earlier mortality.

Causes of death. The major disturbances believed to cause death of the animals are shown in table 3. A majority of the cadmium-fed rats were hypertensive which contributed to progressive mortality of males but less so of females (8, 9). Infections and tumors made up most of the recognizable causes.

The number of spontaneous tumors observed grossly and examined microscopically are shown in table 3 (males 24.2%, females 39.7%). Only one was noted in males given lead, a phenomenon also observed in mice (1). All males with tumors, except one, died after 600 days of age, 84.6% after 800 days and 77% after 1000 days. As 40% of male rats fed lead were alive at 800 days of age, suppression or delay in occurrence of tumors in this group appeared to be real.

Most females with tumors also lived beyond 600 days of age, 55.3% after 1000 days. Twelve of the 18 chromium-fed animals died after this later interval, whereas tumors were fairly evenly distributed from 700 to 1200 days in the other groups. No significant differences appeared among the various groups as to type of tumor. Of the eight in lead-fed females only one appeared to have metastasized.

There were 17 large mammary tumors and 8 of ovary or uterus; 17 tumors in the lung, 12 in the liver, 11 of the thyroid, 8 sarcomata, 6 abdominal neoplasms, 5 fibromata of the skin, 4 neoplasms of the lymphatics or spleen, 4 of the gastrointestinal tract and 3 pheochromocytomata were observed, of which 54 had metastasized. No one tumor was confined to one group.

TABLE 2

Mortality during April, 1963, from epidemic of pneumonia introduced into laboratory [1]

	No. rats in group	No. surviving before epidemic	No. dead in epidemic	Mortality
				%
Males				
Control	52	44	12	27.3
Chromium	54	45	12	26.7
Cadmium	69	34	8	23.6
Lead	62	25	9	36.0
Total	237	148	41	27.7
Females				
Controls	52	39	10	25.6
Chromium	54	51	4	7.8 [2]
Cadmium	58	25	8	32.0
Lead	60	36	5	13.9
Total	224	151	27	17.9

[1] At the time of the epidemic, animals were 750 to 820 days old.
[2] By chi-square analysis differs from control females ($P < 0.05$), cadmium females ($P < 0.025$), all males, and all other groups combined ($P < 0.01$).

TABLE 3

Gross causes of death of rats fed metals

	Control	Chromium	Cadmium	Lead
Males				
Tumors	9	16	13	1 [1]
Metastatic	(6) [2]	(9)	(8)	(1) [3]
Pneumonia	15	14	17	20
Other infections	2	0	2	3
Hemorrhage	1	1	5	2
Neurological disease [4]	(1)	(0)	(3)	(6) [5]
Other	2	5	1	3
Unknown	6	3	10	10
No. autopsied	35	39	48	39
Females				
Tumors	15	18	15	8 [5]
Metastatic	(10)	(8)	(11)	(1) [6]
Pneumonia	10	11	9	14
Other infections	1	2	4	4
Hemorrhage	3	0	2	2
Neurological disease	(0)	(0)	(7) [7]	(5) [8]
Other	2	3	4	1
Unknown	4	1	2	6
No. autopsied	35	35	36	35

[1] Differs from chromium group, $P < 0.025$, and from other groups combined, $P < 0.001$.
[2] Numbers in parentheses not counted in total.
[3] Differs from other groups combined, $P < 0.05$.
[4] Neurological disease includes vestibular disturbances, paralyses, convulsions and "rolling fits."
[5] Differs from chromium group, $P < 0.05$.
[6] Differs from control group, $P < 0.01$, and from other groups combined, $P < 0.005$.
[7] Differs from control and chromium groups, $P < 0.001$.
[8] Differs from control and chromium groups, $P < 0.01$.

Neurological disorders, with one exception, occurred in only cadmium- and lead-fed animals. Twelve had severe vestibular disturbances.

Significant microscopic findings are listed in table 4. The presence of cadmium was usually associated with arteriolar sclerosis in kidney. Sclerosis of small arteries was observed in sections of the heart and in 19 sections of lung, liver and adrenal, largely confined to cadmium-fed animals. Hypertrophy of the left ventricle was also present in the cadmium-fed rats. Cirrhosis of the liver and fatty degeneration and vacuolation of hepatic cells were observed in all groups, especially in older animals, and had sometimes advanced to the point of "hobnail" appearance.

Heart and body weights. Hearts of 15 old, chromium-fed animals and 15 old rats from the control and lead groups were weighed to the nearest milligram. The ratio heart weight-to-body weight $\times 10^4$ differed in the 2 groups at death, being 59.5 ± 2.9 and 49.1 ± 3.0, respectively ($P \sim 0.01$). The mean ratio of 3 hypertensive rats was 54.0. Cardiac hypertrophy was observed grossly in 18 of 29 cadmium-fed animals.

Size of the left ventricle was measured on fixed transverse sections of the heart by a micrometer scale on a microscope stage. Mean thickness in 10 cadmium-fed males was 4.88 ± 0.39 mm and in 10 from other groups, 3.84 ± 0.32 mm ($P \sim 0.025$). Ventricular thickness of 12 cadmium-fed females was 4.23 ± 0.22 mm and of 8 from other groups, 3.45 ± 0.19 mm ($P < 0.01$).

Mean body weights at death were, males: control 280.2, chromium 301.9, cadmium 308.0, lead 308.4 g; females: control 234.8, chromium 220.1, cadmium 226.1, lead 223.8 g.[6] Differences were not significant. Only one female in the chromium group weighed more than 300 g, whereas there were 4 to 7 in the others

[6] During the 4 to 6 weeks prior to death, male rats lost 59 to 96 g in weight (17–28%) in this order: O > Pb = Cd > Cr; females lost 15 to 32 g (12–17%). Calculations of heart-to-body weight ratios using these prior weights reduced values as follows: males, chromium 0.56, cadmium 0.36, lead 0.37, control 0.33; females, chromium 0.47, cadmium 0.46, control 0.39. Differences in these ratios from death weight values were 0.10 to 0.13 in males and 0.04 to 0.08 in females.

TABLE 4

Significant microscopic findings in organs of rats of both sexes

	Control	Chromium	Cadmium	Lead
Kidney, no. examined	31	19	46	20
Arteriolar sclerosis: slight	2	4	3	3
moderate	0	0	13	0
marked	0	0	19 [2]	0
Glomerular changes: [1] slight	0	1	3	1
moderate	2	2	7	0
marked	1	1	3	1
Pyelonephritis: slight	6	2	1	2
moderate	2	1	1	0
marked	1	1	1	1
Liver, no. examined	21	15	20	6
Cirrhosis: slight	4	1	5	2
moderate	5	2	6	1
marked	4	4	5	0
Heart, no. examined	17	17	29	4
Enlarged	1	1	18 [3]	0
Arteriosclerosis	0	0	17 [3]	0

[1] Thickening of basement membrane of glomerular capsule or of tuft, hyalinization of glomerulus.
[2] Total moderate and marked, significantly different from controls, $P < 0.0005$, and from other groups combined, $P < 0.0005$.
[3] Significantly different from other groups combined, $P < 0.0005$.

heavier than 300 g. Two to 4 males in each group weighed 400 g or more. At 21 months of age, mean weight of males was 401.5 to 466.1 g and of females 258.0 to 274.5 g, the chromium groups being the heaviest and the controls the lightest (2). Peak weights were achieved between 500 and 700 days of age. Therefore, the effect of chromium on body weight did not persist to the end of life.

Blanching of the incisor teeth. Considered a sign of cadmium toxicity in rats, this change was observed in 23 animals with a mean age at death of 1142 days, none of which was younger than 1049 days and 17 of which were older than 1095 days (3 years). It was noted in 13 chromium-fed, 5 cadmium-fed, 2 lead-fed and 3 control animals, of which 15 were female and 8 male. Inasmuch as 29 chromium-fed, 15 cadmium-fed, 9 lead-fed and 13 control rats were alive at 3 years of age and as the incidence of blanching increased from 14% at 33 to 36 months to 29% at 39 to 42 months, this phenomenon appeared to be a function of aging and was obviously unrelated to the metal given.

Tissue concentrations of metal. In tables 5, 6 and 7 are shown mean concentrations of these metals in 5 organs, compared with the values observed for American adults by Tipton and Cook (4). Rats given these doses attained concentrations within human ranges, although mean values for chromium were higher and for cadmium lower than those of adult man, resembling ranges observed in infants and children (10, 11). Individual variations in groups of animals were wide.

The intake of water by mature male rats was 6.8 ± 0.5, and by females 7.5 ± 0.3 g/100 g body weight/day, measured over a year's time ($P < 0.025$). No differences were noted in the cadmium groups, males ingesting approximately 34.0 and females 37.5 mg metal/100 g body weight/1000 days. Lead-fed males took 7.9 ± 0.3 and females 10.2 ± 0.4 g water/100 g/day ($P < 0.005$), or 39.4 and 51.2 mg metal/100 g/1000 days, respectively. Chromium-fed males ingested 6.4 ± 0.4 g water/100 g/day, or 32 mg metal/1000 days, whereas females drank 9.2 ± 0.7 g/100 g/day ($P < 0.01$), or 45.9 mg chromium/100 g/1000 days. Therefore, females always took more water on a weight basis (and more metal) than males, and those fed lead and chromium voluntarily increased their intakes above those of the controls.

Accumulation of cadmium. The liver and kidney of young rats appear to accumulate cadmium. Five male animals less than 3 months of age accumulated 0.041 ±

TABLE 5

Cadmium in tissues of rats in two age groups [1]

	Controls						Cadmium-fed					
	< 2 Years old			> 2 Years old			< 2 Years old			> 2 Years old		
	No.	Found	Cd	No.	Found	Cd	No.	Found	Cd	No.	Found	Cd
			μg/g wet wt			*μg/g wet wt*			*μg/g wet wt*			*μg/g wet wt*
Males												
Kidney	14	0	—	19	0	—	21	21	2.07	12	12	1.54
Liver	9	0	—	16	1	0.01	21	21	1.05	19	19	2.24
Heart	12	0	—	17	0	—	21	16	0.16	24	14	0.09
Lung	8	0	—	27	0	—	7	7	0.17	19	17	0.06
Spleen	4	0	—	24	0	—	11	7	0.10	21	16	0.18
Mean [2]								89%	0.71		82%	0.82
Females												
Kidney	15	0	—	27	0	—	13	13	1.44	23	23	2.33
Liver	12	0	—	17	0	—	13	13	0.91	19	19	0.39
Heart	16	0	—	21	2	0.02	9	8	0.12	23	3	0.47
Lung	8	0	—	21	3	0.04	9	9	0.40	19	9	0.07
Spleen	12	0	—	21	0	—	9	9	0.47	23	23	0.25
Mean [2]								98% [3]	0.67		66% [3]	0.70

[1] Adult Americans had the following median concentrations (and 80% ranges) by spectrographic methods (4): kidney, 32 (17–56); liver, 2.3 (0.8–4.4); heart, detected in 3 of 140 samples, < 0.6; lung, < 0.6, present in one-half of 141 samples; spleen, < 0.7 μg/g, detected in 18 of 143 samples.

[2] Means of concentrations show gross trends and are not to be construed as otherwise meaningful.

[3] Incidences differ by chi-square analysis ($P < 0.0005$); incidence in all hearts of cadmium-fed rats (53.2%) is significantly lower than that in kidneys or livers ($P < 0.0005$).

TABLE 6

Lead in tissues of rats in two age groups [1]

	Controls						Lead-fed					
	< 2 Years old			> 2 Years old			< 2 Years old			> 2 Years old		
	No.	Found	Pb	No.	Found	Pb	No.	Found	Pb	No.	Found	Pb
			μg/g wet wt			*μg/g wet wt*			*μg/g wet wt*			*μg/g wet wt*
Males												
Kidney	8	6	0.23	28	11	0.05	8	8	1.54	23	12	0.13
Liver	24	16	0.16	28	21	0.06	11	11	0.54	29	20	0.22
Heart	6	4	0.63	11	8	0.30	8	6	0.18	26	9	0.07
Lung	8	7	0.26	28	12	0.11	8	6	0.26	29	8	0.11
Spleen	5	4	0.36	24	14	0.09	8	8	0.56	29	26	0.24
Mean [2]		73%	0.33		66%	0.12		91% [3]	0.62		55% [3]	0.15
Females												
Kidney	23	9	0.55	28	19	0.13	15	15	1.10	15	12	0.16
Liver	32	12	0.54	32	12	0.10	14	13	0.78	15	12	0.02
Heart	13	8	0.52	32	19	0.26	13	8	0.45	15	11	0.22
Lung	21	5	0.56	23	12	0.06	13	8	0.70	15	10	0.08
Spleen	20	15	0.23	32	11	0.09	5	5	1.07	15	12	0.33
Mean [2]		45% [3]	0.48		50% [3]	0.13		82% [3]	0.82		76% [3]	0.16

[1] Seven wild rats had: kidney, 2.04; liver, 0.28; heart, 0.24; lungs, 0.91; spleen, 0.80 and testes, 0.15 μg/g. Adult Americans had the following median concentrations (and 80% ranges) by spectrographic methods (4): kidney, 1.0 (0.4–2.2); liver, 1.7 (0.8–3.3); heart, 0.06 (0.06–0.2); lung, 0.5 (0.05–5.5); spleen, 0.4 (0.09–1.2). Lead was present in all tissues except in one-half of the hearts.

[2] Means of concentrations are given only to show gross trends and are not to be construed as otherwise meaningful.

[3] Differs from comparable incidence in same sex by chi-square analysis ($P < 0.0005$). Incidence in hearts of lead-fed rats (54.1%) is significantly lower than that in spleens (89.3%), $P < 0.0005$, or livers (80.9%), $P < 0.01$.

TABLE 7

Chromium in tissues of rats in two age groups [1]

	Controls						Chromium-fed					
	< 2 Years old			> 2 Years old			< 2 Years old			> 2 Years old		
	No.	Found	Cr	No.	Found	Cr	No.	Found	Cr	No.	Found	Cr
			μg/g wet wt			μg/g wet wt			μg/g wet wt			μg/g wet wt
Males												
Kidney	18	17	0.12	4	3	0.22	29	28	0.14	30	30	0.21
Liver	6	6	0.06	5	4	0.08	32	30	0.05	28	28	0.04
Heart	9	9	0.49	4	4	0.47	21	21	0.45	27	27	0.39
Lung	16	16	0.43	15	14	0.25	28	26	0.44	28	28	0.20
Spleen	16	12	0.20	25	25	0.62	35	35	0.65	20	20	0.60
Mean [2]		92%	0.26		94%	0.33		97%	0.35		100%	0.29
Females												
Kidney	9	9	0.81	4	4	0.19	13	13	0.37	16	16	0.09
Liver	22	17	0.14	7	3	0.13	13	13	0.07	23	23	0.07
Heart	10	10	0.46	8	8	0.06	13	13	0.46	16	16	0.16
Lung	14	9	0.84	11	3	0.09	13	13	0.35	16	16	0.18
Spleen	4	1	(4.40)	4	4	0.10	13	13	1.33	17	17	0.21
Mean [2]		78% [3]	0.56		65% [3]	0.11		100% [3]	0.52		100% [3]	0.16

[1] Adult Americans had the following median concentrations (and 80% ranges) by spectrographic methods (4): kidney, 0.01 (< 0.001–0.06) present in 117/142 cases; liver, 0.09 (< 0.001–0.05) present in 115/146 cases; heart, 0.02 (0.001–0.10) present in 125/140 cases; lung, 0.14 (0.03–0.6) in all cases; spleen, 0.007 (0.001–0.04 μg/g) present in 113/143 cases.

[2] Means of concentrations show gross trends and are not to be construed as otherwise meaningful.

[3] Differs from comparable incidence by chi-square analysis ($P < 0.0005$).

0.005 μg/g kidney/day, giving concentrations which approximated those in older animals (1.5–2.6 μg/g). For the first 3 to 5 days of exposure the rate was about triple that amount. In liver, cadmium increased at a rate of 0.038 ± 0.001 μg/g day, approximating adult concentrations by 3 months of age.

In the 4 young females analyzed, renal rate of accumulation averaged 0.076 and hepatic 0.03 μg/g tissue/day. These rates kept pace with, but did not exceed, growth rates thereafter. Heart muscle of young rats did not contain cadmium. Single weanling animals of both sexes exposed for 3 to 5 days showed 0.14 to 0.64 μg/g renal and 0.2 to 0.41 μg/g hepatic cadmium. Cadmium did not accumulate in the kidneys and livers of rats older than 2 years compared with their younger counterparts (table 5). Ranges in kidneys of mature rats were 1.5 to 8.55 μg/g; and in livers, 0.17 to 2.27 μg/g. Red blood cells of 26 of 42 cadmium-fed rats contained 0.25 μg/g; cadmium was not detected in cells of 16 others nor of 26 control animals.

Accumulation of lead. As the diet was slightly contaminated, lead was present in the controls as well as in those given the metal, especially in heart (table 6). Lead-fed younger animals accumulated it in kidney and liver, approximating human concentrations. Unexpectedly, rats over 2 years of age had tissue levels of lead about equal to those not given it, and every tissue contained less than did those of animals under 2 years of age. No lead was noted in red blood cells except for a trace in one female.

In exposed rats younger than 3 months of age, lead accumulated in liver at the rate of 0.02 to 0.03 μg/g/day, and in unexposed rats at 0.014 μg/g/day. Somewhat less lead was observed in the livers and kidneys of adult breeders born in our laboratory than in breeders obtained from a commercial laboratory.

Lack of accumulation of chromium. Differences in tissue concentrations of control and treated animals were little apparent, except for a higher prevalence of chromium in treated females (table 7). There was a tendency for lower values to occur in older females than in younger, both treated and controls, and no accumulation with age was evident. Red blood

cells of males and females fed chromium, however, contained much, 1.42 and 2.34 μg/g, respectively; controls had 0.72 μg/g.

Most infant rats contained chromium (table 8), whereas the livers of their mothers were often deficient in this metal. For example, the whole bodies of 7 stillborn rats and 10 formed embryos contained 0.12 to 0.13 μg/g chromium; none was detected in the livers of their 2 mothers; in a third there was only 0.03 μg/g postpartum. As the diet was low in chromium, it is possible that deficiency resulting from pregnancy was only slowly restored. Therefore, oral glucose tolerance tests (2.5 g in 10 ml water) were performed on 16 brood females after their breeding ages; the test was performed on 16 other animals not receiving chromium; 4 excreted glucose. Of 23 given the metal, one was positive. Twenty-two control rats not given chromium were shipped by air express in wooden cages to Dr. Walter Mertz, Washington, D. C. All showed markedly delayed uptakes of glucose after intravenous injection, a deficiency rapidly restored by feeding chromium. Uptake by tissues was especially low in 8 females too old to breed. These results are reported elsewhere (13).

Metals in tumors. Chromium was observed in 12 of 15 mammary or subcutaneous tumors at 0.02 to 0.41 μg/g, mean 0.15 μg. Lead occurred in 4 tumors (0.01 to 0.06 μg/g) in animals given lead; it was not detected in 4 not given the metal. Cadmium was present in 6 tumors of animals given the metal, at a range of 0.01 to 0.07 μg/g and not in 4 others. Traces of cadmium, but not of lead, occurred in a bladder calculus and in abdominal fluid.

Urinary changes. No metal affected pH of the urine significantly, the means being 6.12 in males and 5.85 in females. Moderate proteinuria, however, appeared greater in rats fed cadmium. Of 84 control and chromium rats tested, 15 had virtually no urinary protein; of 19 given cadmium all had 30 mg or more; 14, 100 mg or more and 3, > 1000 mg/100 ml. Mean proteinuria in the cadmium group was 266 ± 78.1, in the control and chromium groups combined, 151 ± 19.3 ($P < 0.025$), and in the lead group, 148 ± 30.0 mg/100 ml.

Metals in wild animals. Compared with laboratory rats given cadmium, renal concentrations of this metal in wild animals native to this area were similar (11). A coyote and a woodchuck, however, had

TABLE 8

Metals in whole bodies of infant rats and in livers of their mothers from 2 breeding sources

	Cadmium		Lead		Chromium	
	No. present/total	Conc	No. present/total	Conc	No. present/total	Conc
		μg/g wet wt		*μg/g wet wt*		*μg/g wet wt*
Bred in laboratory						
Formed embryos	0/10	0.0	10/10	0.17	3/3	0.13
Stillborn	0/7	0.0	2/2	0.03	9/14	0.12
1–3 days old	0/8	0.0	5/11	0.10	7/7	0.07
5–15 days old	0/8	0.0	1/5	0.70	8/10	0.15
Mean	0/33	0.0	18/28	0.16	27/34	0.09
Adult breeders [1]						
Liver	0/4	0.0	2/2	0.10	1/3	0.03
Bred by supplier [2]						
Stillborn	1	0.03	1	0.13	1	0.04
14 days old	1	0.02	1	0.0	1	0.02
28 days old	2/2	0.50	2/2	0.83	—	—
Mean	4/4	0.26	3/4	0.59	2/2	0.03
Adult breeders [1]						
Liver	2/2	0.26	3/4	0.27	2/2	0.0

[1] Cadmium was not detected in 75 kidneys or 53 livers of rats bred in our laboratory. It was present in the kidneys of 38 and the livers of 44 adult rats obtained as weanlings from the supplier at mean concentrations of 0.06 and 0.09 μg/g respectively, persisting as long as 1100 days.

[2] Rockland Farms, New City, New York.

TABLE 9

Metallic environmental contaminants to which rats or their tissues may be exposed

Item	Cd		Pb		Cr	Remarks
	µg/g wet wt	*µg/liter*	*µg/g wet wt*	*µg/liter*	*µg/g wet wt*	
Food						
Experimental diet (this study)	0.0		0.05–0.22		0.07–0.17	
Commercial diet no. 1 [1]	0.1		1.9		trace	ref. (20)
Commercial diet no. 2 [2]	0.09		1.7		2.3	ref. (20)
Commercial diet no. 3 [3]	0.09–0.2		—		trace	
Commercial diet no. 4 [4]	—		1.9		trace	ref. (20)
Commercial diet no. 5 [5]	0.09		0.0		4.24	
Water						
Deionized twice	0.0		0.0		0.0	bulk deionizer [6]
Deionized once		4.25	—		0.0	bulk deionizer [6]
Forest spring, polyethylene pipe, black		0.25		3.3	0.0	
Tap, laboratory		0.06–0.6		2.3–4.5	0.0	
Iron pipe, galvanized, dissolved	45.93		0.92		—	
Copper pipe, dissolved	3.21		2.14		—	
Polyethylene, clear	0.0		0.0		0.0	
Polyethylene, black	0.3		—		—	
Rubber stoppers [7]	—		1.47		—	
Water exposed to rubber stoppers		5.5	~ 0.0		—	0.3 µg Cd/100 cm² stopper/day dissolved
Bedding						
Softwood chips	0.05		1.83		0.11	
Sawdust	0.25		1.6		—	
Laboratory litter [8]	0.08		0.22		—	
Cellophane excelsior, clear	0.0		2.4		—	
Paper towels, brand 1	0.2		0.25		0.04	
Paper towels, brand 2	—		20.5		4.0	
Paper towels, brand 3	~ 0.0		9.78		—	
Plastic varnish, sample 1	0.0		0.25		—	
Plastic varnish, sample 2	0.0		0.57		—	
Air						
Dust from precipitator, 6 months	~ 0.0		20.7		~ 0.0	
Snow, melted, near precipitator	0.0			2.70	—	
100 m in forest	0.0			0.80	—	
Suburbs, 25 m from road		0.38		6.00	—	
Near automobile exhaust		0.98		1858.0	—	
Balcony 10-m high	0.0		0.0		—	

[1] Rockland Rat Diet, A. E. Staley Manufacturing Company, Decatur, Illinois.
[2] Purina Rabbit Pellets, Ralston Purina Company, St. Louis.
[3] Purina Laboratory Chow, Ralston Purina Company.
[4] Borden's Dog Meal, The Borden Company, New York.
[5] Wayne Dog Food, Allied Mills, Chicago.
[6] Culligan, Inc., Northbrook, Illinois.
[7] A. H. Thomas Company, Philadelphia.
[8] P. J. Schweitzer Division, Kimberly-Clark Company, Windom, Minnesota.

only 0.02 to 0.06 μg/g in similar tissues. The kidneys of a beaver showed 1.51 and of a pet rabbit raised with commercial rabbit pellets, 0.53 μg/g cadmium.

Chromium was present in the tissues of wild rats at concentrations similar to those of control laboratory rats, 0.05 to 0.65 μg/g, with the highest concentrations in spleen. Seven other wild animals contained in their tissues 0.16 to 0.48 μg/g, again with highest values in spleen. Lead in the kidneys of 11 wild animals averaged 0.81 and in 11 livers, 0.91 μg/g, within the ranges of laboratory rats given the metal.

Precautions necessary to avoid metallic contamination. The need for precautions to exclude extraneous metallic contamination is indicated in table 8. Immature rats bred or born in our laboratory were free of cadmium, whereas those obtained as weanlings were not. Renal cadmium in weanlings obtained from the supplier apparently persisted throughout life; our own rats did not accumulate cadmium from food or water. Thus it was possible to ascertain the source of animal by analyzing the kidneys for cadmium. There was also more lead and less chromium in rats obtained alive than in those born in our laboratory.

The difficulties of obtaining a cadmium- and lead-free environment have been outlined (2) and may be illustrated by table 9. The diet was repeatedly analyzed; it usually showed no detectable cadmium, although rare samples contained 0.01 and one, 0.02 μg/g, probably the result of inadvertent contamination. We have calculated that the intake of cadmium by rats under these conditions amounted to about 3 μg/year, of which 2 μg came from contact of water with rubber stoppers in water bottles and 1 μg from contaminated food. This amount was not large enough to show in the kidneys.

Lead could not be excluded completely owing to contamination of seed rye (0.2 ppm) and wood chips (1.83 ppm) used as bedding. Although the intake of air into the animal quarters was close to a forest on top of a hill 30 m (99 feet) from a private road, there was intermittent daily traffic of personnel with resultant motor vehicle exhaust gases. Lead showed in melted snow and in 6-month collections of dust from electrostatic air precipitators (table 9). Lead in the rat diet varied during the 4 years of the experiment from 0.05 to 0.22 ppm. The major source of environmental contamination appeared to be in motor vehicle exhausts, although there was a small amount of natural lead in virgin soils (14). Chromium, on the other hand, although present, caused no great problem; stainless steel, of which the tops of the animal cages were made, contributed traces to urine but little or none to water.

Reproduction. Approximately 1000 rats have been bred in this laboratory with the cadmium-free diet and no abnormalities in offspring or mothers have been noticed. Approximately 50 brood females, born in this laboratory, have produced offspring; analyses of representative kidneys have failed to show cadmium.

DISCUSSION

The results here reported in rats generally confirm those for mice (1, 3). At organ concentrations similar to those of adult human beings, lead exerted a continuously toxic effect at all ages and in both sexes in terms of mortality and life span. Insofar as could be determined, this effect was manifest largely by susceptibility to spontaneous infections and was not caused by malignant tumors, which were rare, or by overt poisoning. Similar effects of lead on mortality and suppression of tumors were confined to male mice (1). This sub-clinical toxicity was achieved with intakes by males of approximately 40 μg and by females of 52 μg/100 g body weight/day. Human beings consuming average diets have been calculated to ingest 0.43 μg/100 g body weight/day in food and water (14). Thus the rat exposed to this amount of lead accumulates in 2 years, concentrations which it takes man 50 years to achieve. These data indicate that about 0.14 to 0.18% of the amount of lead ingested by rats is retained in tissues, whereas in man retention amounts to about 1.8% of the smaller amounts in food and water. In the organs analyzed, tissues of younger rats contained approximately 50.5 μg lead/100 g; those of man, 106 μg/100 g (4).

Furthermore, in lead-exposed rats the concentration in every tissue decreased from younger to older animals by one-half to one-fourth or less. This same phenomenon was observed in mouse kidneys and livers (1). As analyses were made on animals dying spontaneously, it is possible that those which had accumulated lead died, whereas those which, for some unexplained reason, did not accumulate lead, survived. Therefore, individual susceptibility or adaptability to the toxic effects of lead by both rats and mice may account for this phenomenon, operative even in those receiving a low-lead intake. The extent to which this form of "selection" was manifest is evidenced by the similarity of the mean concentration of lead in the organs of the oldest control and lead-fed animals; except for spleen they are in the same low ranges (table 6).

This phenomenon was not evident in the chromium-fed animals. Concentration of chromium in males did not differ significantly from that of their controls, although there was a tendency for females to lose chromium with age, whether fed it or not. Red blood cells, spleens and hearts generally had the highest values and livers the lowest (males, $P < 0.001$). All animals showed chromium in one or more tissues; the small amount in food undoubtedly supplied the tissues of the controls. Homeostasis was evident from the data. Whereas there was no analytical evidence of complete chromium deficiency, older females may have been marginally deficient, as shown by lower prevalence in tissues, by disturbance in tissue uptake of glucose and by susceptibility to infections in animals not given the metal. Although we could demonstrate no change in median life span, more were alive at 3 years of age than in the control groups. The absence of any signs of toxicity from this dose is noteworthy.

From these and other studies it has become apparent that chromium is an essential trace element for certain mammals in terms of normal metabolic processes: 1) chromium increased growth and mature weights of rats and mice (2, 3); 2) no signs of sub-clinical toxicity or microscopic abnormalities were observed in more than 200 chromium-fed animals; 3) rats marginally deficient in chromium exhibit abnormal glucose metabolism (13, 15); and 4) the life span of male mice (1) and longevity of male rats are extended when chromium is added to a marginal diet. Although no one, to our knowledge, has produced complete chromium deficiency in animals, and those with marginal deficiencies do not appear to suffer seriously therefrom in life span or mortality, the weight of evidence is in favor of a physiological role for this element, which acts in microgram or sub-microgram quantities (15, 16).

The behavior of rats fed cadmium differed from those fed chromium. At renal concentrations considerably less than those in adult Americans, toxicity manifest by increased mortality occurred during most of the lives of males and 6 months of the lives of females. A large number became hypertensive, exhibited renal arteriolar sclerosis, left ventricular hypertrophy and some increase in proteinuria. As is well known, kidney and liver accumulated cadmium, but unlike human tissues (11), there was no significant increase with age or duration of exposure. The prevalence of cadmium in heart was significantly lower than in kidney or liver, analagous to the human situation, although unlike the human, this metal appeared in 77% of lungs and 86% of spleens. As in the case of lead, it is possible that animals accumulating cadmium died younger than those accumulating less; curves of concentrations with age in mice (1) and man (11) are consistent with this hypothesis.

Although cadmium has been present in the kidneys of all animals tested (11), except newborn infants and the rats and mice reported by us (1, 11), there is no evidence that this metal is other than an environmental contaminant: 1) cadmium is not essential for growth of rats or mice; 2) cadmium is not essential for reproduction of rats or mice; 3) the life span of cadmium-free rats and mice is longer than that of animals fed the metal; 4) no abnormalities have appeared in cadmium-free rats and mice; and 5) toxicity in terms of survival of rats and male mice has appeared with low oral doses and low renal concentrations of cadmium (1–3).

Therefore, cadmium does not fulfill any of the criteria of an essential micro-nutrient even though it is widely present in the environment.

The classic experiments of McCay et al. (17, 18) extended the life span of rats by restricting the intake of food. Median life span of their males was about 870 days and of females, about 940 days. Their results have been confirmed by Berg and Simms (19, 20). Although analyses of the diet used by McCay et al. are not available, Berg's diet[7] contained several extraneous elements, including cadmium at 0.1 ppm (11), lead at 1.9 ppm and arsenic at 13.8 ppm.[8] Because of the pronounced effect of small doses of lead on life span, the possibility arises that the effects of restriction of food may have been partly the result of the restriction of lead or of another contaminating trace element and not wholly the result of the food itself. The deposition of lead and cadmium in rat tissues occurs at the fastest rate in young animals, and all commercial animal diets analyzed contained both metals (11, 14). Berg and Simms (20) showed lower mortality in rats whose food was restricted 46% than in those fed ad libitum. Calculations from their data indicate that at life expectancies, total consumption of lead was 9.1 and 8.9 mg/100 g body weight by males and 10.6 and 10.7 mg/100 g body weight by females, respectively, although males had an increased life expectancy of 200 days and females of 364 days with restricted food intake. Kibler et al. (21) subjected male rats, receiving a commercial diet, to a cold environment; 60% greater food intakes and significantly higher mortality resulted with rats living at 9° than at 28°. Calculations from data of these investigators show that at the mean age of deaths, intake of lead at 1.9 ppm would be 32.1 mg/rat (5.1 mg/100 g) in the group living at 28° and 35.1 mg/rat (7.4 mg/100 g) in the group living at 9°, despite a life span of 201 days and 156 g less body weight of the animals living in the cold. With diets ad libitum, containing 0.2 ppm lead, our male rats consumed approximately 29.8 mg lead/100 g body weight by their median life span, or 729 days, when given the metal in water, and 1.0 mg in 978 days when not; females ingested 38.6 mg/100 g body weight in 727 days with lead and 1.2 mg in 945 days when not receiving lead. Therefore, experiments relating life span to food consumption should probably take into account the intake of extraneous trace elements that may influence survival.

Although the long median life span of our control rats may be inherent in the strain, the ages of the last survivors did not differ from those reported for Sprague-Dawley males by Berg and Harmison (22), (1200 days), Verzár (23), (1170 days) and by McCay et al. (17, 18) for restricted rats (about 1225 days). The principal difference lay not in maximal longevity but in median life span. For Sprague-Dawley male rats, Jones and Kimeldorf (24) reported 696 days, Berg and Harmison (22) 750 days and Verzár (23), 705 days. Gilbert and Gilman (25) reported for Wistar rats, 750 days and Kibler et al. (21) for Holtzman rats, about 670 days, ages similar to those of our lead-fed males, 729 days. The median life spans of our control and chromium-fed animals were 7 to 9 months longer, and their normal sigmoid-shaped curves fell sharply at a considerably later interval than did those published by Jones and Kimeldorf (24) and McCay et al. (18), resembling rats restricted as to food (17, 18, 20). Therefore, these Long-Evans rats did not differ from other common strains in maximal longevity, but under the conditions of this experiment could be made to shorten their median life span according to the intake of 2 extraneous trace elements, cadmium and lead, and to lengthen them by restriction of these elements, even when fed ad libitum. Thus, our low-metal diet essentially reproduced the extension of life span resulting from a low intake of food (fig. 4).

[7] Rockland Rat Diet, A. E. Staley Manufacturing Company, Decatur, Illinois.

[8] Data of R. G. Keenan, 1958, Principal Spectrochemist, U. S. Public Health Service, Occupational Health Field Headquarters, Cincinnati, Ohio. Personal communication. Purina Rabbit Pellets (Ralston Purina Company, St. Louis) also contained 1.7 ppm Pb and 2.9 ppm As, whereas Borden's Dog Meal (The Borden Company, New York) contained 1.9 ppm Pb and 10.1 ppm As. In a previous analysis made in 1954, Keenan found 14.8 ppm lead in Purina Dog Chow and 94 ppm in Borden's Dog Meal, amounts much larger than those ingested by our rats given lead in drinking water.

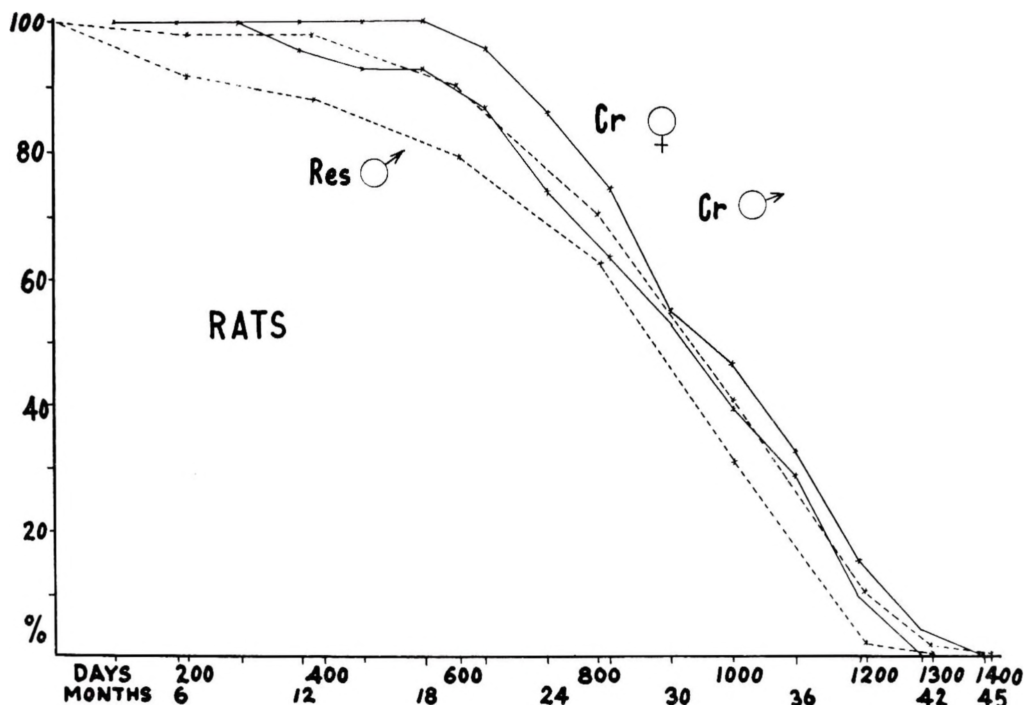

Fig. 4 Survival curves of chromium-fed rats receiving the low-metal diet ad libitum compared with curves of rats restricted as to food intake reported by McCay et al. (17), reproduced by Comfort (23). Hatched lines are those of restricted (Res) males and females, from left to right. Solid line on left is curve of 46 chromium-fed males; on right of 50 chromium-fed females. No significant differences appear between rats of same sex at any interval.

Furthermore, the feeding of chromium not only increased resistance to a pulmonary infection in female rats, but in both sexes increased maximal and mean longevity beyond any we have seen reported, except perhaps for 14 females of Berg and Simms which were not carried beyond 1200 days (20), and a few females of McCay et al. living up to about 1350 days of age (18). This effect of chromium was not demonstrable in mice, although it increased the median life span of males (1).

Also, the usually shorter life span of male than female rats was not evident at any age except in the oldest animals. The 3 ages at death of all males differed from those of all females by only 12 days or less. Apparently this difference between the sexes is not necessarily inherent and can be influenced by exclusion of certain metallic contaminants. Female rats are more resistant than are males to the renal toxicity of mercury (26) and female mice to the innate toxicity of cadmium, lead and titanium (1). When several trace elements were severely restricted, sex differences in mortality largely disappeared in rats. In mice given chromium the median life span was only 30 days longer in females than in males, whereas those not given it showed a difference of 152 days (1).

We must emphasize that comparisons of the groups here reported are complicated by lack of certain basic information when these experiments were begun in 1960. The group fed chromium may be considered "normal" in view of the effect of this metal on growth (2), survival (1) and glucose metabolism (13). Controls were deficient in cadmium and low in lead, as planned, and were probably marginally deficient in chromium. Therefore, comparisons may be made between chromium-fed and controls, cadmium-deficient and cadmium-fed, and low-lead and lead-fed animals, with the reservation that no conclusion can be drawn as to the influence of cadmium or lead in animals receiving adequate amounts of chromium.

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Influence of Medium-chain Triglyceride (MCT) on Cholesterol Metabolism in Rats [1]

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ABSTRACT Medium-chain triglyceride (MCT) is a liquid, saturated fat composed almost entirely of triglycerides of C_8 and C_{10} fatty acids. The effect of MCT on serum and liver cholesterol levels of normal and cholesterol-fed rats and upon hepatic lipogenesis in rats was compared with coconut oil and corn oil. Normal rats fed 20% MCT exhibited lower serum and liver cholesterol levels than did control rats or rats fed 20% coconut oil or corn oil. In rats fed 2% cholesterol and 0.5% cholic acid and 20% MCT, serum cholesterol levels were about one-half of levels observed in rats fed cholesterol-cholic acid and either coconut or corn oil. Liver cholesterol levels in the MCT-fed rats were about 65% of levels in the other 2 groups. The serum β-lipoprotein cholesterol was elevated to the same extent in all 3 groups. The α-/β-lipoprotein cholesterol ratio is 0.10. When hypercholesteremic rats were fed cholesterol-free diets containing 20% of MCT, coconut or corn oil for 2 weeks, serum and liver cholesterol levels decreased at the same rate in all 3 groups. In vivo hepatic lipogenesis from acetate in MCT-fed rats was lower than that observed in rats fed corn oil and equal to that observed in rats fed coconut oil. Fatty acid synthesis was significantly higher in the MCT group. In liver slices, cholesterol synthesis from acetate was lower in rats fed coconut oil than it was in the MCT or corn oil groups. When mevalonate is the precursor, cholesterogenesis was equal in all 3 groups. Fatty acid synthesis was highest in liver slices of MCT-fed rats.

The availability of medium-chain triglyceride (MCT), a liquid fat of iodine value 0.2 which consists primarily of glycerides of caprylic (C_8) and capric (C_{10}) acids, has stimulated research on the effect of this fat upon serum cholesterol levels in a number of species. It has been observed that MCT is less cholesterolemic than are saturated triglycerides containing longer chain fatty acids when fed to rats (1–3), dogs (4) or man (5, 6). This effect may be due to the different mode of lipolysis and absorption of fats and fatty acids with a chain length of 10 carbon atoms or less (7, 8). We have compared the effects of MCT with those of coconut and corn oil upon the serum and liver cholesterol levels of cholesterol-fed rats and have also compared the effects of these fats on in vivo and in vitro hepatic lipogenesis in rats. Our results are the basis of this report.

METHODS

All studies were carried out in male Wistar-strain rats weighing 150 to 160 g. The percentage composition of the basal diet was: casein, 30; salt mixture (USP XIII), 4; cellulose, 2;[2] dextrose, 44; and the oil under study (MCT, coconut oil or corn oil), 20. Adequate vitamin supplementation to the diet included (per kg diet): (in grams) choline, 1; inositol, 1; and (in milligrams) *p*-aminobenzoic acid, 300; niacin, 100; vitamin K, 10; thiamine, 2; riboflavin, 4; pyridoxine, 4; Ca pantothenate, 10; ascorbic acid, 25; folic acid, 2.5; α-tocopherol, 10; and (in micrograms) vitamin D_2, 500; vitamin B_{12}, 50; and biotin, 25. Cholesterol (2%) and cholic acid (0.5%) were added to the diet at the expense of the dextrose. Control rats were maintained with laboratory chow.

In the experiment in which the effects of the various fats upon cholesterol-induced hypercholesterolemia was studied, the rats were maintained with the specific diets for 3 weeks then decapitated. A 1-g aliquot of each liver was dissolved in 15% alcoholic KOH and the cholesterol content of a petroleum ether extract of the saponification mixture was determined. The serum α- and β-lipoproteins were separated by dextran sulfate precipitation (9). All

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[2] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

cholesterol determinations were carried out using the method of Mann (10).

For the biosynthesis experiments the rats were maintained with the 20% fat diets for 7 days. In one series of experiments the animals were given intraperitoneal injections of sodium acetate-1-^{14}C (1 μc/100 g) and killed 4 hours later. One-gram aliquots of each liver were taken for cholesterol determination. The remaining liver was also saponified, the cholesterol digitonide was prepared from the extracted non-saponifiable material and assayed for radioactivity in a Packard Tri-Carb Liquid Scintillation Spectrometer (11). The fatty acids were obtained by petroleum ether extraction after acidification of the aqueous portion remaining after extraction of the non-saponifiable material. The extracts were dried over anhydrous sodium sulfate and aliquots taken for determination of radioactivity. Aortas were pooled, dissolved in KOH and cholesterol and fatty acids isolated in the same fashion. In another series of experiments the animals were killed after they had received the experimental diet for one week and the livers were excised quickly and sliced free-hand. The slices (0.5 mg) were incubated for 4 hours under 100% oxygen in 5 ml phosphate buffer, pH 7 containing 0.006 M $MgCl_2$ and 0.03 M nicotinamide and either 1 μc of sodium acetate-1-^{14}C or DL-mevalonolactone-2-^{14}C. The reaction was stopped by addition of hot alcoholic KOH and cholesterol and fatty acids were analyzed as described above.

RESULTS AND DISCUSSION

1. *Influence of fats on hypercholesterolemia.* In table 1 we have summarized the data relating to the effects of MCT, coconut oil and corn oil upon serum and liver cholesterol levels of rats rendered hypercholesterolemic by simultaneous administration of cholesterol and cholic acid. In all cases a severe hypercholesterolemia resulted. When MCT was present in the diet the increases in serum and liver cholesterol were significantly smaller than those observed under the influence of either of the other longer chain fats. The α-/β-lipoprotein cholesterol partition was the same in all 3 groups, so that in all cases a marked increase in the β-lipoprotein cholesterol was observed.

The group maintained with cholesterol, cholic acid and 20% corn oil was larger than the others and, after having been fed the diet for 3 weeks, the surviving rats were returned to their normal laboratory ration or to cholesterol-free diets containing 20% of MCT, coconut oil or corn oil. The purpose of this experiment was to determine the course of the various regimens upon the previously established hypercholesterolemia. The serum-liver cholesterol pool was calculated (12) for these groups of animals. In the first week following cessation of the cholesterol supplement the decrease in serum cholesterol was most marked in the MCT group but of all the groups, that maintained with laboratory chow showed the most rapid decrease towards normal levels. In all cases the

TABLE 1

Effect of diets containing 2% cholesterol, 0.5% cholic acid and 20% of various oils on serum and liver lipids of rats (3-week feeding)

Group	Survival ratio	Wt gain	Liver wt	Liver cholesterol	Serum cholesterol			
					Total	α-Lipoprotein	β-Lipoprotein	α-/β-Lipoprotein
		g	*g*	*mg/100 g*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*
MCT [1]	5/8	83	13.5	603 ± 131 [2,3]	149.2 ± 25.8 [2,4]	15.0	134.2	0.11
Coconut oil	6/8	92	14.6	933 ± 27	334.6 ± 52.4	34.2	300.4	0.11
Corn oil	8/8	79	14.3	929 ± 36	333.8 ± 42.2	28.8	305.0	0.09
Control	8/8	92	10.4	95 ± 5	46.8 ± 4.0	25.7	21.1	1.22

[1] MCT indicates medium-chain triglyceride.
[2] SE.
[3] MCT vs. coconut oil or corn oil, $P < 0.05$.
[4] MCT vs. coconut oil or corn oil, $P < 0.01$.

marked decrease in serum and liver cholesterol levels is indicative of the ability of the rat to readjust rapidly from the effects of hypercholesterolemia. By the second week all 4 groups of rats were normocholesterolemic but liver cholesterol had attained normal levels only in the group fed the relatively low fat diet. The liver cholesterol levels observed in the groups fed coconut and corn oil approach the levels observed in another feeding experiment in which these fats (20%) were added to the basal, cholesterol-free diet for only one week. The liver cholesterol levels of the rats fed 20% MCT for one week were lower than those observed in the rats returned to MCT for 2 weeks following maintenance on a hypercholesterolemic regimen (table 2). This difference in liver cholesterol levels may reflect the different mode of transport of this fat and indicate a relative inability to clear the liver of cholesterol.

2. *Hepatic lipogenesis.* We first compared in vivo biosynthesis of cholesterol and fatty acids in rats fed 20% of MCT, coconut oil or corn oil for 7 days. The average serum and liver cholesterol levels of these groups are presented in table 3. The results are consistent with those of Kaunitz (1–3). The biosynthesis data are presented in table 4. The weights of the pooled aortas were comparable (115 to 140 mg) for all 4 groups. The incorporation of radioactivity into aorta fatty acids was highest in the control and MCT groups, whereas aorta cholesterol specific activity was highest in the MCT and corn oil groups. The fatty acid radioactivity (count/min/mg aorta) was: MCT, 65; coconut oil, 33; corn oil, 24; control, 58. Aorta cholesterol specific activities were: MCT, 275; coconut oil, 180; corn oil, 240; control, 170. These results are comparable with those reported by Reiser et al. (13).

The order of fatty acid specific activity in the pooled aortas of various groups resembles that observed in the livers, i.e., fatty acid specific activities of the MCT and control group aorta fatty acids are

TABLE 2

Serum and liver lipids of hypercholesterolemic rats after return to diets containing 20% of various oils [1]

Group	No. rats	Wt gain	Liver wt	Liver cholesterol	Serum cholesterol	Serum-liver cholesterol pool		
						Serum	Liver	Total
		g	*g*	*mg/100 g*	*mg/100 ml*	*mg*	*mg*	*mg*
Week 1								
MCT [2]	4	30	16.1	458 ± 38 [3]	93.4 ± 7.6 [3]	7.7	73.7	81.4
Coconut oil	4	32	17.2	401 ± 51	135.2 ± 20.3	11.3	69.0	80.3
Corn oil	4	34	14.4	486 ± 35	115.9 ± 6.7	8.8	70.0	78.8
Control	3	34	12.5	303 ± 49	73.0 ± 9.2	5.3	37.9	43.2
Week 2								
MCT	4	43	17.0	261 ± 38	58.2 ± 5.5	5.3	44.4	49.7
Coconut oil	4	34	18.1	207 ± 38	54.9 ± 4.9	4.9	37.5	42.4
Corn oil	4	55	19.0	265 ± 15	56.3 ± 6.0	5.4	50.4	55.8
Control	3	65	15.4	179 ± 27	56.7 ± 2.9	5.3	27.6	32.9

[1] Average values for hypercholesterolemic rats: liver cholesterol: 929 mg/100 g; serum cholesterol: 334 mg/100 ml.
[2] MCT indicates medium-chain triglyceride.
[3] SE.

TABLE 3

Serum and liver lipids of rats fed 20% of various oils for 7 days (5 rats per group)

Group	Wt gain	Liver wt	Liver cholesterol	Serum cholesterol
	g	*g*	*mg/100 g*	*mg/100 ml*
MCT [1]	21	7.1	140 ± 17 [2,3]	54.0 ± 4.9 [2,4]
Coconut oil	18	7.6	191 ± 23	77.6 ± 2.2
Corn oil	15	6.6	236 ± 9	85.1 ± 7.7
Control	28	6.8	179 ± 23	66.9 ± 3.0

[1] MCT indicates medium-chain triglycerides.
[2] SE.
[3] MCT vs. corn oil, $P < 0.01$.
[4] MCT vs. coconut or corn oil, $P < 0.01$; control vs. MCT, coconut oil, corn oil, $P = 0.05$.

TABLE 4

Lipogenesis in livers of rats fed 20% of various fats for 7 days (5 rats per group)

Diet	% Incorporation of sodium acetate-1-^{14}C	
	Cholesterol	Fatty acid
MCT [1]	0.61 ± 0.03 [2,3]	1.45 ± 0.03 [2,4]
Coconut oil	0.54 ± 0.17	0.89 ± 0.14
Corn oil	1.26 ± 0.37	1.00 ± 0.19
Control	1.44 ± 0.29	1.31 ± 0.38

[1] MCT indicates medium-chain triglyceride.
[2] SE.
[3] Control vs. MCT or coconut oil, $P < 0.05$.
[4] MCT vs. coconut oil, $P < 0.01$; MCT vs. corn oil, $0.01 < P < 0.05$.

comparable to each other and are, in turn, considerably higher than those of the coconut and corn oil groups. This similarity is not observed in the specific activities of the aortic cholesterol of the various dietary groups. It is not possible to ascertain from our data whether the differences in aortic lipid specific activities are due to differences in biosynthesis *in situ* or to mode of transport and deposition of circulating cholesterol and fatty acids. Our observations and those of Reiser (13) suggest the importance of devising experiments which can identify clearly the origin of the radioactive lipid which is recovered from the aorta.

Two separate experiments were carried out in which lipogenesis from acetate and mevalonate by rat liver slices was compared. In the first experiment only cholesterol biosynthesis was compared. When acetate was the precursor, more radioactivity was incorporated into cholesterol by liver slices from rats fed MCT than by slices from either of the other 2 experimental groups. When mevalonate was the precursor there were no differences in the amount incorporated into cholesterol by any of the 4 groups studied. In the second experiment, we compared both cholesterol and fatty acid biosynthesis and found cholesterogenesis to be of the same order as observed in the first experiment. Fatty acid synthesis was highest in the MCT-fed group. The data are presented in table 5.

The effects of saturated and unsaturated fats on hepatic cholesterogenesis have been compared by several groups of workers and our results with coconut and corn oils are consistent with the majority of the results which are summarized in table 6. Usually, the extent of cholesterol biosynthesis is greater in livers of rats fed unsaturated fat when acetate is the precursor. Our observation that this difference does not appear when mevalonate is the precursor confirms the report of Wilson and Siperstein (18). In general, lipogenesis in the livers of rats fed MCT resembles that observed in the control animals.

TABLE 5

Lipogenesis by liver slices of rats fed 20% of various fats for 7 days (4 rats per group)

Diet	% of radioactivity recovered			
	Cholesterol		Fatty acid	
	Acetate	Mevalonate	Acetate	Mevalonate
Experiment 1				
MCT [1]	0.20 ± 0.02 [2,3]	0.50 ± 0.03	—	—
Coconut oil	0.09 ± 0.01	0.48 ± 0.02	—	—
Corn oil	0.15 ± 0.03	0.43 ± 0.03	—	—
Control	0.25 ± 0.03 [4]	0.64 ± 0.04 [5]	—	—
Experiment 2				
MCT	1.80 ± 0.12	2.78 ± 0.07	3.39 ± 0.34 [6]	0.032 ± 0.007
Coconut oil	0.78 ± 0.26 [7]	2.19 ± 0.16	2.27 ± 0.22	0.020 ± 0.004
Corn oil	1.61 ± 0.12	2.21 ± 0.08	1.58 ± 0.14	0.019 ± 0.005
Control	1.68 ± 0.06	2.66 ± 0.11	2.74 ± 0.21	0.021 ± 0.006

[1] MCT indicates medium-chain triglyceride.
[2] SE.
[3] MCT vs. coconut oil, $P < 0.01$.
[4] Control vs. coconut oil, $P < 0.01$; vs. corn oil, $P < 0.05$.
[5] Control vs. MCT, coconut oil, corn oil, $P < 0.05$.
[6] MCT vs. coconut oil, $P < 0.05$; MCT vs. corn oil, $P < 0.01$.
[7] Coconut oil vs. MCT, corn oil, control, $P < 0.05$.

TABLE 6
Effects of dietary fat on hepatic cholesterogenesis in rats

Rat strain	Type of experiment	Precursor	Results	Reference
USC	slice	acetate	cottonseed oil > coconut oil	(14)
	homogenate	acetate	corn oil > coconut oil	(15)
	in vivo	acetate	corn oil > coconut oil	(15)
Long Evans	slice	acetate	lard > corn oil	(16)
Osborne-Mendel	in vivo	acetate	corn oil > cocconut oil	(17)
Long Evans	slice	acetate	corn oil > lard	(18)
	slice	mevalonate	corn oil = lard	(18)
Holtzman	in vivo	acetate	lard = safflower oil	(13)

The only experiments comparable to ours in which short chain triglycerides have been fed are those reported by Reiser et al. (13). They observed that in vivo cholesterol biosynthesis from acetate was highest in rats fed tripalmitin, but was higher in rats fed tricaprylin (C_8) or tricaprin (C_{10}) than in rats fed trilinolein or safflower oil. Cholesterol synthesis in livers of control rats was greater than that in any group except the group fed tripalmitin. These results are in general agreement with the data shown in table 5. Fatty acid biosynthesis as reported by Reiser et al. (13) was greatest (over 5% recovery of administered ^{14}C) in rats fed C_4, C_6 or C_8 triglycerides, ranged between 1.4 and 2.7% recovery in rats fed C_{10}, C_{12}, C_{14} or C_{16} triglycerides and then decreased markedly as fats containing fatty acids of chain length beyond C_{16} were administered. The recovery of radioactivity in fatty acids of rats fed lard was 0.54% and in those fed safflower oil it was 0.26%. These data are also comparable to our observations.

The cholesterol content of the livers of the MCT-fed rats was considerably lower than that of the other 2 experimental groups and this may partially explain the observed enhancement in cholesterol biosynthesis. The great increase in fatty acid biosynthesis in the livers of the rats fed MCT is noteworthy. It could be argued that the generally lower liver lipid observed in these animals (as compared with those fed coconut or corn oils) renders them essentially normal in this respect and thus permits fat biosynthesis to proceed at a normal rate. Another possibility is that the presence of pre-formed C_8 and C_{10} fatty acids presents a ready substrate for activation and chain elongation. If this is true, the labeling pattern in the fatty acids isolated from livers of rats fed MCT and that were administered acetate-^{14}C should be different from the pattern of labeling in the liver fatty acids of the other groups. This possibility is under investigation.

The work which we have described covers only one aspect of the influence of MCT on cholesterol metabolism. The effects of MCT upon serum and liver cholesterol levels may also depend upon alterations in rates of cholesterol degradation and excretion. Elucidation of the influence of MCT upon these parameters of cholesterol metabolism is required for a complete understanding of its action.

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Chemical Pathology of Acute Amino Acid Deficiencies

VIII. INFLUENCE OF AMINO ACID INTAKE ON THE MORPHOLOGIC AND BIOCHEMICAL CHANGES IN YOUNG RATS FORCE-FED A THREONINE-DEVOID DIET [1]

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ABSTRACT Young rats of the Sprague-Dawley strain were force-fed for 3 days purified diets devoid of threonine and containing low (9%), normal (16%) or high (28%) levels of amino acids. With feeding the experimental diets, the ratio of carbohydrate (dextrin) to amino acids became 8:1 in the low, 4:1 in the normal, and 2:1 in the high amino acid diets. All animals developed fatty liver with a periportal distribution, increased hepatic glycogen, and atrophy of the pancreas, submaxillary gland, stomach, spleen, and thymus. The results indicate that the level of amino acid intake did not alter appreciably the pathologic changes caused by the threonine deficiency.

This investigation is the eighth of a series concerned with the study of the morphologic and biochemical tissue changes in young rats force-fed purified diets devoid of single essential amino acids. In some of the earlier studies we investigated the importance of the quantity of total diet consumed (1–3) as well as the dietary content of carbohydrate (4) and fat (5) in influencing the induction and severity of pathologic changes.

The present study was undertaken to determine whether alterations in the amino acid content of a purified diet devoid of threonine would influence the pathologic changes due to threonine deficiency. In an earlier study (6), we observed that rats force-fed for 3 days an amino acid-free diet showed no pathologic changes in the liver, whereas the rats fed the regular 16% amino acid diet devoid of threonine developed an increase in liver lipid with a periportal distribution and an increase in liver glycogen. It, therefore, seemed worthwhile to determine whether increased (28%) or decreased (9%) amounts of amino acids in the purified diet devoid of threonine would influence the pathologic changes. In addition, by changing the quantity of amino acids in the diets of these experiments, the ratio of carbohydrate to amino acids in the purified diets became altered. Since a number of reports (7–9) have stressed the importance of the ratio of carbohydrate (calories) to protein in nutritional deficiencies, especially those relating to kwashiorkor, the possible significance of such alterations in experimental studies was tested in our present investigation. These results are presented and discussed in this paper.

METHODS

Male and female rats of the Sprague-Dawley strain, one month old, and weighing, on the average, 73 g were used. The animals were maintained with a commercial chow [2] for 4 days before the experiments were begun. In all experiments several groups of rats, each of the same sex, age and weight were used.

The basal regular calorie diet was based upon that used by Forbes and Vaughan (10) and was similar to that used in our own earlier experiments (1, 2, 4). The percentage composition was as follows: essential amino acids, 9.2; non-essential amino acids or L-glutamic acid, 8.1; salt mixture (11), 4; vitamin-sucrose mixture

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[2] Wayne Lab-Blox, Allied Mills, Inc., Chicago.

(1), 5; corn oil,[3] 5; cod liver oil, 1.5; and dextrin, 67.2. L-Glutamic acid was substituted for the non-essential amino acids in some experiments without any change in the results.

Animals were divided into 3 major groups (table 1) according to the amino acid content in the diet: 1) low (9%) amino acid; 2) normal (16%) amino acid; and 3) high (28%) amino acid. By force-feeding the animals, the daily intake of amino acids was altered by decreasing or increasing the quantity of amino acids while the daily intake of the other dietary components was kept constant. The animals of the low amino acid group received one-half and those of the high amino acid group, 2 times the amount of amino acids received by the animals of the normal amino acid group (table 1). Thereby the ratio of carbohydrate (dextrin) to amino acids became as follows: low amino acid group, 8:1; normal amino acid group, 4:1; and high amino acid group, 2:1. These changes in amino acid content also altered the total quantity of daily diet and calorie intake so that the rats of the low amino acid group received 6.4 g and 26.7 kcal/day; rats of the normal amino acid group, 7.0 g and 29.4 kcal/day; rats of the high amino acid group, 8.2 g and 34 kcal/day. In several experiments, 2 additional groups, isocaloric low amino acid and isocaloric high amino acid, were studied. The diets for these 2 groups were made isocaloric (29.4 kcal/day) to the diet of the normal amino acid group by changing slightly both the dextrin and amino acid composition of the diet, yet preserving the same ratio of dextrin to amino acid as in the low and high amino acid groups, without changing the other dietary components (table 1). Animals fed the threonine-devoid diet received the complete diet devoid of threonine with dextrin substituted for the missing amino acid.

Rats were force-fed according to the method of Shay and Gruenstein (12) using plastic tubes. All rats were force-fed the normal amino acid complete diet for one day before beginning the experimental diets. The rations were blended with distilled water so that each milliliter of diet mixture contained from 0.46 to 0.59 g diet, depending on the type of diet. The rations were fed 3 times daily, at 8:30 A.M., and 1:00 P.M., and 5:30 P.M. for 3 days. The animals received, depending on the type of diet, an average daily feeding of from 0.9 to 1.1 g ration/10 g initial body weight. All rats had free access to water.

Rats were housed in individual wire cages with raised bottoms and kept in an air conditioned room maintained at 25.5°. Rats were weighed at the beginning and end of each experiment. On the morning of the fourth day the rats were anesthetized with ether and exsanguinated, approximately 16 hours after the last feeding. In 3 experiments, each animal received an intraperitoneal injection of an aqueous solution of radioactive amino acid, uniformly labeled with C^{14}, three hours before killing. The organs were weighed fresh. In paired organs, the right organ was weighed. For histologic study, pieces of tissue from certain organs were fixed in Zenker-formal solution and in 10% formalin. Paraffin sections were routinely stained with hematoxylin and eosin. Frozen sections of liver after formalin fixation were stained with oil red O. The methods used for chemical analyses have been described in detail in earlier studies (2–6). In the 3 experiments where amino acid incorporation into organ protein was studied, rats were force-fed the control or experimental diets for 3 days and then 3 hours before killing, each animal received an intraperitoneal injection of an aqueous solution of radioactive amino acid, uniformly labeled with C^{14}. The following dosages of radioactive amino acid were administered: in one experiment, 0.03 mg (1.6 μc) of L-valine, in another experiment, 0.005 mg (1.7 μc) of L-leucine, and in a third experiment, 0.02 mg (5.0 μc) of L-leucine. Aliquots of liver, pancreas, and gastrocnemius muscle homogenates were precipitated with 10% trichloroacetic acid (TCA) and were subsequently washed 4 times with 5% TCA and once each with 95% ethanol, ethanol-ethyl ether mixture (3:1), and ethyl ether. Ten milligrams of the corresponding nonradioactive amino acid was added to the third TCA wash in order to dilute out any radioactive amino acid that might have remained adherent to

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

TABLE 1

Diet intake, body weight changes, and organ weights of rats force-fed complete or threonine-devoid diets containing different amounts of amino acids

Group	Daily diet intake			No. rats	Changes in body wt [1]	Liver	Gastrocnemius muscle	Spleen
	Calories	Dextrin	Amino acids					
	kcal	*g*	*g*		*g*	*g*	*mg*	*mg*
Low (9%) amino acid								
Complete diet	26.7	5.05	0.61	14	+3.5 ± 0.9 [2]	2.53 ± 0.05 [2]	384 ± 14 [2]	244 ± 18 [2]
Threonine-devoid diet	26.7	5.09	0.57	14	+1.2 ± 0.8	3.00 ± 0.08 [3]	366 ± 7	188 ± 16 [4]
Normal (16%) amino acid								
Complete diet	29.4	5.05	1.21	13	+6.4 ± 0.6	2.69 ± 0.12	393 ± 15	256 ± 18
Threonine-devoid diet	29.4	5.12	1.14	14	−0.1 ± 0.4 [3]	3.38 ± 0.10 [3]	346 ± 11 [4]	181 ± 16 [3]
High (28%) amino-acid								
Complete diet	34.0	5.05	2.32	12	+9.1 ± 0.9	2.98 ± 0.07	460 ± 10	313 ± 21
Threonine-devoid diet	34.0	5.19	2.38	8	+0.2 ± 1.8 [3]	4.07 ± 0.22 [3]	383 ± 27 [4]	183 ± 38 [3]
Isocaloric low amino acid								
Complete diet	29.4	5.62	0.70	9	+3.8 ± 0.5	2.90 ± 0.15	433 ± 25	285 ± 29
Threonine-devoid diet	29.4	5.66	0.66	9	+0.9 ± 0.8 [3]	3.32 ± 0.18	386 ± 21	198 ± 15 [4]
Isocaloric high amino acid								
Complete diet	29.4	4.28	2.08	6	+8.5 ± 0.5	3.04 ± 0.10	474 ± 17	333 ± 20
Threonine-devoid diet	29.4	4.40	1.96	10	+1.9 ± 0.6 [3]	3.67 ± 0.15 [3]	382 ± 9 [3]	166 ± 11 [3]

[1] Initial body weights ranged from 68.1 g to 77.1 g.

[2] Mean ± SE.

[3] $P < 0.01$ (highly significant).

[4] P between 0.01 and 0.05 (probably signficant).

but not incorporated into the tissue protein. The dried protein powder was plated on Whatman no. 540 filter paper and counted in a windowless flow counter. The protein in the counted sample was weighed. The counts were corrected by a self-absorption curve. Acid-soluble radioactivity (the total radioactivity in the first 2 TCA washes) was determined by plating aliquots of the washes on stainless steel planchets after concentrating the solution to a small volume.

RESULTS

In table 1 are summarized the changes in the weights of the whole body and the weights of the liver, the right gastrocnemius muscle and the spleen in rats of the low, normal and high amino acid groups force-fed for 3 days. Rats fed the high amino acid complete diet gained more weight than those fed the normal amino acid complete diet which in turn gained more than those fed the low amino acid complete diet. In all groups the rats fed the complete diets gained more weight than those fed the threonine-devoid diets. The weights of the pancreas, the kidney and the adrenal gland of the animals were not significantly different in the control and experimental groups. The average weight ranges of these organs were as follows: pancreas, 372 to 445 mg; kidney, 349 to 469 mg; and adrenal gland, 15.6 to 18.8 mg.

The mean liver weight of rats of all 3 groups fed the threonine-devoid diet was significantly greater than that of animals fed the complete diets. The gastrocnemius muscle of rats fed the normal and high amino acid threonine-devoid diets weighed significantly less than did the muscle of rats fed the comparable complete diets. The spleen of animals of all 3 groups weighed significantly less in those fed the threonine-devoid diets than in those fed the complete diets.

Biochemical changes

Liver lipid, glycogen, and protein content of animals in the low, normal, and high amino acid groups are summarized in table 2. In all groups, liver lipid and glycogen were significantly increased in animals fed the threonine-devoid diets over that in animals fed the complete diets. Liver protein increased slightly, whereas gastrocnemius muscle protein decreased somewhat in rats fed the threonine-devoid diet in comparison with those fed the complete diet of all groups. Pancreatic amylase activity expressed as Smith and Roe units (13) was relatively low in animals fed the control or experimental diets of the low amino acid group. In the normal and high amino acid groups pancreatic amylase activity was significantly lower in the rats fed the experimental diets than in those fed the control diets. Pancreatic trypsin activity expressed as Schwert and Takenaka units (14) was similar in control and experimental animals of all groups.

In table 3 are presented the results of radioactive incorporation of amino acid into protein of liver, pancreas and gastrocnemius muscle of rats force-fed for 3 days the complete and threonine-devoid diets in three experiments. The results are expressed as radioactivity per total organ rather than as specific activity (radioactivity per unit weight of protein) in order to correct for any difference in size of the non-radioactive-protein pool diluting the radioactive proteins. Also, the total nonprotein acid-soluble radioactivity was determined at the time of killing in these experiments. Since this value gives a rough index of the pool size of the labeled precursor amino acid which influences the extent of incorporation into protein, it was used to obtain a ratio (total radioactivity in organ protein/total radioactivity in acid-soluble fraction of total organ) which gives a relative expression of the extent of incorporation of isotope into organ protein.

Hepatic protein synthesis, as measured by incorporation of radioactive amino acid into liver protein, increased in all but one instance in rats force-fed the threonine-devoid diets in comparison with those force-fed the complete diets. This increase, when using the ratio values, consisted of an average of 21% in the low amino acid group; 63% in the isocaloric low amino acid group; 51% in the normal amino acid group; and 34% in the isocaloric high amino acid group.

The results of pancreatic protein synthesis, as determined by incorporation of ra-

TABLE 2

Analyses of liver, gastrocnemius muscle, and pancreas of rats force-fed complete or threonine-devoid diets containing different amounts of amino acids

Group	No. rats	Liver			Gastrocnemius muscle protein	Pancreas		
		Total lipid	Glycogen	Protein		Protein	Amylase	Trypsin
		mg/liver	*mg/liver*	*mg/liver*	*mg/muscle*	*mg/pancreas*	*units × 10⁻³/pancreas*	*units × 10⁻³/pancreas*
Low (9%) amino acid								
Complete diet	14	113 ± 6 [1]	31 ± 7 [1]	558 ± 29 [1]	83.4 ± 6.0 [1]	54.4 ± 3.4 [1]	8.5 ± 0.9 [1]	12.7 ± 1.4 [1]
Threonine-devoid diet	14	142 ± 12 [2]	92 ± 14 [3]	563 ± 27	80.8 ± 3.0	59.0 ± 3.6	7.2 ± 1.3	14.0 ± 1.7
Normal (16%) amino acid								
Complete diet	13	137 ± 6	25 ± 7	588 ± 26	95.5 ± 6.0	61.0 ± 4.4	11.0 ± 1.4	14.6 ± 1.9
Threonine-devoid diet	14	189 ± 16 [3]	115 ± 13 [3]	715 ± 88	85.0 ± 5.0	66.5 ± 4.3	6.3 ± 0.7 [3]	14.2 ± 1.8
High (28%) amino acid								
Complete diet	12	143 ± 4	18 ± 6	619 ± 42	103.1 ± 9.0	63.3 ± 3.6	14.7 ± 2.3	16.2 ± 2.2
Threonine-devoid diet	8	217 ± 17 [3]	164 ± 41 [3]	763 ± 78	94.8 ± 8.4	51.2 ± 5.2	5.1 ± 1.0 [3]	11.5 ± 2.1
Isocaloric low amino acid								
Regular calorie, complete diet	9	148 ± 8	56 ± 15	622 ± 35	103.1 ± 5.0	45.9 ± 3.6	4.8 ± 0.6	10.5 ± 1.5
Regular calorie, threonine-devoid diet	9	173 ± 14	92 ± 25	683 ± 40	90.3 ± 3.6	56.8 ± 5.7	6.0 ± 1.3	18.1 ± 2.7 [2]
Isocaloric high amino acid								
Regular calorie, complete diet	6	139 ± 4	61 ± 17	699 ± 36	116.0 ± 2.0	62.6 ± 2.3	12.4 ± 1.4	15.5 ± 2.5
Regular calorie, threonine-devoid diet	10	204 ± 23 [2]	147 ± 24 [2]	710 ± 142	89.5 ± 4.9 [3]	65.1 ± 4.9	8.3 ± 1.3 [2]	18.8 ± 1.3

[1] Mean ± SE.
[2] P between 0.05 and 0.01 (probably significant).
[3] $P < 0.01$ (highly significant).

TABLE 3

Incorporation of radioactive amino acids into protein of liver, pancreas and gastrocnemius muscle of rats force-fed complete or threonine-devoid diets containing different amounts of amino acids

Radioactive amino acid administered	Group	No. rats	Liver protein		Pancreatic protein		Muscle protein	
			(A) Total radioactivity [1]	(A) Relative to acid-soluble radioactivity [2]	(B) Total radioactivity [1]	(B) Relative to acid-soluble radioactivity [2]	(C) Total radioactivity [1]	(C) Relative to acid-soluble radioactivity [2]
			count/min × 10^{-3}		count/min × 10^{-3}		count/min	
L-Valine [3]	Low amino acid							
	Complete diet	3	30.4 ± 2.6 [4]	41.5 ± 6.2				
	Threonine-devoid diet	3	41.4 ± 5.6	58.5 ± 9.8				
	Normal amino acid							
	Complete diet	2	32.0 ± 8.3	38.7 ± 5.7				
	Threonine-devoid diet	2	58.1 ± 9.5	42.1 ± 9.4				
L-Leucine [5]	Low amino acid							
	Complete diet	3	29.8 ± 1.6	56.2 ± 2.0	6.4 ± 0.7	24.0 ± 7.0	612 ± 144	16.3 ± 2.4
	Threonine-devoid diet	3	37.5 ± 3.7	55.6 ± 3.7	11.7 ± 3.9	32.1 ± 7.9	397 ± 52	8.9 ± 1.2 [6]
	Normal amino acid							
	Complete diet	3	28.3 ± 2.6	46.4 ± 9.0	6.2 ± 1.1	24.1 ± 5.1	587 ± 65	10.2 ± 1.2
	Threonine-devoid diet	3	31.7 ± 10.6	60.3 ± 15.2	4.0 ± 0.7	18.4 ± 5.7	174 ± 33 [7]	5.5 ± 0.6 [6]
L-Leucine [8]	Isocaloric low amino acid							
	Complete diet	2	151.0 ± 12.0	60.7 ± 14.2	11.4 ± 2.2	24.5 ± 2.7	2988 ± 139	18.7 ± 2.2
	Threonine-devoid diet	2	220.1 ± 13.7 [6]	99.1 ± 18.5	17.6 ± 2.2	30.4 ± 6.9	1935 ± 196 [6]	18.7 ± 0.3
	Normal amino acid							
	Complete diet	2	91.3 ± 3.7	67.5 ± 7.2	17.9 ± 4.2	46.8 ± 0.1	5249 ± 751	37.0 ± 2.3
	Threonine-devoid diet	3	261.4 ± 75.4	145.2 ± 52.1	31.8 ± 7.3	60.4 ± 19.7	1513 ± 219 [7]	9.8 ± 1.2 [7]
	Isocaloric high amino acid							
	Complete diet	2	130.7 ± 4.5	96.1 ± 42.8	25.4 ± 1.4	55.1 ± 7.9	3996 ± 478	19.4 ± 4.4
	Threonine-devoid diet	3	259.1 ± 70.1	128.6 ± 32.6	23.1 ± 3.2	44.2 ± 7.0	1247 ± 38 [7]	14.5 ± 3.0

[1] Radioactivity in total organ protein.

[2] Total radioactivity relative to acid-soluble radioactivity:

$$\frac{\text{(Total radioactivity in organ protein)}}{\text{(Radioactivity in acid-soluble fraction of total organ)}}$$

[3] 1.6 μc (0.03 mg) C^{14}-L-valine.

[4] Mean value ± SE of the mean.

[5] 1.7 μc (0.005 mg) C^{14}-L-leucine.

[6] P between 0.05 and 0.01 (probably significant).

[7] $P < 0.01$ (highly significant).

[8] 5 μc (0.02 mg) C^{14}-L-leucine.

dioactive amino acid into pancreatic protein, were variable. In 2 groups there were slight decreases, and in 3 groups there were slight increases in experimental animals when compared with those in control animals.

Muscle protein synthesis, as determined by incorporation of radioactive amino acid into gastrocnemius muscle protein, revealed large decreases in the experimental animals compared with the controls. The results are in obvious contrast to most of the incorporation results in the liver of the same animals.

Morphologic changes

Normal amino acid. The observations in the animals fed the complete and threonine-devoid diets were identical to those described in earlier reports (1,4–6). In brief, while no pathologic changes were observed in the aniamls fed the complete diet, the animals fed the threonine-devoid diet developed periportal fatty liver, excess hepatic glycogen, and atrophy of the pancreas, submaxillary gland, stomach, thymus and spleen.

Low amino acid. The livers of the animals fed the threonine-devoid diet had a mild-to-moderate degree of fatty change with a periportal distribution, whereas the livers of the animals fed the complete diet were normal in most cases, but a few animals had a minimal degree of periportal fatty change. The pancreas and submaxillary gland of most of the experimental animals showed moderate atrophy of the glandular cells, whereas these organs of the control animals showed mild glandular atrophy in only a few animals. The thymus, spleen, and stomach showed moderate atrophic changes in the experimental animals but these organs were normal in the control animals.

High amino acid. Animals fed the threonine-devoid diet showed the following morphologic changes: moderate-to-marked fatty liver with a periportal distribution and atrophy of the pancreas, submaxillary, stomach, thymus, and spleen. The same organs were all normal in the animals fed the complete diet.

In summary, the morphologic changes of the different organs were very similar in the animals fed the threonine-devoid diets of the low, normal and high amino acid groups. One minor difference was that the livers of the animals force-fed the high amino acid threonine-devoid diet showed a somewhat more marked degree of fatty change than that observed in the livers of animals of the other 2 experimental groups.

Isocaloric low and high amino acid groups

In several experiments, groups of animals were fed low or high amino acid complete diets and threonine-devoid diets which were made isocaloric to the normal amino acid diets by minor changes in the dextrin and amino acid composition of the diets but without changing the other dietary components (table 1). However, the ratios of dextrin to amino acids in the diets of the 2 isocaloric groups were maintained the same as those in the diet of the corresponding low and high amino acid groups. The results of these 2 additional groups are summarized in tables 1, 2, and 3. The results are comparable to those observed with the isocaloric normal amino acid group. The only differences were in pancreatic enzymes where the animals fed the isocaloric low amino acid diet had lower amylase levels in both control and experimental groups and had increased trypsin levels in the experimental groups as compared with the controls. The morphologic changes in the isocarloric low and high amino acid control and experimental animals were similar to those described earlier for the low and high amino acid control and experimental animals.

DISCUSSION

The results of this study indicate that young rats force-fed for 3 days a threonine-devoid diet containing 9, 16, or 28% amino acids develop fatty liver with a periportal distribution, increased hepatic glycogen, and atrophy of the pancreas, submaxillary, stomach, spleen and thymus. Neither a decrease in amino acid content of diet from 16 to 9% nor an increase in amino acid content of diet from 16 to 28% appreciably alters or protects against the pathologic changes resulting from the deficient diet. On chemical analysis, the liver had increased total lipid, glycogen, protein and

amino acid incorporation into protein, the gastrocnemius muscle had decreased protein content and decreased amino acid incorporation into protein (except in the isocaloric low amino acid group), and the pancreas had decreased amylase content (except in the low amino acid groups) in the experimental animals in comparison with control animals regardless of level of dietary amino acids.

Animals force-fed for 3 days the low (9%) amino acid complete diet developed few, if any, pathologic changes. In contrast, other studies (15–19) in which animals were fed ad libitum for weeks or months protein-deficient diets, composed of natural foodstuffs or purified diets low in protein, have shown many pathologic changes due to protein deficiency. The difference between our results and those of others can probably be attributed to 2 factors: 1) the shorter duration of our experiments, and 2) the use of a low but balanced amino acid mixture in our diet. The importance of the second factor can be illustrated by 2 examples. Rats force-fed for 3 days protein-deficient (6–9% protein) diets composed of poor-quality plant proteins develop pathologic changes similar to those observed in the experimental animals of the present study (20), whereas rats force-fed diets containing 8% high quality protein (lactalbumin or casein) under similar conditions failed to develop any lesions.[4] In addition, Platt et al. (21) reported pathologic changes in rats force-fed for 6 days diets containing 2% and 5% casein but not with diets containing 10% or 15% casein. Thus, although pathologic changes attributable to protein deficiency develop in rats force-fed for 3 days diets devoid in an essential amino acid or containing poor-quality proteins, such changes do not develop in similar short-term experiments in which animals are force-fed a low, yet balanced, amino acid or protein diet.

The observation of enhanced liver protein synthesis along with decreased skeletal muscle protein synthesis in rats force-fed the threonine-devoid diets containing different quantities of amino acids is similar to that noted earlier with threonine (2, 22) or with other single essential amino acid deficiencies (23). Thus, the level of dietary amino acids in the deficient diets does not appear to influence the indicated effects on liver and muscle protein synthesis. Also, gastrocnemius muscle protein content of animals fed the low amino acid complete diet was lower than in any of the other control groups, including the isocaloric low amino acid group (table 2). This is probably due to the lower caloric intake of animals in the low amino acid group and is consistent with the results described by Munro (24) which indicate that under certain conditions increased calories added to a low protein diet can improve nitrogen balance and thereby reduce the loss of body protein which is predominantly skeletal muscle protein.

Previous experience with our kwashiorkor-like experimental model in young rats has demonstrated that the quantity of diet consumed (1–3), as well as the dietary content of carbohydrate (4), is important in the induction of the pathologic changes due to single essential amino acid deficiencies. The results of the present study emphasize how little influence the ratio of carbohydrate to amino acids (8:1, 4:1, or 2:1) has upon the development of this pathologic picture and thereby strengthen the conclusion that the quantity and carbohydrate (caloric) intake of a deficient diet is far more important than the amount of carbohydrate relative to amino acids and that ratio alterations become important only when they are secondary to changes in the quantity of diet or carbohydrate consumed. 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. However, in kwashiorkor, an infant disease considered primarily to be the result of protein deficiency, the diet intake has been reported to be high in carbohydrate as well as low in protein (25–27). Also, some clinical reports (7–9) have stressed the importance of the ratio of carbohydrate to protein in the diet of these children. Further clinical studies are necessary to clarify the importance of the quantity and of the ratio of carbohydrate to protein of deficient diets in the pathogenesis of kwashiorkor.

[4] Unpublished data, H. Sidransky.

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Metabolic Patterns in Preadolescent Children

XVI. RIBOFLAVIN UTILIZATION IN RELATION TO NITROGEN INTAKE [1]

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ABSTRACT The riboflavin content of food, urine and feces was determined for 35 preadolescent girls fed controlled, adequate diets, the protein content of which, 18 to 88 g/day, constituted the experimental variable. Riboflavin in whole blood was determined on 24 subjects. Nitrogen intake and urinary riboflavin showed significant, positive correlation. About one-half the riboflavin intake was excreted in the urine. Correlation of intake and outgo of riboflavin was not significant. Mean fecal riboflavin values decreased sharply when nitrogen intake was reduced from 14.12 to 2.91 g/day. At the same time, the ratio of sucrose to lactose, in grams per day, increased from 104/31 to 195/3. Urinary creatinine and urinary riboflavin showed significant positive correlation as did riboflavin retention and energy balance. Riboflavin values in whole blood did not appear to be affected by differences in protein intake in 1956 when one-half the subjects were given about 2 g of protein and the other half about 3 g of protein/kg of body weight. In 1958 riboflavin blood values decreased from a mean of 21.3 to 11.4 μg/100 ml when protein intake was reduced to less than 1 g/kg of body weight but not so low as to prevent a retention of 0.3 g nitrogen/day.

The Southern Regional Cooperative Research Project on Nutrition includes the investigation reported here on riboflavin intake and output, blood content and interrelationship with nitrogen intake.

PROCEDURE

Description of the plan and organization of the cooperative project and details of conducting it have been published (1). Three studies have been made on the metabolism of riboflavin, using as subjects girls, 7 to 9 years old, maintained with diets in which the nutrient content was planned in accordance with the recommended allowance of the National Research Council (2) with the exception of protein, the intake of which was the experimental variable with a range of 18 to 88 g/day. Variation in riboflavin intake occurred from the choice of foods necessitated for regulation of the nitrogen content of the diet.

The relationship of riboflavin intake and nitrogen intake to riboflavin outgo was studied under the following conditions: (a) in 1954, with 11 subjects for 16 four-day periods when protein intake was within the range recommended by the National Research Council; (b) in 1956, with 12 subjects for 14 four-day periods when the protein intake provided an average of about 2 g of protein/kg of body weight for one-half the subjects and about 3 g for the other half; and (c) in 1958, when 12 subjects received about 0.8 g of protein/kg of body weight for the first 5 six-day periods and about 0.6 g for the last 3 periods.

Naturally occurring foods commonly acceptable to preadolescent children were used for all diets. The only supplementation in 1954 and 1956 was that of the usually enriched bread and cereals and vitamin D milk. For the diet highest in protein and consequently, made up of foods high in riboflavin, bread and cereals were not enriched. In 1958, the low protein diets restricted the choice of foods to an extent that required supplementation of minerals and vitamins. Bread was fortified with Ca, P, Fe, thiamine, riboflavin and niacin. Mono-calcium orthophosphate was added

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TABLE 1

One-day sample menus

	Diet no.						
	1	2	3	4	5	8	8'[1]
	g	*g*	*g*	*g*	*g*	*g*	*g*
Breakfast							
Orange juice, frozen	60		50		50	120	
Pineapple juice, frozen		120	120	120	120		
Wheat cereal,[2] enriched		20		20			
Sugar, dark brown	14	14		14			
Jelly	10	10		10			
Cream				30			
Egg			45		45		
Bread	20	20	20	20	20		
Margarine	5	5	5	5	5	20	
Milk	244	150	150	150	150		
Cornflakes	20					14	
Apple butter						20	
Bread, fortified						10	
Coffee cream, 80 g water plus 40 g cream						120	
Snack							
Flavored beverage [3]						5	
Milk			150				
Sugar, brown						14	
Sugar, white						10	
Crackers, graham		14	14	14	14	7	
Fondant						20	
Lunch							
Tomato soup, condensed		60	60	60	60		
Milk	200	200	200	200	200	120	
Tuna fish, oil pack		30	30	30	30		
Bread		40	40	40	40	40	
Lettuce	10	10	10	10	10	15	
Mayonnaise	5	10	10	10	10	25	
Applesauce, canned		100	100	100	100		
Sugar wafer			30		60		
Liver sausage	30					10	
Bun	30						
Coconut bar	20						
Custard, baked	105						
Bread, fortified						40	
Apricots, canned						100	
Flavored beverage [3]						5	
Sugar, brown						14	
Sugar, white						10	
Snack							
Flavored beverage [3]						5	
Raisins	25	25	25	25	25	15	
Lemonade, frozen	100						
Dinner							
Beef roast	30					10	
Rice, converted	50						
Gravy, drippings	20						
Broccoli, frozen	50	50					
Ice cream, vanilla	80						
Roll	25						
Margarine	5	10	10	10	10	20	
Ham, baked		25	75	50	75		
Potato, boiled		50	50	50	50	65	
Beans, lima, frozen			100	75	100		
Gingerbread		40	40	40	40		
Hard sauce				21			

TABLE 1 (*Continued*)
One-day sample menus

	Diet no.						
	1	2	3	4	5	8	8′ [1]
	g	*g*	*g*	*g*	*g*	*g*	*g*
Bread			10		10		
Milk		200	100	200	200		
Squash, summer, frozen						50	
Cake, plain, fortified						50	
Flavored beverage [3]						5	
Sugar, brown						13	
Sugar, white						10	
Snack							
Flavored beverage [3]						5	
Fondant, plain						40	
Raisins	25						
Peanut butter	15						
Jelly	10						
Bread	20						
Other							
Gelatin in flavored beverage [3]					17		
Sugar in flavored beverage [3]		85	14	100	54		
Pineapple juice					34		
Margarine		5		10			

[1] Diet 8′ was the same as diet 8 except that all milk was withdrawn. For exception see text footnote 3.
[2] Ralston, Ralston Purina Company, St. Louis.
[3] Kool Aid, General Foods Corporation, White Plains, New York.

to tapioca, cookies, cake and sherbet. Drisdol, added to breakfast cream, supplied 400 IU vitamin D per subject per day. A mineral and vitamin supplement in gelatin capsules was also included. Supplementary energy was supplied by fondant. Tap water for cooking and drinking was demineralized in a zeolite system.

Details of menus, recipes and calculated food values are given in the Southern Cooperative Series Bulletin no. 64 (1). A sample one-day menu for each diet is shown in table 1.

Food composited per period and urine and feces composited per period per subject were analyzed for riboflavin content. Separate food composites were made if a child could not eat all the diet because of some minor disturbance, such as a cold. Values for vomitus were added to fecal values. Riboflavin analyses were made on whole blood at the beginning and end of the second and third studies.

The Conner-Straub method (3) with slight modification was used. All work with riboflavin was carried out under controlled illumination. Determinations were made in triplicate on all samples, blanks and standards, all of them being run through the total process at the same time as a check on the purity of the reagents and reliability of the method. Fluorescence was measured in the DU spectrophotometer with suitable Corning glass filters in the fluorescence attachment.

RESULTS AND DISCUSSION

Riboflavin mean intake of all subjects met or exceeded the National Research Council's recommended allowance of 1.3 mg/day for children 7 to 9 years old (2). Intake may be synonymous with absorbed riboflavin in this study because apparent digestibility, reported by James (4) as relatively low in the 1958 study with a low intake of nitrogen, did not affect fecal riboflavin values. Any food residues, therefore, that might have been in the feces, were unimportant sources of riboflavin and constituted no loss. Although riboflavin synthesized by bacteria probably did not add to amounts absorbed because synthesis occurs in man in the part of the intestinal tract where absorption is not likely to occur (5, 6), there might be a possibility of absorption if synthesis should occur earlier in the tract (7), although there appears to be no evidence that riboflavin-synthesizing bacteria are present in the small intestine in man.

Urinary riboflavin shows less variation than intake. In table 2, the mean values for intake and outgo are grouped according to the 7 diets described previously (1). Since the intake is considerably above the recommended allowance of the National Research Council (2), the high urinary excretion is, perhaps, to be expected. Although some deviations from mean values were wide, all of our subjects met the criteria for adequate riboflavin intake represented by Morley et al. (8) as being 26% of the intake excreted in the urine. The percentage range in this study, 45 to 68, is high but possibly such variation is normal in children. Coryell et al. (9) pointed out that in their study of nutritional status of children large differences in fasting-hour and load test excretion occurred among children of the same age and sex, living in the same environment. Stearns et al. (10) report 0.971 mg as the mean urinary riboflavin excretion for 74 children. It is practically the same as values observed in this study for 1958 but lower than those for 1954 and 1956. Jansen and Jansen (11) state that when the body is saturated with riboflavin, between 30 and 60% of the intake is excreted. Morrison and Campbell (12) reported a urinary excretion of 58 to 63% to be fairly constant in normal men.

Riboflavin retention and energy balance data reflect the role of riboflavin in oxidation processes. Since it appears fairly well established that riboflavin in the amounts fed the subjects in these studies is completely absorbed (12) and riboflavin synthesized in the intestinal tract is probably not absorbed (5, 6), riboflavin retention is used here to denote the difference between intake and urinary riboflavin. The correlation coefficient for riboflavin retention and energy balance [2] is 0.68, significant at less than 0.1% by the *t* test (13).

Relation of riboflavin and nitrogen metabolism, whether expressed as milligrams per day or percentage of intake, is indicated by urinary riboflavin variation in the same direction as nitrogen intake, although to a less extent. Nitrogen balance data (4) when protein intake in 1958 was about a third of the recommended allowance of the National Research Council, show that the subjects stored the amount of nitrogen considered necessary for normal growth.[3] Since, at the same time, urinary riboflavin decreased, it may indicate lower metabolic demands for riboflavin with lower nitrogen turn-over. If protein intake had been deficient, an increase in urinary riboflavin might have been expected.

The balance between riboflavin released from protein-bound riboflavin of body tissues and the proportion of ingested riboflavin retained for conversion to nucleotides and phosphates, is in this study, not subject to the stress of deficiency of either nitrogen or riboflavin such as accounts for the relationships reported by Oldham et al. (14). Mean increase of body surface area of 0.004 m^2, 0.004 m^2 and 0.022 m^2 for the 64, 56 and 48-day experimental periods, respectively, of the 1954, 1956 and 1958 studies (1), are evidence of good growth in size. Growth, whether manifest in size or other less easily measured tissue development, depends on ribosomal functioning that is efficient only when a balance is maintained of the protein and RNA fractions. Data shown here appear to indicate that such a balance exists (15). The correlation coefficient for nitrogen intake and urinary riboflavin is 0.70, significant at less than 1% by the *t* test (13). The correlation coefficient for riboflavin intake and urinary riboflavin is not significant.

Creatinine and riboflavin in urine exhibit a relationship comparable to that of creatinine and nitrogen in urine. According to James (4) the ratios of nitrogen to creatinine in urine correlate closely with nitrogen intake. The same is true for the ratios of creatinine to riboflavin. The correlation coefficient for creatinine and riboflavin in urine for the total number of subjects is 0.90, significant at less than 1% by the *t* test (13). Mean values for ratios of micrograms of riboflavin to grams of creatinine excreted vary only slightly between high and low protein intake, 2006 (diet 5) to 2066 (diet 8′). This indicates liberal riboflavin intake (16) and possibly,

[2] Unpublished data used with the permission of the Louisiana Agriculture Experiment Station, Baton Rouge, Louisiana.

[3] Three exceptions occurred in 1958. Subjects 25 and 30 failed to store 0.3 g nitrogen daily in period 6 but stored it thereafter when they increased their caloric intake by 200 kcal. Subject 35 did not store 0.3 g in period 1, in 1958, but did so when nitrogen intake was increased from 3.53 to 3.88 g (4).

TABLE 2

Mean daily riboflavin intake and output, nitrogen intake, calorie balance and creatinine output

Diet no.	Year	No. of subjects	Riboflavin				Nitrogen intake [1]	Urinary creatinine [2]	Calorie balance [3]
			Intake	Urine	Urine	Feces			
			mg	*mg*	*% of intake*	*mg*	*g*	*mg*	*kcal*
1	1954	11	1.84 ± 0.15 [4]	1.25 ± 0.18	68	2.40 ± 0.41	10.45	612	1801
2	1956	3	1.99	1.06	53	2.19	7.70	459	1815
3		3	1.98	1.26	64	2.57	11.64	555	1823
4		3	2.41	1.21	50	2.23	9.39	523	2199
5		3	2.19	1.29	59	2.65	14.12	643	2242
2–5		12	2.14 ± 0.17	1.21 ± 0.16	56	2.41 ± 0.26	10.71	545	2020
8	1958	12	2.07 ± 0.00	0.98 ± 0.05	47	0.43 ± 0.06	3.53	469	2065
8'		12	2.22 ± 0.03	0.99 ± 0.09	45	0.42 ± 0.06	2.91	479	2120

[1] From James (4).
[2] From Southern Cooperative Series Bulletin no. 64 (1).
[3] Unpublished data used with the permission of Louisiana Agriculture Experiment Station.
[4] Standard deviations of observations.

since the ratios are so uniform, confirms the adequacy of the low protein diets.

Fecal riboflavin mean values grouped according to diets (table 2) are correlated with nitrogen intake to an even closer extent than urinary riboflavin. It is unlikely that fecal riboflavin is the result of its excretion as a metabolic end product into the intestinal tract. Näjjar et al. (17) observed no increase in fecal riboflavin after intravenous injection of 5 to 20 mg of riboflavin. The correlation of nitrogen intake and fecal riboflavin may indicate a close relationship reflected not only in intermediary metabolism but also in intestinal permeability to some non-nitrogenous split product of protein which may affect bacterial synthesis. The correlation coefficient is 0.96, significant at less than 1% by the *t* test (13).

Bacterial synthesis of riboflavin in man has been clearly established (17, 18). It probably accounts for most of the fecal riboflavin reported here. Activity of intestinal flora depends on dietary constituents, which may explain the dissimilarity of degree of synthesis. Fat content of the diets is so nearly uniform that it cannot account for differences in fecal riboflavin. Caloric adjustments, where necessary, were made by the addition or subtraction of carbohydrate, chiefly sucrose. The 1958 diets contained more sucrose than the 1954 or 1956 diets and less lactose (table 3). The low fecal values for riboflavin may have been the result of several factors, among them the low lactose, high sucrose and low protein intake. The effect of lactose intake on fecal riboflavin in the rat is noted by Morgan et al. (19), Mannering et al. (20) and De and Roy (21) who reported microbial synthesis of riboflavin to be highest when the carbohydrate of the diet was lactose and lowest when it was sucrose.

Blood riboflavin values range from 13.8 to 22.4 μg/100 ml of whole blood at the beginning of the 1956 study and from 14.9 to 18.7 μg/100 ml at the end of the study. Comparison of mean values (table 4) for the 4 groups given diets containing different amounts of protein, shows that neither riboflavin nor nitrogen intake at the levels of the 1956 study appear to affect the blood content of riboflavin. In

TABLE 3

Carbohydrate content of the diets and riboflavin in feces

Diet no.	1	2	3	4	5	8	8′
No. of subjects	11	3	3	3	3	12	12
Total carbohydrates,[1] g/day	242	279	228	339	300	296	296
Sucrose,[2] g/day	48	106	57	147	104	194	195
Lactose,[3] g/day	30	31	31	31	31	6.2	2.7
Fecal riboflavin, mg/day	2.40	2.19	2.57	2.23	2.65	0.43	0.42

[1] From Southern Cooperative Series Bulletin no. 64 (1).
[2] Calculated: includes amounts listed as sugar in menus and in prepared recipes. From Southern Cooperative Series Bulletin no. 64 (1).
[3] Calculated: includes lactose in fluid milk, in milk used in prepared recipes and added in capsules with vitamin-mineral supplements in 1958. From Southern Cooperative Series Bulletin no. 64 (1).

TABLE 4

Mean riboflavin content of whole blood, mean nitrogen and riboflavin intake

Diet no.	No. of subjects	Nitrogen intake [1]	Riboflavin intake	Blood riboflavin	
				Initial	Final
		g/day	*mg/day*	*μg/100 ml*	*μg/100 ml*
			1956 (56 days)		
2	3	7.70	1.99	16.1	18.4
3	3	11.64	1.98	19.1	16.4
4	3	9.39	2.41	18.1	16.5
5	3	14.12	2.19	16.5	17.3
2–5	12	10.71	2.14 ± 0.17 [2]	17.4 ± 2.5	17.1 ± 1.3
			1958 (48 days)		
8 and 8′	12	3.30	2.12 ± 0.02	21.3 ± 2.5	11.4 ± 2.1

[1] From James (4).
[2] Standard deviations of the observations.

1958, when protein intake was markedly lower than in 1956, blood riboflavin values decreased from a mean of 21.3 at the beginning of the study to 11.4 μg/100 ml at the end. Variance of the mean values for the 2 years, 4.46/1.58, is significant at the 5% level by the F test (22).

The difference between the calculated values for riboflavin content of the pre-experimental diets (1) and those determined by analysis of the experimental diets was comparatively small, 2.03 to 2.12 mg/day. The corresponding difference in nitrogen from a calculated pre-experimental intake of 11.93 to the analyzed experimental 3.30 g/day suggests that the decrease in blood riboflavin values may be functionally associated with the low nitrogen intake. The range for blood riboflavin at the beginning of the 1958 study was 16.1 to 25.7 μg/100 ml and at the end, 48 days later, 8.9 to 14.6 μg/100 ml. Morley et al. (8) report a range of 4.3 to 14.6 μg for 59 women unrestricted as to diet and a lower range, 6.6 to 10.6 μg/100 ml for 7 normal women whose protein intake was kept at 60 g/day. Präger et al. (23) observed 15.9 μg/100 ml whole blood as the mean value for 70 adults.

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Effects of Zinc Deficiency on Plasma Proteins of Young Japanese Quail [1]

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ABSTRACT One-day-old Japanese quail were fed either a low zinc diet, the same diet supplemented with zinc, or a protein-inadequate diet containing zinc. After 4 weeks small samples of blood were collected from the wing vein of each bird after 7, 24 and 48 hours of fasting. Hematocrits and concentration of total plasma protein were determined; the plasma proteins were resolved by disc electrophoresis in polyacrylamide gel. Birds fed the low zinc diet grew slowly and exhibited the characteristic symptoms of zinc deficiency, whereas those receiving zinc grew and developed normally. Birds fed the protein-inadequate diet with zinc grew slowly, but appeared normal otherwise. During fasting, all birds lost weight and the total plasma proteins decreased; these effects were greatest in the zinc-deficient birds. Hematocrits were not affected by experimental treatment. The plasma protein patterns of the zinc-deficient birds were normal after 7 hours of fasting. At 24 hours, the patterns of more than one-half of these birds deviated from normal and at 48 hours the patterns of all the zinc-deficient birds were markedly changed. The plasma protein of birds receiving dietary zinc and those fed the low protein diet were normal after 7 and 24 hours of fasting. At 48 hours, minor changes were observed in a small proportion of birds in each of these groups. The data suggest a possible role of zinc in the metabolism of one or more plasma proteins.

The young Japanese quail (Coturnix coturnix japonica) has been found to be very sensitive to the omission of dietary zinc (1). At 4 weeks of age the deficiency was characterized by slow growth, abnormal feathering, labored respiration and an incoordinate gait. In this and other species, the effects of zinc deficiency suggest a role of zinc in protein metabolism. It was decided to investigate the electrophoretic pattern of the plasma proteins of zinc-deficient quail as a step toward relating the gross effects of the deficiency to the metabolic functions of zinc.

A small decrease in total plasma proteins of zinc-deficient rats was reported (2). A decrease of total serum proteins was observed in zinc-deficient chicks at 4 weeks of age; however, there was no change in the albumin globulin ratio (3). An increase in serum globulin and a decrease in albumin of zinc-deficient swine were reported (4). Zinc-deficient lambs had slightly low serum albumin levels and markedly elevated levels of gamma globulins, which were possibly caused by infection at the site of skin lesions (5).

In preliminary experiments with the quail, the plasma protein electrophoretic pattern of zinc-deficient birds fasted overnight was different from that of birds fasted 4 to 6 hours. In the studies reported herein, the distribution of plasma proteins from zinc-deficient birds was markedly different from that of zinc-fed birds after 24 and 48 hours of fasting.

EXPERIMENTAL

One-day-old Japanese quail of both sexes were maintained as described previously (1). Some of the birds were fed a purified soybean protein diet (1) with zinc omitted from the salt mixture; other birds were fed the diet supplemented with 75 mg of zinc/kg of diet supplied by zinc carbonate. A protein-inadequate diet was also fed. It had the same composition as the zinc-supplemented diet except that the proportion of soybean protein was reduced from 35 to 25%, glycine from 1 to 0.5% and DL-methionine from 0.6 to 0.4%.

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[1] Part of these data was presented at the meeting of the American Institute of Nutrition in Chicago, Illinois, April, 1964.

These decreases were counterbalanced by additions of glucose so that the concentrations of all other dietary constituents remained the same. All diets contained 0.02% of 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline. All birds had continuous access to food and demineralized water until 28 days of age, at which time food was removed for 48 hours.

Blood was collected from the wing vein in heparinized microhematocrit tubes after 7, 24 and 48 hours of fasting.[2] The hematocrits were read and the plasma was stored in small polyethylene tubes at −15°. Total protein of the plasma was determined by the method of Lowry et al. (6). Disc electrophoretic separation of the proteins in 3 μliters of plasma was carried out in a 7.5% polyacrylamide gel[3] by the procedure of Ornstein and Davis.[4] Each tube containing the gel had an inside diameter of 5.8 mm. A constant current of 2.5 milliamperes per tube was applied until the Bromphenol blue tracking dye had migrated about 3.5 cm in the lower gel. Proteins in the gel were stained with Amido black 10B.

RESULTS

Data on body weight, hematocrit and plasma proteins are presented in table 1. The zinc-deficient birds grew slowly and had other signs of deficiency reported previously (1). They were the only group with significant mortality. The birds fed the protein-inadequate diet grew much more slowly than the zinc-fed birds receiving adequate protein; otherwise they appeared normal.

Fasting for a period of 7 hours produced little weight change in any group, but after 48 hours all groups had lost considerable body weight. The zinc-deficient birds lost 33%, and the zinc-fed birds lost 22% of their weights at the beginning of fasting; the difference is statistically significant at $P < 0.01$. Birds fed the inadequate protein diet lost 24% of their initial body weight.

[2] The conclusions drawn from the data in this paper were also supported by check analyses of plasma in blood obtained by heart puncture.

[3] Reagents for electrophoresis were purchased from Canal Industrial Corporation, Bethesda, Maryland.

[4] Ornstein, L., and B. J. Davis. Disc electrophoresis. Part II. Materials and Methods. Preprinted by Distillation Products Industries, Rochester, New York.

TABLE 1

Effect of zinc deficiency upon body weights, hematocrits and plasma proteins of 4-week-old quail fasted for 48 hours

Length of fast	Body wt	Hematocrit	Total plasma proteins	Decrease [1] in protein band 7	Absence [2] of protein band 7
hr	*g*	*%*	*g/100 ml*	*%*	*%*
No added dietary zinc (14/23) [3]					
0	43 ± 1.5 [4]	—	—	—	—
7	41 ± 1.8	42 ± 1.4 [4]	3.72 ± 0.120 [4]	0	0
24	36 ± 1.6	42 ± 0.9	2.61 ± 0.212	61	23
48	29 ± 1.3	39 ± 1.5	1.68 ± 0.337	100	73
75 mg zinc/kg of diet (17/21)					
0	73 ± 2.2	—	—	—	—
7	72 ± 2.0	38 ± 0.5	3.01 ± 0.100	0	0
24	67 ± 1.9	37 ± 0.5	2.61 ± 0.072	0	0
48	57 ± 2.3	38 ± 0.8	2.39 ± 0.189	29	0
Inadequate protein diet (5/6)					
0	53 ± 4.1	—	—	—	—
7	50 ± 4.1	39 ± 1.2	2.91 ± 0.96	0	0
24	46 ± 3.7	34 ± 2.4	2.66 ± 0.13	0	0
48	40 ± 3.7	35 ± 3.0	2.39 ± 0.48	20	0

[1] Percentage of birds with a decrease in protein band 7.

[2] Percentage of birds with no protein band 7.

[3] Number of birds surviving 7 to 28 days/number of birds at 7 days. Two zinc-deficient birds died between 24 and 48 hours of fasting. Data on zinc-deficient and zinc-supplemented birds are the average of 3 experiments; in one experiment blood was not collected at 7 hours. Data with inadequate protein diet are from 1 experiment.

The mean hematocrit value of the zinc-deficient birds at 7 hours was slightly higher than that of either of the other 2 groups; however, this difference was not statistically significant. The hematocrit value did not change during fasting.

After 7 hours of fasting, the mean plasma protein value for the zinc-deficient birds was significantly higher ($P < 0.01$) than that for the birds receiving zinc. The value for birds fed the inadequate protein diet was similar to that for the control birds fed zinc. All birds had appreciably lower concentrations of plasma proteins after 48 hours of fasting. The decrease in the zinc-deficient group was significantly greater than that of the birds fed zinc ($P < 0.01$).

The normal plasma protein pattern obtained by disc electrophoresis is illustrated by the pattern obtained with the zinc-fed birds after 7 hours of fasting (fig. 1). Nine bands were usually observed in the patterns obtained from normal birds. Until the protein bands are further characterized, they have been arbitrarily identified by number. Two bands were sometimes observed in each of the regions designated as bands 2, 3, 4, 5, 7 and 9. Band 8 was almost certainly albumin. It was present in the largest amount and migrated ahead of all other major components. It was not precipitated by 26.8% sodium sulfite, used either alone or with ether.

Typical effects of fasting upon the plasma protein patterns are shown in figure 1. At 7 hours, the pattern of the zinc-deficient birds was normal. At 24 hours, band 7 was markedly decreased and sometimes, as in this case, it migrated closer to band 8.[5] At 48 hours most of the bands appeared weaker and no protein-staining material was observed at the usual location of band 7. Neither the addition of zinc to the plasma nor dialysis against the electrophoresis buffer affected the pattern. The typical plasma protein pattern of zinc-supplemented birds was not markedly altered during fasting. The patterns of the birds fed the inadequate protein diet were similar to those of the zinc-supplemented birds.

The proportion of the birds showing these changes are shown in table 1. At 24 hours, only the zinc-deficient birds showed any decrease in band 7. In the pattern of a fourth of these birds, band 7 was no longer visible. At 48 hours, all of the zinc-deficient birds had a marked decrease in band 7 and in 73% band 7 was not present. A small proportion of zinc-supplemented and protein-inadequate birds had a moderate decrease in band 7.

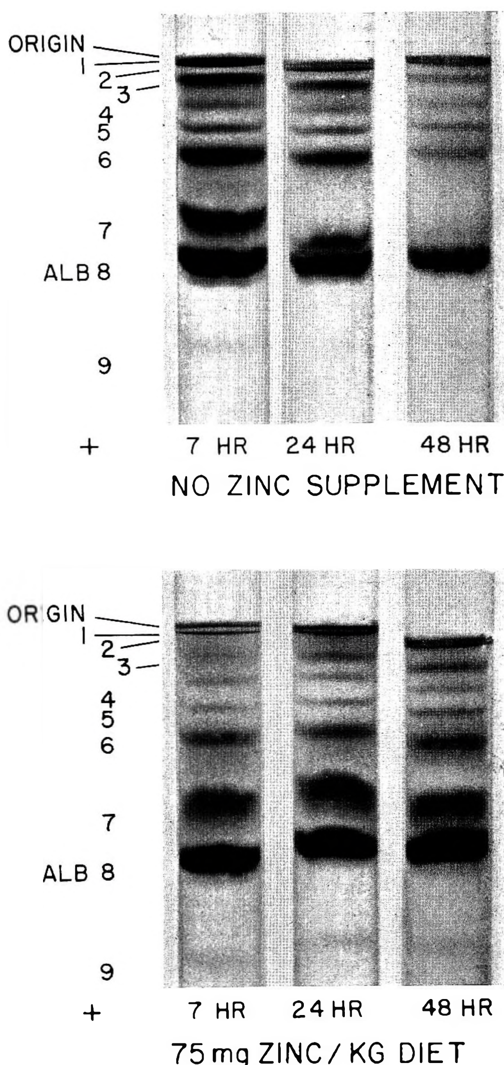

Fig. 1 Effects of zinc deficiency on the plasma protein patterns of quail after fasting for 7, 24 and 48 hours.

[5] Under standardized conditions, each protein band migrates at a characteristic rate in relation to the tracking dye. This relative rate for albumin was usually about 0.73 to 0.77 and for band 7 about 0.62 to 0.65. This relationship provides a useful parameter for band identification.

DISCUSSION

The changes in the plasma protein pattern that appeared during fasting of zinc-deficient birds were quite marked. Whether these reflect a specific effect of zinc in the breakdown of protein metabolism has not been established. Some support for specificity of zinc action is derived from the fact that the response of birds fed the inadequate protein diet was similar to that of the normal control birds fed zinc. However, the growth of the protein-inadequate birds was not quite as suppressed as that of the zinc-deficient birds. It is possible that the changes observed in the zinc-deficient birds might also have occurred in the zinc-fed birds had fasting been continued longer. Hemodilution can be excluded as a factor that influenced the results obtained during fasting because the hematocrit values remained unchanged.

It is possible that band 7 contained more than one protein moiety. Preliminary extraction and fractionation studies on the quail plasma indicate that band 7 may be composed of a lipoprotein fraction and possibly a post-albumin fraction. Recently Kaminski and Gajos (7) described a lipoprotein band in duck serum that migrated closely behind albumin during starch gel electrophoresis. The possibility must be considered that fasting may cause changes in the mobility of some protein fractions.

In general, the serum albumin and α_1-globulins have been observed to decrease most rapidly under sudden demands of starvation or protein malnutrition (see reviews 8, 9). These observations came from studies involving a wide variety of experimental subjects and conditions and using techniques of variable accuracy and sophistication. In the present study, albumin did not decrease sharply upon initiation of fasting, whereas band 7 was very sensitive to fasting in the zinc-deficient birds. Precise evaluation of the biochemical significance of these data must await identification and quantitation of the protein fractions.

After 7 hours of fasting, the higher concentration of plasma proteins in the zinc-deficient birds, as compared with zinc-supplemented birds, is different from data obtained in the rat (2) and chick (3). It is possible that the blood volume of the quail was decreased by zinc deficiency; however, there is no apparent explanation for the species variation.

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Effect of Dietary Protein on the Development of Vitamin K Deficiency in the Rat [1,2]

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ABSTRACT The role of dietary protein in vitamin K deficiency was studied in adult male rats by assay of coagulation factors dependent on vitamin K. Normal distribution of these data permitted quantitation and statistical evaluation. The older concept that the source and preparation of dietary protein determines prothrombin levels was confirmed. Greatest deficiency was observed in rats fed diets containing solvent-extracted soy or beef protein. In addition, the deficiency observed with several dietary proteins was alleviated by feeding a supplement of amino acids. In rats fed commercial soy protein, normalization of prothrombin was achieved by the addition of approximately 0.25 μg of vitamin K_1/g of diet; in rats fed "laboratory" casein, less than 0.05 μg/g was needed. The data obtained provide no evidence of a correlation between changes in body weight and vitamin K deficiency under the conditions tested.

During studies of the hemorrhagic diathesis induced in rats by diets containing irradiated beef (1–4), our interest was directed toward the protein portion of the diet. This interest was based in part on early observations of Ansbacher (5) that the type of dietary protein used was an important factor in the development of vitamin K deficiency. More recently, Johnson et al. (6) studied commercially available preparations of casein and soy protein and concluded that Labco [3] casein is not entirely devoid of vitamin K. Mameesh and Johnson (7) devised a diet containing soy protein which produced severe vitamin K deficiency in rats in less than 10 days. To explore further the effect of protein, we have made a quantitative comparison of the effect of several available proteins on deficiency of vitamin K in the rat. In addition, the effect of dietary supplements of amino acids was determined.[4]

For our studies current unpublished information was available through biannual progress reports to The Surgeon General of contractors in the irradiated foods program. Richardson [5] reported decreased incidence of hemorrhage after feeding irradiated soy protein supplemented with either a complete mixture of amino acids or a mixture of L-cystine, L-glutamic acid, and glycine. Subsequently, he [6] concluded that high protein diets increase the vitamin K requirement of male rats.

METHODS

Although rats of the St. Louis University strain do not readily develop internal hemorrhages and die as a result of feeding vitamin K-deficient diets, an effect on prothrombin concentration is observed.[7] Details of the procedure used in these studies have been reported (3). Unless otherwise specified, the purified diet used contained 21% protein, 43% cornstarch, 22% glucose monohydrate,[8] 5% corn oil [9] and a supplement of vitamins and minerals as

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[2] Preliminary reports of the studies contained in this paper were presented at the meeting of the American Chemical Society, St. Louis, March, 1961, and the Federation of American Societies for Experimental Biology and Medicine at Atlantic City, April, 1962.

[3] Labco Casein, The Borden Company, New York.

[4] While these studies were in progress Mameesh et al. (4) reported that prolonged prothrombin times and hemorrhagic deaths occurred in non-coprophagic rats fed a vitamin K-deficient diet containing amino acids as the only source of protein.

[5] Richardson, L. R., A long range investigation of the nutrition properties of irradiated foods, General Progress Report no. 17 to the Office of the Surgeon General, Department of the Army, Washington, D. C., May 1961.

[6] Richardson, L. R., A long range investigation of the nutrition properties of irradiated foods, Final Report to the Office of the Surgeon General, Department of the Army, Washington, D. C., September, 1961.

[7] Hemorrhagic deaths due to vitamin K deficiency have been observed in our strain of rat under special experimental conditions (3). For further discussion of the influence of strain see Mellette (8).

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

[9] Mazola, Corn Products Company.

already described (3). Each diet contained 50 IU of vitamin A acetate/g to enhance dietary effects on prothrombin concentration (3). Groups of 10 adult male rats 13 weeks of age were fed experimental diets for 2 weeks; prothrombin in cardiac blood was measured in triplicate at the end of each week by the method of Hjort et al. (9). This method may also detect changes in Factor X (10, 11) but prothrombin and Factor X are both dependent upon vitamin K and the data obtained have been an excellent indicator of the vitamin K status of the animal. Studies of vitamin K deficiency using this analytical method have been reported previously (3, 8, 12). In the present study, aliquots of a pool of plasma taken from stock rats maintained with commercial laboratory chow [10] were analyzed with each group of experimental samples. Average results are expressed as a percentage of the activity of the pooled plasma which is the reference standard. Separate examination of 45 stock rats gave an average of 99 ± 1% of the reference standard. Throughout this study these numbers represent the mean plus or minus the standard error. Individual values less than 85% were considered deficient.

To provide a basis for later statistical analyses, distribution of the 45 values obtained from stock rats as well as the distribution of data obtained from 9 experimental groups was determined. Due to differences in diet, average prothrombin values for these groups ranged from 46 to 101% of the reference standard. The results shown in table 1 indicate that the individual data are distributed normally. On this basis the *t*-test for non-paired experiments was used to analyze differences between experimental groups.

RESULTS

Results obtained with several vitamin K-deficient diets are shown in table 2. Among the deficient animals those fed casein or lactalbumin had the highest levels of prothrombin, whereas lowest levels were observed in rats fed soy or beef protein. Rats fed protein from fresh beef were more deficient in prothrombin than those fed protein from irradiated beef ($P < 0.001$). Rats fed egg albumin or wheat gluten had similar levels of prothrombin, which were lower than those of rats fed the milk proteins. The diet containing zein was not supplemented with amino acids so that vitamin K deficiency in this instance was accompanied by loss of weight due to inadequate dietary protein. Rats fed the vitamin K-deficient ration without any source of dietary protein lost weight rapidly and exhibited hypoproteinemia as well as moderate hypoprothrombinemia. Total plasma protein determined with Biuret reagent was 87% of

[10] Purina Laboratory Chow, The Ralston Purina Company, St. Louis.

TABLE 1

Distribution of prothrombin values in 10 groups of rats [1]

Group	Area under the curve [2]						
	$\bar{x} - 3$ SD	$\bar{x} - 2$ SD	$\bar{x} -$ SD	$\bar{x}$	$\bar{x} +$ SD	$\bar{x} + 2$ SD	$\bar{x} + 3$ SD
1	0.000	0.022	0.133	0.577	0.888	0.955	1.000
2	0.000	0.050	0.175	0.450	0.850	1.000	
3		0.000	0.175	0.550	0.850	0.975	1.000
4	0.000	0.025	0.125	0.425	0.875	1.000	
5	0.000	0.075	0.150	0.400	0.925	1.000	
6		0.000	0.250	0.475	0.875	1.000	
7		0.000	0.150	0.550	0.850	0.975	1.000
8		0.000	0.231	0.513	0.846	0.948	1.000
9		0.000	0.125	0.500	0.800	1.000	
10	0.000	0.025	0.075	0.500	0.850	0.975	1.000
Average [3]	0.000	0.020	0.159	0.494	0.861	0.983	1.000
Normal [4]	0.001	0.023	0.159	0.500	0.841	0.977	0.999

[1] Average prothrombin values for these groups ranged from 46 to 101% as described in the text. Each group provided approximately 40 individual values. Data from stock rats are those of group 1.
[2] These values are given as the area under the distribution curve (total area = 1) from minus infinity to the intervals described; $\bar{x}$ = mean, SD = standard deviation.
[3] Average distribution of individual values for all groups (404 total observations).
[4] Normal distribution.

TABLE 2

Effect of vitamin K-deficient diets on adult male rats

Diet [1]	Prothrombin	Normal values [2]	Wt gain
	%		g/day
Casein (Labco)	81 ± 3 [3]	19/40	2.0
Lactalbumin (Whitson)	86 ± 3	11/20	3.2
Soy protein (ADM)	46 ± 3	1/40	2.2
Beef protein	47 ± 3	1/40	2.6
Egg albumin	55 ± 6	2/20	2.3
Gluten (NBC)	58 ± 5	1/20	1.5
Zein (NBC)	64 ± 4	1/20	−1.9
Beef protein (irradiated)	66 ± 3	6/39	2.6
Protein-free [4]	74 ± 3	2/20	−3.4

[1] Each diet contained 21% protein, as indicated in the text. Labco casein and Whitson lactalbumin were obtained from the Borden Company, New York; soy protein was ADM C-1 Assay Protein, Archer-Daniels-Midland, Minneapolis; corn zein and wheat gluten were obtained from Nutritional Biochemicals Corporation, (NBC), Cleveland; raw powdered egg albumin was obtained from Anheuser-Busch, Inc., St Louis; beef protein was prepared from cooked ground meat by thorough extraction with alcohol and petroleum ether. Beef used in these studies was purchased by the Office of the Quartermaster General. Beef was irradiated at 5.58 megarads by Co^{60} at the Arco, Idaho, or Savannah River, Georgia, Plants of the Atomic Energy Commission.

[2] Number of prothrombin concentrations greater than 85% of the reference standard per total observations.

[3] Mean ± SE.

[4] Deleted protein was replaced with an equal amount of glucose monohydrate.

that in rats fed 21% casein (5.16 ± 0.10 and 5.90 ± 0.07 g/100 ml, respectively).

The effect of the quantity of dietary protein was next studied by determining levels of prothrombin in rats fed several proteins at 10.5 and 35% of the diet (table 3). The greatest differences occurred in rats fed casein and soy protein. Prothrombin was similarly high in rats fed either 21 or 35% of casein but lower in rats fed 10.5% casein ($P < 0.05$). In studies with soy protein, an opposite effect was observed, i.e., prothrombin deficiency was more severe with each increase of dietary protein.

Following these studies, experiments were continued to obtain information concerning the amount of vitamin K necessary for normal coagulation activity in rats fed 21% Labco casein or ADM [11] soy protein. The results are shown in table 4. The vitamin K_1 requirement of rats fed 21% soy protein was approximately 0.25 μg/g of diet; rats fed casein required less than 0.05 μg/g. Rats fed soy diets containing 0.125 μg of vitamin K_1/g (diet 6) had prothrombin levels similar to those fed 21% casein without added vitamin K (diet 1). Thus, the deficiency in rats fed soy protein is corrected to that observed with Labco casein by the addition of 0.125 μg of vitamin K_1/g of diet (approximately 0.6 μg/g of protein).

Previous investigators have emphasized the importance of thorough extraction of dietary protein for the development of vitamin K deficiency. Results observed in rats fed extracted casein and soy protein are shown in table 5. Samples of GBI [12] and NBC [13] casein were examined since these preparations are extracted with hot alcohol by the manufacturers. Prothrombin was lower in rats fed extracted Labco casein ($P < 0.05$) and markedly lower in rats fed GBI or NBC casein. ADM soy protein which is notably effective in the development of vitamin K deficiency pro-

[11] ADM C-1 Assay Protein. Archer-Daniels-Midland, Minneapolis.

[12] General Biochemicals Incorporated, Chagrin Falls, Ohio.

[13] Nutritional Biochemicals Corporation, Cleveland.

TABLE 3

Effect of varying concentrations of protein in vitamin K-deficient diets

Dietary protein [1]	Dietary concentration of protein					
	10.5%		21%		35%	
	Prothrombin	Wt gain	Prothrombin	Wt gain	Prothrombin	Wt gain
	%	g/day	%	g/day	%	g/day
Casein	63 ± 4(40) [2]	2.6	81 ± 3(40)	2.0	88 ± 3(19)	2.6
Soy	53 ± 4(40)	1.7	46 ± 3(40)	2.2	27 ± 4(20)	2.6
Gluten	62 ± 4(20)	0.0	58 ± 5(20)	1.5	55 ± 5(20)	2.7
Zein	69 ± 4(20)	−2.7	64 ± 4(20)	−1.9	57 ± 4(20)	−3.2

[1] Diets were as described in text except that protein was varied and was compensated for by appropriate changes in glucose monohydrate. Casein was Labco (The Borden Company, New York). Data for 21% protein are also given in table 2.

[2] Mean ± SE. Number of observations are shown in parentheses.

TABLE 4

Prothrombin levels in rats fed vitamin K_1 in diets containing 21% Labco[1] casein and ADM[2] soy protein

Dietary protein	Diet no.	Vitamin K_1	Prothrombin	Normal values[3]
		μg/g	%	
Casein	1	0	81±3[4]	19/40
	2	0.02	97±2	17/20
	3	0.05	116±4	20/20
Soy	4	0	46±3	1/40
	5	0.05	64±3	1/20
	6	0.125	85±5	9/20
	7	0.250	100±2	19/20

[1] Labco Casein, The Borden Company, New York.
[2] ADM C-1 Assay Protein, Archer-Daniels-Midland, Minneapolis.
[3] Number of prothrombin concentrations greater than 85% of the reference standard per total observations.
[4] Mean ± SE.

TABLE 5

Effect of extracted proteins in vitamin K-deficient diets

Dietary protein[2]	Extracted		Unextracted[1]	
	Prothrombin	Wt gain	Prothrombin	Wt gain
	%	*g/day*	%	*g/day*
Casein (Labco)[3]	67±5(20)[4]	2.2	81±3(40)	2.0
Casein (NBC)[5]	47±5(20)	1.5		
Casein (GBI)[6]	50±3(20)	2.6		
Soy (ADM)[7]	29±3(19)	1.0	46±3(40)	2.2

[1] These data are also shown in table 2.
[2] Each diet contained 21% of the indicated protein.
[3] The Borden Company, New York; extracted continuously with ethanol for one week at room temperature.
[4] Mean ± SE. Number of observations are shown in parentheses.
[5] Nutritional Biochemicals Corporation, Cleveland; extracted by the manufacturer.
[6] General Biochemicals Incorporated, Chagrin Falls, Ohio; extracted by the manufacturer.
[7] ADM C-1 Assay Protein, Archer-Daniels-Midland, Minneapolis; continuously extracted with ethanol for one week at room temperature.

duced still lower levels of prothrombin after extraction with ethanol ($P < 0.001$).

Since coprophagy may supply some of the vitamin K required by the rat (6, 13), the lowest prothrombin level observed in these studies (29% after feeding extracted soy protein) is probably higher than the minimal level which may occur during total deprivation of vitamin K. To estimate the effect of intestinal sources of vitamin K under our experimental conditions, rats were fed 1% sulfadiazine in diets containing either Labco or GBI casein. This drug has been shown to deplete fecal vitamin K in the rat (14). Labco and GBI casein were selected for this experiment to ascertain their possible content of vitamin K; however, prothrombin levels were very low in rats fed either protein (table 6). Furthermore, no vitamin K could be detected by chick bioassay in feces collected during the second week of feeding either diet. Feces collected from rats fed Labco casein without sulfadiazine contained approximately 16 μg of vitamin K activity/g (dry weight).

In another series of experiments, vitamin K deficiency was studied in rats fed diets containing a wholesome mixture of amino acids.[14] The mixture was fed either as a supplement to several dietary proteins or as the sole source of amino nitrogen. The results shown in table 7 indicate that rats fed soy protein, gluten or zein supplemented with amino acids had higher prothrombin levels than were observed after feeding diets containing the respective proteins alone (table 3). Although supplemental amino acids had a prothrombinogenic effect, rats fed the amino acid mixture alone were deficient

[14] The composition of the amino acid mixture is shown in a footnote to table 7. It is based in part on data and references in Block and Bolling (16) concerning the amino acid composition of casein and on other data for casein supplied by Nutritional Biochemicals Corporation and General Biochemicals Incorporated.

TABLE 6

Prothrombin levels in rats fed diets containing 18% casein and 1% sulfadiazine[1]

Dietary protein	Prothrombin	Wt gain
	%	g/day
Casein (Labco)[2]	12±1[3]	0.6
Casein (GBI)[4]	12±2	0.2

[1] The diets were prepared according to Kornberg et al. (15) except that salts 446 were used in place of salts 550.
[2] The Borden Company, New York.
[3] Mean ± SE.
[4] General Biochemicals Incorporated, Chagrin Falls, Ohio.

TABLE 7

Effect of amino acid supplementation in vitamin K-deficient diets

Dietary protein[1]	Prothrombin	Normal values[2]	Wt gain
	%		g/day
Soy	87±5[3]	11/20	1.6
Gluten	96±3	17/20	2.1
Zein	100±6	16/20	1.4
None[4]	47±5	2/20	1.6

[1] Diets were as indicated in text, except that 10.5% protein was supplemented with an equal amount of the following mixture of L-amino acids: cystine, 0.35; methionine, 2.92; arginine·HCl, 3.53; histidine·HCl, 2.73; lysine·HCl, 7.28; tyrosine, 5.45; tryptophan, 1.12; phenylalanine, 5.36; threonine, 4.04; valine, 6.58; leucine, 10.10; glycine, 1.83; isoleucine, 6.35; alanine, 2.53; proline, 7.57; glutamic acid, 21.21; aspartic acid, 5.97; and serine, 5.07.
[2] Number of prothrombin concentrations greater than 85% of the reference standard per total observations.
[3] Mean ± SE.
[4] Diet contained 21% of the amino acid mixture as its only source of protein.

in prothrombin. These results confirm Richardson's report[15] of the antihemorrhagic effect of supplemental amino acids but indicate that this effect does not occur in the absence of dietary protein.

Because some variation in weight gain occurred throughout these studies, it seemed worthwhile to examine the degree to which the change of body weight in individual animals may be correlated with prothrombin concentration. Correlation coefficients for these variables in each group of 20 animals maintained with 12 different experimental rations are shown in table 8. The degrees of correlation were low; furthermore, positive (direct) and negative (inverse) correlations occurred with apparent random. Similar data were obtained for 4 groups of rats that lost weight during the experiment (average weight loss from 1.9 to 3.4 g/day). Correlation coefficients for these groups were −0.17, −0.30, +0.06, and −0.09. These data provide no evidence to support the existence of an association between prothrombin concentration and weight gain under the conditions tested.

TABLE 8

Coefficient of correlation between prothrombin concentration and weight gain in 12 experimental groups

Exp. no.	Prothrombin	Wt gain	r[1]
	%	g/day	
1	101	1.4	+0.17
2	93	1.9	−0.17
3	81	2.0	−0.09
4	75	2.8	−0.45
5	72	4.5	+0.33
6	69	1.2	+0.06
7	65	2.6	−0.02
8	63	2.6	−0.24
9	53	1.6	−0.33
10	46	2.7	−0.19
11	45	2.6	+0.32
12	23	1.7	−0.41

[1] Each correlation coefficient (r) was calculated from data obtained with 20 animals. With 20 pairs of data the coefficient indicating correlation at the 5% level of significance is 0.444; at the 1% level it is 0.561.

The effect of changes in body weight on prothrombin concentration was examined further in studies of the effect of weight loss due to restricted feeding. Under otherwise standard assay conditions, rats were restricted to approximately one-half the amount of vitamin K-deficient casein ration which would have been consumed ad libitum. These animals lost an average of 2.2 g/day and had an average prothrombin level of 85 ± 3%. This level of prothrombin is not different from that observed in rats fed the same diet ad libitum (81 ± 3%, table 2). Rats fed a similarly restricted amount of the vitamin K-deficient soy ration lost an average of 1.9 g/day and had an average prothrombin level of 54 ± 5%. This level of prothrombin also is probably not different ($P > 0.05$) from that observed in rats fed the same diet ad libitum (46 ± 3%, table 2). Thus, weight loss due to caloric restriction did not influence significantly the prothrombin levels which resulted in rats fed these diets ad libitum.

[15] See footnote 5.

DISCUSSION

From the foregoing data and those of earlier investigators the severity of vitamin K deficiency in the rat depends clearly on the type of dietary protein used. Milk proteins, casein and lactalbumin are least desirable proteins for producing the deficiency. Lower levels of prothrombin were obtained after feeding soy or beef protein and the deficiency was most pronounced in rats fed diets containing extracted soy protein.

The lower levels of prothrombin observed after feeding extracted casein and soy protein suggest that these proteins may be contaminated with substances active as vitamin K. Such contamination is possible since the sources of these proteins, soybeans and milk, have been shown to contain prothrombinogenic activity (17, 18). However, the amount of vitamin K in the purified proteins is difficult to assess. Increasing dietary levels of soy protein resulted in greater prothrombin deficiency (table 3), thus precluding an estimate of the amount of vitamin K in this protein. By comparison with the effects of soy protein, Labco casein appears to contain approximately 0.6 μg of vitamin K_1/g; however, this value is probably high. In unpublished experiments with chicks, bioassay of Labco casein revealed that this protein contains less than 0.15 μg of vitamin K_1/g. This lower estimate is also supported by the failure of casein to provide an effective amount of vitamin K in rats fed sulfadiazine.

The effects of vitamin K deficiency on blood coagulation may be minimized if a deficiency in protein occurs simultaneously. This view is supported by the results obtained with a protein-free ration (table 2). Despite a decrease in total plasma protein, only moderate hypoprothrombinemia occurred. In contrast with this, rats that were fed at the highest levels of dietary protein (with the exception of casein) had the greatest deficiency in prothrombin (table 3). The data presented in table 8 and in the text indicate that these effects are not correlated with observed changes in body weight.

A role for dietary protein in the development of vitamin K deficiency is further indicated by the results obtained after feeding amino acids. As shown in table 7, prothrombin was elevated after feeding a supplement of amino acids with several dietary proteins. Rats fed the same mixture of amino acids as their sole source of protein had low levels of prothrombin. The basis for these results is not clear; however, they may be related to the differences in prothrombin levels observed in rats fed different dietary proteins.

The effect of dietary proteins and amino acids on vitamin K deficiency may be due in part to changes in the intestinal supply of vitamin K. Except in those experiments in which sulfadiazine was fed, no attempt was made to limit the availability of vitamin K synthesized in the gut. However, Johnson et al. (6) have observed that coprophagic habits of the rat may vary in response to the diet and have correlated these changes with vitamin K nutrition. The diet may also influence intestinal synthesis and absorption of vitamin K but there is little experimental precedent on which to propose specific changes in this respect. Although macro-nutrients may influence the synthesis of vitamins, little has been reported on the effects of dietary protein (19). Furthermore, according to Gershoff (19), there is little evidence that macro-nutrients directly affect the absorption of most vitamins in healthy animals and man.

Statistical analyses of the data presented in this report indicate that hypoprothrombinemia in rats can be studied quantitatively and support the view that young adult male rats provide reliable data for bioassay of vitamin K. Under conditions used here, prothrombin levels in rats fed 21% ADM soy protein were proportional to vitamin K_1 up to the minimal dietary requirement of approximately 0.25 μg/g. The minimal requirement for normalization of prothrombin in rats fed Labco casein was less than 0.05 μg/g.

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Amino Acid Imbalance [1] and Tryptophan-niacin Metabolism

I. EFFECT OF EXCESS LEUCINE ON THE URINARY EXCRETION OF TRYPTOPHAN-NIACIN METABOLITES IN RATS

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ABSTRACT The effect of feeding excess leucine on the urinary excretion of tryptophan-niacin metabolites and nitrogen was studied in young and adult rats. Urinary excretion of quinolinic acid and N′methylnicotinamide was increased in both young and adult rats when L-leucine was added at 1.5% level to a 9% casein diet. Quinolinic acid excretion was more markedly affected in young rats, whereas N′-methylnicotinamide excretion was more affected in adult rats. Isoleucine counteracted the effect of leucine in young rats. Nitrogen excretion increased on leucine feeding in adult rats but not in young rats. Adult rats fed a jowar (*Sorghum vulgare*) diet tended to excrete relatively more N′-methylnicotinamide and niacin than when fed a wheat diet.

The effect of leucine feeding on the urinary excretion of tryptophan-niacin metabolites in human subjects and pellagrins has been reported by Gopalan and Srikantia (1) and Belavady et al. (2). Feeding leucine caused a significant increase in quinolinic acid excretion and a decrease in 6-pyridone of N′-methylnicotinamide excretion in normal subjects besides altering the excretion of other metabolites to varying degrees (2). Leucine is present in a relatively high concentration in the millet jowar (*Sorghum vulgare*). Amino acid imbalance due to an excess of leucine in jowar has been suggested as a possible factor in the development of pellagra which is endemic in certain population groups that subsist principally on this millet (1).

Amino acid imbalance in certain types of diets has been shown to cause an increase in niacin requirement in rats and chicks (3). Addition of relatively low levels of gelatin, acid hydrolyzed protein or certain amino acids to a niacin-free casein diet has been shown to cause growth retardation in rats (4–8), which can be corrected by niacin or tryptophan. These observations have been extended to non-casein diets (9) and diets based on amino acid mixtures by Koeppe and Henderson (10). Lyman and Elvehjem (11) have shown that niacin is more effective in correcting the imbalance than tryptophan itself, indicating that imbalance affects the efficient conversion of tryptophan to niacin. Sauberlich and Salmon (12) showed, however, that growth retardation in animals due to imbalance caused by feeding casein-gelatin diet could be corrected by tryptophan and not by niacin alone.

These observations would point to a disturbance in tryptophan-niacin metabolism brought about by an amino acid imbalance in the diet, the mechanism of which is not clear. A detailed investigation of the effect of feeding excess leucine on tryptophan-niacin metabolism in experimental animals was considered necessary to throw some light on the mechanism of the observed action of leucine in humans. The present paper reports such an investigation with rats.

EXPERIMENTAL

Experiment 1. Weanling rats of either sex, distributed at random into 7 groups of 6 each were used in this experiment. They were fed ad libitum for 4 weeks a basal ration (diet A) containing casein (9% protein) either alone, or supplemented with leucine, niacin or tryptophan in different combinations. The composition of the basal

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[1] The term "amino acid imbalance" has been used in a general sense indicating adverse effect of an excess of an amino acid.

ration is shown in table 1, and additions to the diets are shown in table 2. Weekly weights and food intakes were recorded. At the end of this period the animals were transferred to metabolic cages and urine was collected for 3 consecutive days. An accurate record of food intake during the collection period was maintained.

Experiment 2. Six adult female rats weighing between 130 and 160 g housed individually in metabolic cages were fed in 3 periods the following diets: basal ration (diet A, table 1) containing 9% protein derived from casein during the first and the third periods and basal ration plus 1.5% L-leucine during the middle period. The duration of each period was 10 days. The first 7 days were allowed for stabilization, and urine was collected during the last 3 days. A record of food intake was kept during the collection period.

Experiment 3. In another metabolic experiment similar to experiment 2, 6 adult rats (3 males and 3 females) with an average weight of 200 g were given 2 diets (diets B and C) in which the proteins were derived from jowar (*Sorghum vulgare*) or wheat, respectively. The compositions of the diets are shown in table 1. These 2 diets were fed to 3 rats each during the first period and the diets were interchanged during the second period. The purpose of this experiment was to study tryptophan-niacin metabolism in rats fed these 2 cereals which differ in their leucine content, jowar having a much higher content than wheat.

Methods. Urine was collected in toluene and glacial acetic acid, and stored in cold. Urine was pooled for three days, made up to a known volume, filtered and kept cold. Total and free niacin, N′-methylnicotinamide and quinolinic acid were estimated in all samples of urine. Total urinary nitrogen was estimated in all the samples except those collected in experiment 3.

Niacin was estimated in the diets and urine by the method of Friedemann, and Frazier (13). Total niacin was determined after hydrolyzing the urine with 40% NaOH as described by Swaminathan (14). The N′-methylnicotinamide was estimated by the method of Carpenter and Kodicek (15). Quinolinic acid was estimated by the method of Henderson (16). The conversion efficiency of quinolinic acid to niacin under the experimental conditions used was determined with pure quinolinic acid.[2] The conversion efficiency was found to be 74.6 ± 4.9% and the conversion factor of 1.87 was used in calculating the quinolinic acid content of the urine samples. Recovery of added quinolinic acid to urine was complete. Nitrogen was determined by the macro-Kjeldahl method. Tryptophan was estimated in the diets by the microbiological assay described by Barton-Wright (16) using *Lactobacillus arabinosus*.

TABLE 1

Composition of the basal diets

	Diet A	Diet B	Diet C
Casein	11.0	—	—
Jowar (*Sorghum vulgare*)	—	90.0	—
Wheat	—	—	71.0
Peanut oil	5.0	5.0	5.0
Salt mixture [1]	4.0	4.0	4.0
Vitamin mixture [2]	1.0	1.0	1.0
Cornstarch	79.0	—	19.0
Cystine	0.2	—	—
Choline chloride	0.1	—	—

[1] Wesson, L. G. 1932 Science, 75: 339.

[2] Vitamin mixture: (mg/100 g) thiamine, 20; riboflavin, 30; Ca pantothenate, 200; biotin, 1; folic acid, 2; inositol, 1000; pyridoxine·HCl, 5; vitamin B_{12}, 0.15; p-aminobenzoic acid, 10; vitamin K, 10. In addition: vitamin A, 100 IU/day; vitamin D, 15 IU/day and, tocopherol, 3 mg/day were fed orally.

RESULTS

Experiment 1. Urinary excretion levels of nitrogen, quinolinic acid, total and free niacin and N′-methylnicotinamide in young rats are shown in table 2. Quinolinic acid excretion increased significantly ($P < 0.01$) by nearly 2.5 times when leucine was added at a 1.5% level to a casein diet. Excretion of quinolinic acid also increased when 1 mg/100 g of niacin or 0.1% tryptophan was added to the basal diet. The former increase was significant ($P < 0.05$) whereas the latter was not. The observation that quinolinic acid excretion was increased when niacin alone was added to the basal diet could possibly be explained as being due to feedback inhibition by the dietary niacin of the conversion of quinolinic acid to niacin.

[2] Obtained from L. Light and Company, Ltd., Colnbrook, England.

TABLE 2

Effect of leucine on the urinary tryptophan-niacin metabolites in young rats [1]

Group	Diet	Diet intake/ rat/day	Wt gain/ 4 weeks/ rat	Avg for 3 days: Intake			Avg for 3 days: Excretion				
				Nitrogen	Niacin	Tryptophan	Nitrogen	Quinolinic acid	Niacin: Total	Niacin: Free	N'-methyl-nicotinamide
		g	g	mg	µg	mg	mg	µg	µg	µg	µg
1	Basal diet A (casein)	9.2	77.9	515.6	534.0	38.6	177.4 ± 35.4 [2]	109.2 ± 17.7	70.1 ± 10.9	40.9 ± 8.1	64.0 ± 5.1
2	+ 1 mg/100 g niacin	9.5	79.9	661.7	1134.0	47.2	181.3 ± 44.6	254.1 ± 51.2	83.1 ± 14.7	40.0 ± 9.5	107.3 ± 15.4
3	+ 0.1% DL-tryptophan	9.6	80.3	618.8	635.0	87.8	141.3 ± 26.0	190.2 ± 56.0	85.9 ± 16.6	51.1 ± 9.7	104.9 ± 5.7
4	+ 1.5% L-leucine	9.7	77.3	612.1	555.0	41.7	183.0 ± 23.9	278.6 ± 61.6	106.8 ± 22.9	42.4 ± 11.7	91.9 ± 12.5
5	+ 1 mg/100 g niacin + 1.5% L-leucine	10.0	77.0	685.7	1019.0	44.6	200.6 ± 28.1	196.2 ± 35.4	63.5 ± 12.5	36.8 ± 6.6	143.5 ± 19.0
6	+ 0.1% DL-tryptophan + 1.5% L-leucine	10.2	80.6	568.6	567.0	70.0	171.5 ± 43.5	252.4 ± 36.3	99.1 ± 23.9	32.1 ± 11.4	136.4 ± 21.3
7	+ 1.5% L-leucine + 0.2% DL-isoleucine	9.3	78.5	648.1	579.0	41.9	149.8 ± 30.2	141.2 ± 28.8	82.8 ± 8.9	31.4 ± 6.0	41.6 ± 3.3

[1] Six rats in each group: 9% protein.
[2] SE of mean.

TABLE 3

Effect of leucine on the urinary tryptophan-niacin metabolites in adult rats [1]

Period	Diet	Diet intake/ day/rat	Wt gain/ 10 days/ rat	Avg for 3 days							
				Intake			Excretion				
									Niacin		
				Nitrogen	Niacin	Trypto-phan	Nitrogen	Quinolinic acid	Total	Free	N′-methyl-nicotinamide
		g	g	mg	μg	mg	mg	μg	μg	μg	μg
1	Basal diet A [2] (casein)	16.2	11.0	800.4	740.6	53.5	49.0 ± 2.2 [3]	56.0 ± 9.4	43.7 ± 5.5	22.4 ± 5.7	61.2 ± 8.2
2	Basal diet + 1.5% L-leucine	10.4	8.0	588.4	474.7	34.2	117.1 ± 9.3	66.1 ± 24.1	111.3 ± 23.7	53.1 ± 18.6	353.9 ± 113.3
3	Basal diet A	20.2	3.0	997.5	906.6	66.7	167.8 ± 12.8	56.7 ± 12.0	84.7 ± 9.1	47.4 ± 7.9	184.2 ± 8.1

[1] Six rats in each group: 9% protein.
[2] Contains 1 mg/100 g niacin.
[3] SE of mean.

TABLE 4

Urinary tryptophan-niacin metabolites in adult rats fed jowar and wheat diets [1]

Period	Diet	Diet intake/ day/rat	Wt gain/ 10 days/ rat	Avg for 3 days					
				Intake		Excretion			
							Niacin		
				Niacin	Trypto-phan	Quinolinic acid	Total	Free	N′-methyl-nicotinamide
		g	g	mg	mg	μg	μg	μg	μg
1	Jowar basal diet B	19.4	11.8	1.2	37.2	159.5 ± 55.9 [2]	233.2 ± 80.0	32.3 ± 5.4	91.2 ± 19.5
2	Wheat basal diet C	19.9	15.5	1.3	62.0	169.7 ± 37.4	178.2 ± 53.8	39.8 ± 0.5	63.1 ± 10.8

[1] Six rats in each group: 9% protein.
[2] SE of mean.

The N′-methylnicotinamide excretion increased to an equal and significant extent when niacin ($P < 0.05$) or tryptophan ($P < 0.01$) was added to the basal diet. An increase in the excretion of this metabolite was also observed when leucine was added to the basal diet or to the basal diet containing added niacin or tryptophan. These increases were not highly significant perhaps due to wide scatter in the individual values.

One observation was that addition of 0.2% isoleucine to the basal diet containing leucine significantly counteracted the increased excretion of quinolinic acid ($P < 0.05$) and N′-methylnicotinamide ($P < 0.001$) caused by leucine.

The differences observed in the excretion of total niacin among the different groups were not statistically significant. Free niacin excretion was not effected by the different additions to the basal diet. Leucine also did not affect nitrogen excretion in the young rats. This observation appears to correlate with the observed lack of growth retardation in young rats fed the diet containing leucine is the present study.

Experiment 2. Figures for urinary excretion of tryptophan-niacin metabolites in adult rats in experiment 2 are shown in table 3. There was an increase in the urinary excretion of nitrogen, quinolinic acid, total and free niacin and N′-methylnicotinamide accompanied by decreased food intake when leucine was incorporated into the diet at a level of 1.5%. These increases were highly significant when adjustments were made for differences in food intake and the results were analyzed according to analysis of covariance. The observed increase in nitrogen excretion on leucine feeding in adult rats was in contrast with the behavior of the young rats in which nitrogen excretion was not affected by leucine feeding. The results obtained in the adult rats with respect to nitrogen excretion agree with the observations of Deshpande et al. (18) and Kumta et al. (19), who demonstrated that an amino acid imbalance caused a decrease in nitrogen retention in adult rats. After withdrawal of leucine, urinary excretion of the different metabolites decreased, but the values did not return to the original level (i.e., first period). The persistence of a higher level of excretion of the metabolites even after leucine withdrawal might be partly due to a marked increase in food intake after leucine withdrawal. On the other hand, the higher level of excretion might indicate that the effect of leucine was not reversed within the short period of 8 days after its withdrawal. Metabolic alterations caused by leucine feeding would thus appear to extend beyond the period of leucine feeding.

Experiment 3. Urinary excretion levels of quinolinic acid, total and free niacin and N′-methylnicotinamide in adult rats fed jowar and wheat diets are shown in table 4. The intake of tryptophan was significantly lower with jowar diet than the wheat diet, whereas the intake of niacin was of the same order with the 2 diets. A higher level of excretion of N′-methylnicotinamide and total niacin with the jowar diet than with the wheat diet was not statistically significant.

DISCUSSION

The foregoing results indicate that feeding excess leucine affected the urinary excretion of niacin-tryptophan metabolites in rats. Inclusion of leucine at a level of 1.5% in the basal ration caused a significant increase in the excretion of N′-methylnicotinamide in adult rats and to a less significant extent in young rats. Quinolinic acid excretion was increased to a significant extent in both young and adult rats. Niacin (total and free) excretion was increased significantly in adult rats but not in young rats. Belavady et al. (2) observed that in human subjects leucine feeding markedly increased the excretion of quinolinic acid and reduced the excretion of 6-pyridone of N′-methylnicotinamide. Rosen and Perlzweig (8) have reported an increased excretion of N′-methylnicotinamide in rats by gelatin-induced amino acid imbalance. Truswell et al. (20), however, did not observe any effect on the excretion of N′-methylnicotinamide in female rats on the addition of L-leucine to the diet. However, the level of leucine used by these authors was possibly too low to show any demonstrable effect. A somewhat reduced excretion of N′-methylnicotinamide in rats maintained with a 9% casein diet to which threonine was added

has also been reported by Pearson and Phornphiboul.[3] Pearson and Song[4] have confirmed this and showed that in addition to N′-methylnicotinamide, the excretion of 3-hydroxy anthranilic acid, kynurenine and quinolinic acid was less in the group fed threonine.

These results suggest that dietary amino acid excess could cause a disturbance in the tryptophan-niacin metabolism. A comparison of our results with those of Pearson and co-workers[5] indicate that the qualitative nature of such a disturbance depends upon the amino acid which is fed in excess. These differences could be due possibly to the differences in the biochemical changes brought about by excess of threonine and leucine. Even in the case of a particular amino acid fed in excess, the metabolites affected appear to be influenced both qualitatively and quantitatively by the age of the animals. The differences may be related to the differences in the relative needs of tryptophan for growth and maintenance of young and adult rats.

Addition of isoleucine to the diet overcame the increase in the excretion of quinolinic acid and N′-methylnicotinamide in leucine fed young rats. Harper and coworkers (21, 22) have observed an antagonistic relationship between leucine and isoleucine with respect to the growth of rats. The present observation may be an instance of true antagonism. However, Gopalan and Srikantia (1) failed to observe an antagonistic effect of isoleucine on leucine in human subjects.

Concerning the mechanism underlying the observed effect of excess leucine on tryptophan niacin metabolites, there are 2 possibilities: one a generalized effect and the other specific interference in the chain of reactions of tryptophan-niacin metabolism. The generalized effect might be mediated through increased diversion of dietary tryptophan through the niacin pathway following a failure on the part of the tissue to utilize imbalanced amino acids for protein synthesis. Increased nitrogen excretion in adult rats fed leucine suggest an impaired utilization of amino acids. But other observations as lack of growth retardation in young rats fed leucine, and the absence of uniform increase in the various metabolites on leucine feeding, do not support this mechanism. Experimental evidence for the impaired utilization of tryptophan in threonine-induced imbalance is contradictory (23, 24). Further studies are necessary to evaluate the effect of amino acid imbalance on tryptophan utilization.

Some of the specific effects of excess leucine that might explain the present observation in rats and those of Belavady et al. (2) in humans are: (a) inhibition of conversion of quinolinic acid to niacin or more appropriately to niacin ribonucleotide (25, 26); (b) a block in the incorporation of niacin into nicotinamide nucleotide; (c) an increased breakdown of nicotinamide nucleotides; and (d) a decreased oxidation of N′-methylnicotinamide to the 6-pyridone of N′-methylnicotinamide. It has been shown now in humans that in vitro synthesis of nicotinamide nucleotide by erythrocytes is significantly reduced with leucine feeding.[6] The effects, if any, of excess leucine on other loci of tryptophan-niacin metabolism are under investigation.

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Some Aspects of Glucose Metabolism of Chromium-deficient Rats Raised in a Strictly Controlled Environment

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ABSTRACT Intravenous glucose tolerance and glucose oxidation in vitro was measured in chromium-deficient rats raised under conditions which allowed strict control of trace element contaminations. A severe impairment of glucose removal rates was observed in all animals. Subsequent chromium supplementation resulted in significant increases of rates toward normal values. Oxidation of glucose in vitro by adipose tissue of chromium-deficient rats was lower than that of supplemented animals, both in the presence and absence of insulin. These results indicate a more severe degree of chromium deficiency than that observed in rats raised in an ordinary environment.

Impairment of glucose tolerance has been described as an early symptom of a deficiency in "glucose tolerance factor," a dietary agent, identified as trivalent chromium (1, 2). These observations were made on rats maintained under ordinary conditions with various purified rations and laboratory chows. Although the rates of glucose removal from blood were significantly lower than those of rats fed a natural ration, the impairment varied within groups of animals. No depression of growth rates was detected in these animals with marginal chromium deficiency and, under these conditions, the effect of chromium was restricted to various aspects of metabolism (1, 3, 4).

However, rats and mice raised in an environment allowing strict control of trace element contaminations responded to more pronounced chromium deficiency with impaired growth and survival, and supplementation with 5 ppm of the element had a beneficial effect on both parameters (5–7). To investigate a possible relationship between these 2 independent observations an experiment was designed to measure some aspects of glucose metabolism in rats grown under low-chromium conditions.

EXPERIMENTAL

Rats of the Long-Evans strain were bred in a specially built laboratory on a hilltop in southern Vermont, under precautions designed to eliminate, insofar as possible, extraneous metallic contamination. The conditions have been described in detail (6). In brief, spring water, hardness 18 ppm, was passed through a commercial water softener and bulk demineralizer[1] and then further demineralized by a laboratory type of apparatus[2] to a resistance of approximately 5 million ohms. To this water were added the essential trace metals, zinc and copper (as acetate), cobalt (as chloride), manganese (as citrate) and molybdenum (as sodium molybdate), in amounts approximating those in commercial laboratory chows (6). This drinking water was supplied to animals in polyethylene bottles with stainless steel drinking tubes. Chromium determinations of water in which these tubes had soaked for several days showed that the steel gave up only negligible quantities of chromium. Forest air was passed through electrostatic precipitators into a sealed room used for animal quarters, made of wood, covered with plastic varnish. Cages were of Acrylic plastic with stainless steel covers.

The diet consisted of 60% seed rye, ground locally, 30% powdered skim milk, 9% corn oil, and 1% sodium chloride, to which were added per kilogram: ferrous sulfate, 100 mg; vitamin A, 5000 IU; vitamin D, 1000 IU; Ca pantothenate, 10 mg;

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[1] Culligan, Inc., Northbrook, Illinois.
[2] Barnstead Bantam, Boston.

niacin, 50 mg; and pyridoxine·HCl, 1.0 mg (6). The chromium content was approximately 0.1 μg/g, wet weight (8).

At an age of 456 to 752 days, 10 rats were shipped by air express to the Walter Reed Army Institute of Research, with free access to potatoes (chromium content 0.006 μg/g, wet weight). They were received within 8 hours and kept in individual cages with a Torula yeast ration (1) and distilled, deionized water for 4 days. At this time, intravenous glucose tolerance tests were performed on 6 rats, after an overnight fast. The 4 remaining rats, too large to fit into available bleeding cages for the in vivo tests, were kept for the in vitro experiments to be described below. The procedure of the glucose tolerance tests was that used previously (9), except that blood samples were obtained at 15, 30, 45 and 60 minutes after the glucose injection and a modified glucose oxidase method (10) was used for the determination. To detect possible spontaneous changes of glucose removal rates, two of the pretested rats continued to receive the water; the other 4 were given a supplement of 5 ppm of chromium (as hexa-aquo chromium trichloride) in the drinking water, and received one intravenous injection of 0.1 μg/100 g of body weight, 2 hours before the final test. After 4 to 7 days, the tests were repeated, and the results were compared with those obtained in the first assay. One week after completion of these experiments, all 10 animals were decapitated, and the response of their epididymal fat tissue, or parametrial fat tissue to various doses of insulin was measured in vitro. The fat tissue was excised quickly and washed for 30 minutes in a glucose-free Krebs-Ringer phosphate medium, pH 7.4 at 37°. At the end of this period, pieces of approximately 100 mg (range 74 to 130 mg) were cut from the tissue and added to 25-ml Erlenmeyer flasks containing 2 ml of the medium, with 1 mg glucose/ml and a sufficient amount of glucose-1-C^{14}. The flasks, containing a suspended cup with hydroxide of Hyamine[3] for the collection of CO_2 were stoppered, aerated and incubated with shaking for 2 hours at 37° in a Dubnoff shaker. The Hyamine was then quantitatively transferred to counting vials with an appropriate scintillator and was counted in a liquid scintillation detector, with proper corrections for background and efficiency.

A second group consisted of 8 female breeder rats (482 days old), treated as described above. It was used for determination of intravenous glucose tolerance and water consumption.

All glassware used was cleaned by ultrasound and rinsed thoroughly with dilute hydrochloric acid and triple-distilled, deionized water. All possible efforts were made to avoid chromium contamination of the system. The animals were treated in accordance with the principles of laboratory animal care as promulgated by the National Society for Medical Research.

RESULTS

The average glucose removal rate of the 6 rats measured in the first experiment was 1.12 ± 0.2% /minute, with a range of 0.53 to 1.64% /minute. During and after the test, all animals voided large amounts of urine which gave a "dark" reaction for glucose (0.5% or more) on Combistix.[4] This was probably due to the excitement of the first testing procedure since we were unable to obtain any urine, during repeated experiments, regardless of supplementation. The response of the rats to the subsequent chromium treatment was striking. Whereas the average rate of the 2 control animals, maintained with water, decreased further, the glucose removal rate of each of the chromium-supplemented rats was improved (fig. 1). The group average of 3.5% /minute is close to that observed in Sprague-Dawley rats of even younger age, raised with a natural wheat-casein ration (1).

A similar response, only quantitatively different, was noted in the second group of 8 rats, consisting of females too old to breed. The initial average removal rate was 0.74% /minute. Because of these very low rates, the *k* values for glucose removal were calculated from the average glucose levels in each group, thus obviating the necessity of taking into account "negative" removal rates which were occasionally observed. Because of the slow appearance

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[3] Packard Instrument Company, La Grange, Illinois.
[4] Ames Company, Elkhart, Indiana.

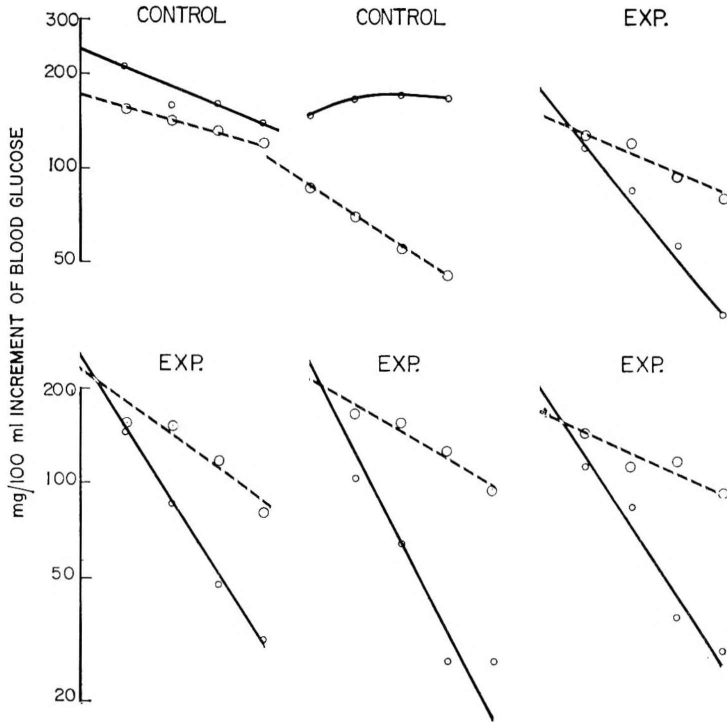

Fig. 1 Intravenous glucose tolerance in 6 rats raised under low-chromium conditions. Measurements of blood glucose, upon intravenous glucose injection, are plotted against time (15, 30, 45 and 60 minutes). After the first test (broken lines) 4 rats (experimental) received 5 ppm chromium (III) in the drinking water and one additional intravenous dose of 0.1 μg/100 g. The 2 remaining rats (control), received no chromium. The solid lines represent the results of the second test. The glucose removal rates, in %/minute, for first and second tests, respectively, were as follows: control 1, 0.53, 0.9; control 2, 1.45, negative rate; experiment 1, 0.86, 2.7; experiment 2, 1.64, 3.58; experiment 3, 1.27, 4.25; and experiment 4, 0.97, 3.36.

of a chromium effect, the experiment was extended to 11 days. As shown in figure 2, the average glucose removal rate of the chromium-supplemented group (4 rats) increased from 0.51 to 1.39%/minute; that of the water controls (4 rats) decreased consecutively from 0.97 to 0.61 and 0.18% /minute. This deterioration of glucose tolerance was not accompanied by significant weight changes. The average weight increased slightly, except for one rat that lost weight, probably because of an infection, but that still responded to chromium.

That the increase of glucose removal rates by chromium is correlated with a direct effect of the element on the peripheral tissue was demonstrated in the in vitro studies (table 1). The tissue of chromium-deficient rats had lower metabolic activity as measured by CO_2 production from labeled glucose in the presence of 3 different levels of insulin, but also in its absence. The difference in activity of the tissues without insulin may be related to the greater efficiency of the tissue-bound, endogenous hormone of the chromium-supplemented rats. These experiments again show a pattern of activity, similar to, but more pronounced than, that observed in "conventional" animals. One phenomenon, however, not previously observed under the conditions of marginal chromium deficiency, was the difference in water consumption between deficient and supplemented animals. The water intake, measured individually and daily for 7 days, was 16.2 ± 1.3 and 25.8 ± 1.7 ml/day for chromium-supplemented rats and controls, respectively ($P < 0.01$).

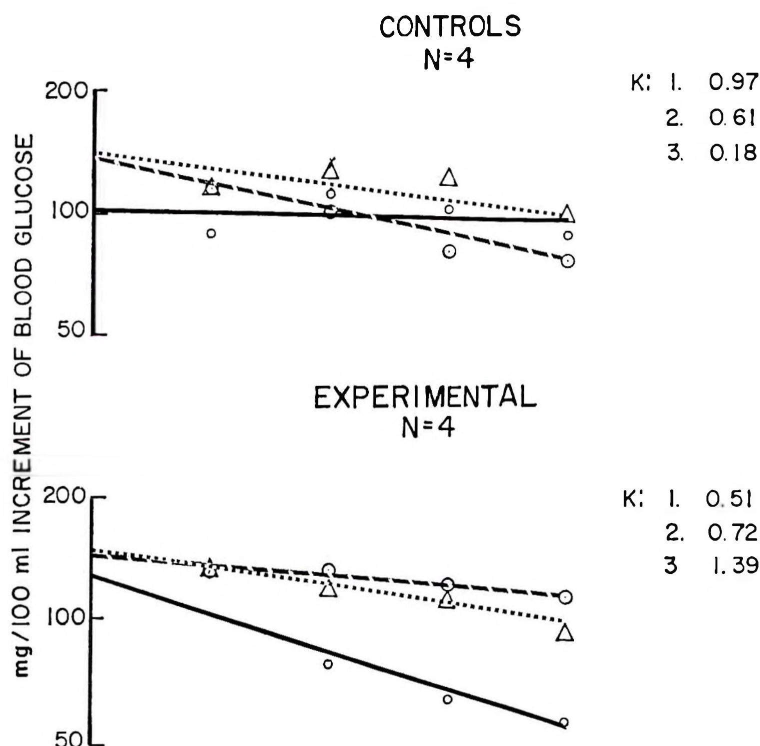

Fig. 2 Average glucose tolerance tests of low-chromium rats (old breeders). Increment blood glucose plotted against time (15, 30, 45 and 60 minutes). Broken lines: first test, dotted lines; second test after 7 days, solid lines, after 11 days. At completion of first test, 4 rats (experimental) received 5 ppm chromium (III) in the drinking water and one dose of 0.1 μg/100 g, intravenously, 2 hours before each following test. Four rats served as controls and received no chromium. The *k* values of the average glucose removal rates are shown with each group for the 3 tests.

TABLE 1

$C^{14}O_2$ production from glucose-1-C^{14} by adipose tissue of rats in vitro

Supplement	No. of rats	Insulin (μU/flask)			
		0	200	500	1000
		mμmoles CO_2/00 mg tissue			
None	5	10.7 ± 0.8 [1]	17.9 ± 2.5	15.4 ± 1.1	18.7 ± 2.0
5 ppm chromium	5	19.9 ± 1.3	28.3 ± 7.7	33.9 ± 8.0	38.9 ± 7.0

[1] Mean ± SE of mean.

DISCUSSION

The uniform and severe impairment of intravenous glucose tolerance in these animals suggests a degree of chromium deficiency exceeding by far that observed in rats raised under ordinary condition. In the latter, glucose removal rates averaged around 2.7%/minute (1), as compared with a normal value of approximately 4.0%/minute. That the impairment noted in the present studies was related to chromium deficiency is shown by the uniform response to supplementation with the element. The progressive deterioration of the control animals (fig. 2) warrants discussion. The virtual absence of glucose removal during a one-hour experiment has rarely been observed by us in thousands of glucose tolerance tests. It indicates a severe disturbance of glucose

metabolism, not only of peripheral uptake, but probably also of the homeostatic function of the liver, as suggested by the increase of blood sugar during the first 30 minutes of the test (fig. 2). Although this increase is indicated by a difference in blood sugar levels of only 20 mg/100 ml, we can almost certainly eliminate the possibility of technical errors as a cause. This type of response to a glucose load could be considered diabetic; yet, the fasting blood glucose levels were not elevated in the animals. Only the increased water consumption may be suggestive of a diabetic state.

The progressive deterioration of glucose tolerance in the control animals, although prevented by chromium supplementation, may be related to additional dietary factors. The Torula yeast diet, used during the tests described here, contains suboptimal amounts of cystine. Sulfur amino acids have been implicated in the action and metabolism of insulin (11). However, the significant effect of chromium speaks for this element as the main factor involved, and the pronounced impairment in the controls of the second group as well as the slower onset of the chromium effect may indicate a more severe chromium deficiency than was present in the first group. The livers of female breeder rats obtained postpartum contain little or no chromium (8). These animals, through their repeated pregnancies, may have become more depleted than the others.

It is possible that airborne chromium may have been responsible for the difference in the degree of chromium deficiency observed in rats bred in Vermont and in Washington. Air in Washington, D. C. contained 0.08 μg chromium/m^3 (12), whereas this element was not detected in dust electrostatically precipitated from a 6-month collection in the Vermont laboratory.

On the basis of the observations reported here, together with those published previously, it appears justified to describe the following sequence of events. At weaning and during the following few weeks, glucose removal rates of rats are between 4 and 5 %/minute, regardless of diet (9). Immature rats, mice and human beings, as well as snake and chicken eggs, contain considerable chromium (8). With a natural, complete ration or with a chromium-sufficient artificial diet, these rates decrease only little with age, to around 3.5 to 3.8 %/minute, whereas low-chromium diets cause a significant impairment of glucose tolerance, to rates of approximately 2.7%/minute (1, 9). At this time, growth is not depressed, and chromium supplements have no effect on weight gain. When chromium deficiency becomes more severe, as in the animals used for this experiment, glucose tolerance becomes more severely deranged, and significant retardation of growth and decreased longevity is observed (5, 6). The alternative of this being the result of the disturbed glucose metabolism or of both being only symptoms of a more basic disturbance is under study. Available evidence suggests that chromium enhances the effects of insulin, not only on glucose metabolism but also in other systems, by facilitating the initial reaction of the hormone with its acceptor sites on membranes (13, 14).

The results of these and of previous studies emphasize the need for strict control of external contamination in trace element research which cannot be achieved easily in conventional laboratories. Since chromium and probably other elements in physiologic doses have no pharmacologic action, but only restore deficient functions to normal, the apparent effect depends on the degree of the deficiency. The more severe this can be made, the more profound will be the observed impairment and the effect of resupplementation. At this point, it may become permissible to extrapolate from the various degrees of deficiency to the absolute absence of the element in question, and consider an element which prevents these symptoms as essential, even though an absolute deficiency with immediate and acute pathologic consequences has not been achieved.

With many of the micronutrients, a marginal state of deficiency is all that can be obtained under ordinary conditions. But even here, observation of metabolic changes, however slight, may produce indications for important roles of a given substance.

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Dietary Pectin and Blood Cholesterol [1]

Fausch and Anderson recently suggested species differences in the blood cholesterol response of pectin-fed animals (1). Feeding swine an essentially cholesterol-free diet, these workers observed that pectin produced an elevation in serum cholesterol. These observations seemed inconsistent with the hypocholesterolemic effects of pectin reported for man (2) and for the rat (3). Fausch and Anderson also cite work suggesting that only non-herbivorous animals respond to pectin with lowered blood cholesterol.

Based upon our experience with three species — man, chicken and rabbit — we should like to offer a different interpretation for the observations of Fausch and Anderson: pectin produces a lowering of blood cholesterol only in the presence of dietary cholesterol. The following results are presented in support of this hypothesis:

Species	Dietary cholesterol	Plasma cholesterol	
		Pectin diet	Control diet
		mg/100 ml	*mg/100 ml*
Man	–	165 ± 9	167 ± 6
	+	157 ± 15	191 ± 10
Chicken	–	111 ± 6	96 ± 4
	+	170 ± 10	220 ± 11
Rabbit	+	345 ± 50	478 ± 35

The pectin response of chickens fed a cholesterol-free ration (4) was very similar to that reported by Fausch and Anderson for swine; however cholesterol feeding reversed the response. In our experiments with humans, dietary cholesterol exceeded twice the amount ingested by the subjects studied by Keys et al. (2); concomitantly we observed a correspondingly greater hypocholesterolemic effect from pectin. Finally, we also obtained a lowering of blood cholesterol in an herbivorous species when we fed pectin to rabbits.

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