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TO

THE JOURNAL OF NUTRITION

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Never divide a word at the end of a line, for, it might be set incorrectly with a hyphen. The repeated use of a unit in a sentence may be avoided by stating the common unit used as, "the diet consisted of the following in milligrams: sucrose, 6; etc." Or, in other cases, as "8, 12 and 14%."

A common error occurs in describing the percentage of a compound. Use the form, "15% of calcium" when the percentage is not an adjective. Number all pages consecutively in the following order: title page, text, tables, figures and legends, literature cited and footnotes.

Start each of the following sections on a new page and typed double-spaced:

1. Tables, with appropriate heading (a separate sheet for each table)
2. Explanation of figures, numbered and listed consecutively
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4. Footnotes.

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Acknowledgments. Financial support should be listed as a footnote to the title. Credit for materials should be listed as a footnote in the text. Technical assistance and advice may be cited in a section headed Acknowledgments, which will follow the summary.

Summary. This should represent a brief recapitulation of the problem and results.

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tion of the manuscript under consideration, it is advisable to make a copy of it available to the Editor. When a manuscript is one of a series of papers, the preceding paper should be included in Literature Cited.

Numbers. If possible, avoid using a number as the first word in a sentence. Numbers so used should always be spelled out, but no abbreviations should follow such numbers. Numbers indicating time, weight and measurements are to be expressed in Arabic figures when followed by an abbreviation (e.g., 2 mm; 1 sec.; 3 gm; 100 mm^3). Except as indicated, the numbers *one*, *two* and *three* will be spelled out in the text. All higher numbers will be given in Arabic figures. Use Arabic numerals throughout, including tables.

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The metric system should be used in preference to others for measurements, weights, etc. Temperatures should always be expressed in Centigrade scale. Metric abbreviations listed below are shown in the approved lower-case style, *without periods:*

	Weight		*Length*
kg	kilogram	km	kilometer
gm	gram	m	meter
mg	milligram	cm	centimeter
μg	microgram	mm	millimeter
mμg	millimicrogram	μ	micron
μμg	micromicrogram	mμ	millimicron
		μμ	micromicron
	Volume		
m^3	cubic meter		
cm^3	cubic centimeter		*Area*
mm^3	cubic millimeter	m^2	square meter
l	liter	cm^2	square centimeter
ml	milliliter	mm^2	square millimeter

Symbols. When preceded by a figure, the following symbols are used:

A	angstrom units	°	degree
%	per cent	ppm	parts per million

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ELMER MARTIN NELSON

(1892–1958)

ELMER MARTIN NELSON

ELMER MARTIN NELSON
—*A Biographical Sketch*

(July 5, 1892—December 24, 1958)

Elmer Martin Nelson was a competent biochemist, well-trained in nutrition, who attained eminence in his profession. His career came at a time when animal feeding technics, in which he was specially skilled, led to extraordinary advances in our knowledge of foods and nutrition. His work was done, however, not in a university or pure research center, but in the service of the Food and Drug Administration. This is the government agency charged with the administration of the pure food and drug laws.

Nearly all of Doctor Nelson's published papers, of which there were more than 80, were on subjects determined by the requirements of his official position. They were directed toward the attainment of its goals. He was a member of a team. As a team player he probably would have been quick to point out, if given the opportunity, that his associates deserved much of the credit for the results of any activity in which he participated. Concerning the many programs in the broad field of public health in which he took part, he often mentioned that group opinion is weightier than individual opinion, and no person is solely responsible for the accomplishments of a group. He used to make it clear that even in the scientific decisions he was called upon to render, he sought the advice of others, so that his decisions would be reasonably free of bias and would truly reflect the thinking of the best available experts. But what the Food and Drug Administration has accomplished over the last 30 years, in guiding an enlightened industry in applying the advances of nutrition to improvements in food processing and distribution, can be attributed in no small measure to the work of this stalwart worker in nutrition.

Doctor Nelson's career did not go unrecognized during his lifetime. In 1949 he received the Award of the Grocery Manufacturers' Association for his contributions to progress in foods and nutrition. In 1957 he received the Babcock-Hart Award of the Institute of Food Technologists for his distinguished contributions to food technology. Pleased indeed he was to receive that award because it bore both the name of Babcock, who had started the study of nutrition at Wisconsin, and of Hart, who continued it, and who had been Nelson's teacher, advisor, and friend. But he was even more pleased later, when he learned that the American Public Health Association had awarded one of its outstanding citations to the entire Food and Drug Administration for meritorious service in the field of public health.

Doctor Nelson served two terms as an associate editor of the Journal of Nutrition. He was elected treasurer of the American Institute of Nutrition and, in 1948, he served as its president. In the following year he was named as the Institute's representative at a conference held in London, England, where plans were developed for the establishment of an International Union of Nutrition Sciences; later he was a member of the organizing committee that planned the Fifth International Congress on Nutrition in the United States in 1960. Much earlier, in 1934, Doctor Nelson had served as United States delegate to the International Conference on Vitamin Standardization which was held in London, England. It was at this conference, held under the auspices of the League of Nations, that international standardization of the vitamins was initiated.

Early years

The beginning of his career was simple enough. He was born on July 5, 1892, in Clark, South Dakota, and his parents were of Swedish origin. He had three sisters.

The family lived on a farm until young Nelson was 10 years old. An interesting account of some phases of life on that South Dakota farm is available to us. It was included in the last paper that Doctor Nelson read before an audience. The paper, which has not been published, entitled "Food Facts and Fallacies," was presented before the New Jersey Welfare Council on October 29, 1958. His purpose was to illustrate that simple foods are not incompatible with good nutritional status, but the account also points up the great advances in methods of food processing and distribution that have occurred in the recent past.

"There were no motor trucks in those days," Doctor Nelson observed, speaking of his boyhood, "and there was only one railroad across the state. Fruit trees do not survive the severe winters in this area. At that time fresh fruits and canned fruits and vegetables were not available to us, except for the vegetables grown in our garden. This source of supply is not very dependable because frosts occur as late as the middle of June and again in the middle of August. The closest grocery stores were about 8 miles away, and only in emergencies did we go to town more than once a week. I do not remember eating an orange at any time other than Christmas, but bananas were purchased occasionally during the summer months. We purchased a barrel of apples every fall, and one or two baskets of grapes on my mother's birthday in September. White flour, occasional fresh meat, dried peas and beans, cheese, smoked and salted fish and dried fruits were our other important purchases.

"If weather permitted," he continued, "we had a good garden of peas, carrots, beets, lettuce, cabbages, squash and sweet corn, but these were available only from the middle of July to the middle of August. Potatoes were usually grown in sufficient quantities by making two or three plantings, in the hope that one planting would get the necessary rainfall. We had eggs, but since they were a source of cash income, they were used sparingly. Hogs were slaughtered on the farm and fried salt pork was served at least once a day. Chicken was an important source of meat, which was augmented at times by prairie chicken, wild ducks and wild geese. Our diet was primarily one of bread, potatoes and meat, supplemented with milk and, at times, vegetables but very little fruit. The diet of others in this area was similar, except that they probably had more fresh meat such as beef, mutton, and pork, and fewer vegetables.

"No one," he concluded, "has ever reported that deficiency disease occurred in this area in which I spent my childhood."

The Nelson family moved to Wisconsin and, in February, 1914, after 4 years work in a paper mill, young Nelson entered the State University. Except for a 100-dollar gift from his mother he supported himself entirely throughout his college career. He was over 6 feet tall, and slender. He was given the nickname of "Slim" which old friends continued to use in conversing with him for many years. He received his B.S. degree from the College of Agriculture in 1918, when World War I was in progress, enlisted in the Army of the United States, and served several months in the Medical Corps.

On his discharge from the Army he returned to the University of Wisconsin as a graduate student. He received an M.S. degree in 1919, and continued part-time teaching and course work under the direction of Professor Harry Steenbock. He was awarded the Ph.D. degree in 1923, and then remained at the University for two additional years as an industrial Fellow. During this time, when so many historically important papers emanated from the Wisconsin laboratories, his name appeared among the authors of 9 papers. These papers dealt with the physiology of the "fat-soluble vitamine," with the use of the rat in quantitative determinations of vitamin B, and with some of the studies which ultimately led to the recognition, several years later, of the relationship between ultra-violet irradiation and vitamin D. The name of Mariana T. Sell appears as one of the collaborators of two of these early papers; later, one may notice the name of Mariana T. Nelson—the two young members of the staff were married in 1923. When Doctor Nelson left Wisconsin in 1925 to accept a position with the Soft Wheat Millers' Association in Nashville, Tennessee, someone is said to have re-

marked that his departure involved a double loss to the University. For the rest of his life Doctor Nelson had in his home a sympathetic collaborator who not only performed some of his bibliographic work, but who also on occasion helped in the formulation of solutions to some of the complex problems with which he had to cope.

In the government service

The Nelsons remained in Nashville about a year and then, in 1926, left for Washington. For the remainder of his career, Elmer Nelson was a government employee, a civil servant in the finest sense of the term. He was employed by the United States Department of Agriculture, which at that time contained the division which administered the old Pure Food and Drug Law of 1906. Half his time was spent with regulatory problems and half with research.

During the ensuing 9 years, Doctor Nelson's research activities were varied. With D. Breese Jones he studied the vitamin content of oysters and clams, and the deleterious effects of ethylene treatment on the nutritive value of tomatoes which had been artificially ripened by its use. Considerable attention was devoted to the perfection of methods for the quantitative estimation of the vitamins, especially of vitamins A, B, and D. He was named referee for vitamins by the Association of Official Agricultural Chemists and, for many years, the annual reports of collaborative studies on vitamin methods, which he prepared, provided an interesting and authoritative account of progress being made in this field, so important to the pharmaceutical and food industries. He also was appointed a member of the Vitamin Advisory Board of the United States Pharmacopeia, and served in this capacity for over 20 years.

With D. Breese Jones, Doctor Nelson studied the vitamins of sugar cane juice and related products, and participated in the studies which yielded the key to the identification of selenium as the toxic ingredient of wheat, and of other crops, when grown on certain soils. With Chester D. Tolle and others, he demonstrated the value of menhaden oil and of burbot liver oil as sources of vitamin D, and thereby helped in the establishment of a new industry. Tolle and Nelson also were first to show the value of canned salmon as a food source of vitamins A and D. With Martha M. Eliot and others, Nelson tested the value of salmon oil in the treatment of infantile rickets. He reported on assay procedures for the determination of vitamin D when added to milk, and on dye titrations for the estimation of vitamin C, and their application to the determination of the vitamin C content of orange juice. With Reed Walker, he showed that the apparent vitamin B content of fresh yeast cells was increased by drying, an observation that was later verified by others and shown to be owing to the increased availability of the vitamin in the dried cells. With H. H. Mottern, he studied the effects of lead arsenate sprays on the vitamin C content of oranges; the vitamin content was decreased as a result of this treatment.

It is apparent that during this period Doctor Nelson was active in two fields: the quantitative determination of the vitamins, and the effects of processing and other treatments on the nutritive value of foods. The importance of such studies from the practical point of view was considerable. They came at a time when the public was beginning to give attention to popular accounts of scientific discoveries. The vitamins were beginning to be exploited. There was no television then, but there was radio, and advertising technics were being developed for this new medium of communciation. Few chemists in industry had then had experience with animal feeding methods, and those in the food and pharmaceutical industries were confronted with new problems for which the usual analytical methods would not provide an answer. The development of standardized methods for the estimation of vitamins led, first, to the development of a stabilized vitamin industry, and secondly, to confidence of physicians and the public in the potency of the industry's products. The studies on the effects of processing on the nutritive values of foods focussed attention by the food industry on nutrition. In time, this interest led to the development by others of improved methods of food technology for the preservation of important nutritive properties of processed foods.

Division of Nutrition established

In 1935, Doctor Nelson was designated Chief of a new division, now known as the Division of Nutrition, of the Food and Drug Administration, and at present in the Department of Health, Education and Welfare. He organized the division, secured a competent staff, many of the members of which are still in the service, and developed a program of regulatory work and research, and of consultation with other divisions on all matters pertaining to nutrition. It was at this time that the need for radical revision of the Pure Food and Drug Act of 1906 was being discussed in Congress. The purpose of the old law, as of even earlier laws, was primarily to protect consumers from economic cheats, such as short-weight and the concealment of inferiority, and from the use of poisonous or deleterious materials. There had long been a need for more effective controls and, after prolonged consideration, many of the desired changes were embodied in a new law, the Food, Drug and Cosmetic Act of 1938. This law provided, among other provisions, for the establishment of standards for certain foods, and for informative labeling of articles intended for use as special dietary foods. The new division figured prominently in the administration of these phases of the Act of 1938 which, with its amendments, constitutes the present basic food law of the United States.

Although the food and drug laws are written in terms of acts which are prohibited, and penalties for violations, the Food and Drug Administration has long recognized that effective enforcement is more of an educational than a policing activity. The number of violators of the law is relatively small, and legitimate industry is as much interested as anyone in complying with reasonable rules that are in the public interest. The Food and Drug Administration is a fact-finding body. When violations are encountered that, in the opinion of the Commissioner, require legal action, the information is turned over to the Department of Justice for possible prosecution. The scientific personnel may be called on to serve as expert witnesses in cases that go to trial.

Doctor Nelson made numerous appearances as an expert witness for the government, and prepared for such appearances with great care. He would devote considerable time to the planning of a simple, yet accurate, description of his array of facts, such that a judge and jury could understand. As a result he was a superb witness. Not a single case in which he was involved, which was decided on its scientific merits, was decided against the government. Some of the cases in which he was called upon to offer expert testimony constitute legal landmarks in the history of food and drug law enforcement.

Of course, in the establishment of rules and regulations, and in the interpretation of scientific data, there may be differences of opinion. Doctor Nelson once expressed his philosophy of work in the following words:

"I am convinced that many of the vexing problems that arise in the application of the Food, Drug and Cosmetic Act to the food industry will be solved if we, as scientists, continue to discuss our findings in an informal way. We know that of the new discoveries in this field only those that serve a useful purpose will survive. I believe that we have a common aim in producing and labeling food products to the benefit of the consumer."

His work brought Doctor Nelson into contact with many scientists and others of the food and drug industries. He viewed his function as that of one who is in a position to help apply the newer, worthwhile findings of the laboratory to the benefit of the public, more quickly and effectively than otherwise might be possible. This broad viewpoint, coupled with wise employment of authority, earned for Doctor Nelson the respect and confidence of scientists everywhere The obligations of the Division of Nutrition required the staff to be aware of newer developments in the entire field of nutrition, and in a position to appraise the merit of new developments from the viewpoint of public health. In performing his duties, Doctor Nelson not only made significant contributions to the broad field of public health, but he also brought increased stature to the Food and Drug Administration.

He would go out of his way to be helpful to those who earnestly sought his advice

and help. On one occasion many years ago, there came to his attention the plight of a farmer in Pennsylvania who had lost his entire flock of young turkeys and, in trying to find the cause, he had performed his own assay of vitamin D on the commercial feed he had used. He found the content to be low. Seeking redress, the farmer learned that his assay results were being questioned. He had kept a detailed record of what he had done and what he had observed, and he had also retained the bones of the turkeys and samples of the feed. From a study of the records and examination of the materials, Doctor Nelson came to the conclusion that the work of the amateur investigator contained no flaws. Securing permission to do so, and taking a leave of absence for the purpose, Doctor Nelson went at his own expense to Pennsylvania and presented expert testimony that proved decisive.

The rapid destruction of vitamin D when cod liver oil is mixed with calcium carbonate was first reported in 1932. It was found that vitamin D in its usual forms is also destroyed when admixed with other mineral salts, or with feeds containing a high proportion of minerals; there appears to be an oxidation that is accelerated when the vitamin-containing material is mixed with finely ground materials that permit greater exposure to the air. The last published article that Doctor Nelson wrote was an editorial in the Journal of the American Medical Association; it called attention again to this phenomenon. In recent months, the article related, the laboratories of the Division of Nutrition had examined about 50 commercial preparations, chiefly tablets containing calcium diphosphate and vitamin D—products often recommended for use by pregnant women. Four of these products were completely devoid of vitamin D, 5 had an excess of 100% or more above the amount declared on the label, 20 had an excess greater than would be expected for non-mineral vitamin D products, and the rest were satisfactory. There are several methods of coating the vitamin D to avoid contact with air, some being patented processes. Doctor Nelson refrained from saying that the addition of a large excess of vitamin D was hardly the way to solve the problem, but the inference is there. He did call attention to the fact that J. B. Wilkie, S. W. Jones, and O. L. Kline of the Division of Nutrition had developed a chemical procedure for the assay of vitamin D which, he thought, might serve a useful purpose to manufacturers who desired to develop better controls for this type of preparation.

Councils of the American Medical Association

At the time the editorial on Vitamin D was written Doctor Nelson had been a member of the Council on Pharmacy and Chemistry of the American Medical Association for 23 years. Also, at that time, he held the distinction of being the only nonmedical member of the Council. He served as a consultant to the Council on Foods and Nutrition from about 1936 on, and attended all of its meetings and participated in its deliberations. These associations were mutually advantageous to the Councils and the Food and Drug Administration, as well as helpful to the industries concerned.

In 1935, at about the time when Doctor Nelson was first elected to membership, the Council on Pharmacy and Chemistry had released a report disapproving of what were termed "shotgun vitamin mixtures." This designation alluded to the resemblance of some vitamin mixtures to old-fashioned complex mixtures of many drugs, one of which the user hoped might prove beneficial. The Council's rule against irrational mixtures of therapeutic agents, it might be mentioned, had long served a useful purpose in the consideration of many drugs. But many persons thought that vitamins were in a somewhat different category; they were nutrients, and there was a need for all of the vitamins essential in human nutrition. It was believed, and later demonstrated, that human deficiencies are likely to be multiple in nature, rather than limited to a single nutrient. The conflict in views became reconciled through the simple device of establishing a joint committee on vitamins, representing both Councils and other interested parties. In the activities of this cooperative committee Doctor Nelson played a key role.

While some features of vitamin mixtures were not completely resolved, the unusual opportunities for discussion of problems which the cooperative committee provided, led to the formulation of a number of decisions which had a far-reaching effect. So-called allowable claims for each of the vitamins were developed and made known to industry as a means of guiding advertising claims along lines that were considered appropriate. The Council dropped its introduced generic name of "cevitamic acid" for vitamin C, and accepted the scientific name of ascorbic acid, though the latter name was therapeutically suggestive and therefore in conflict with a rule of the Council for acceptable drugs. Doctor Nelson was instrumental in having questions of nomenclature of the vitamins, many of which were becoming known in those days, referred to committees of the American Society of Biological Chemists, and the American Institute of Nutrition which were established for the purpose, and which collaborated closely with a committee of the American Chemical Society. Through these committees such names as "thiamine" and "niacin" were introduced, to the satisfaction of all parties. Doctor Nelson worked patiently and thoroughly. He often accomplished results without many persons even being aware that he had had a part in obtaining them.

The cooperative committee on vitamins, in perhaps its most fruitful activity, discussed at considerable length questions concerning the fortification of foods with vitamins and other nutrients. Out of the conclusions so reached, the Council on Foods and Nutrition developed sound policies that helped to a considerable degree in guiding the early development of enriched flour and enriched bread, and of margarine fortified with vitamin A, and the establishment of iodized salt on a firm basis.

Regulations for special dietary foods

With all his outside commitments, Doctor Nelson did not neglect his responsibilities to the Food and Drug Administration. One of the important problems which he tackled shortly after the new law of 1938 went into effect, was that of developing regulations for the labeling of special dietary foods. He went about this task in typical fashion. He first secured the appointment of an advisory committee of experts; those appointed for this purpose included H. C. Sherman, E. B. Hart, L. A. Maynard and others. He then thought through his own ideas and reduced them to writing. The resulting statement was submitted to his committee, then thoroughly discussed about a table, revised where indicated, and finally approved by the group. When these views were presented by him later at open hearings, and supported by testimony offered by P. C. Jeans, W. H. Sebrell and others of recognized caliber, they were adopted with little opposition.

Nelson's views, which became the basis of the findings of fact resulting from the hearings, were that products specially designed to meet the nutritional needs of any group of persons should be labeled in terms of what a specified quantity of the product would furnish, expressed as percentages of the dietary requirements. This meant that for the first time, except for some attempts by the League of Nations, an effort would be made to draw up a list of nutrients essential in the diet, and to define the quantitative requirements. In the records of the hearings one may still find Doctor Nelson's easy and straightforward account of his reasoning and the recommendations of his committee, which were in fact his recommendations. It was decided, for what at the time appeared to be good legal reasons, to speak of "minimum requirements" but it was made clear in the testimony that the figures presented were reasonably in excess of the minimum, in order to take care of individual requirements that might be above the average. There was some modification of the figures which he himself proposed, in the light of the testimony of others. But if one were to look into the archives of the hearing clerk in the offices of the Food and Drug Administration, one might be surprised to note the close similarity between the values he proposed in 1940 and the present Recommended Dietary Allowances of the Food and Nutrition Board, except for vitamin C, which is higher in the Recommended Allowances. When one is obliged to make

decisions on the basis of incomplete data, good judgment is required, and Doctor Nelson had good judgment.

The Food and Nutrition Board

Doctor Nelson's services were in demand for important committees concerned with matters of foods and nutrition. It is not surprising therefore that he became one of the original members of the Food and Nutrition Board in 1941. He had met with the organizing group earlier, when the question of improving the nutritive value of flour, by the addition of selected nutrients, first arose. Doctor Nelson listened, and then called attention to the fact that at that very time the Food and Drug Administration was conducting hearings for the purpose of establishing definitions and standards of identity for flour and related products. If the group wished to encourage the production of a nutritionally improved flour, it was important to introduce testimony at the hearings so that provision for the additives could be made in the standards. Arrangements accordingly were made to have introduced at the flour hearings some testimony that would be needed in order to permit the shipping in interstate commerce of a flour to which certain nutrients were added, and for which the name "enriched" was later adopted.

Doctor Nelson's reasoning regarding the levels of nutrients to be supplied by enriched flour illustrates the simple, logical manner in which he approached what were often questions of great complexity. Flour in all its forms, he said at the hearings, was being consumed to the extent of about 6¼ ounces daily, on a per capita basis. This quantity supplies roughly about one-fourth of the caloric requirement. Therefore, he reasoned, it would be logical to think of enriched flour as a product that should supply one-fourth of the daily requirement of the nutrients to be added. This could well be the minimum. Maximal levels to be permitted, he thought, would be the full daily requirement of each nutrient, on the grounds that it would be unnecessary for a food to supply more. Of the nutrients proposed, those to be added should be, first, essential in the diet, and secondly, stable when added to the flour. Of the nutrients proposed, thiamine, riboflavin, niacin, and iron would be required ingredients, because dietary surveys had shown that they could well be increased in the national dietary for all persons of all ages everywhere. Calcium and vitamin D, on the other hand, would be optional ingredients, because surveys had shown that they were needed more by certain age groups than by others, and more by persons in some areas than in others. These views were adopted with little modification.

Had Doctor Nelson not made himself interested in the enrichment of flour, it is quite possible that this important measure in the interest of public health might have floundered, for the initiation of a program is often its most critical period.

Vitamin D-fortified evaporated milk, and vitamin A-fortified margarine, were also defined by the standards-making provisions of the Federal Food, Drug and Cosmetic Act and, in all of these activities, Doctor Nelson participated importantly. Chronologically, the standardization of vitamin D-fortified evaporated milk occurred before a Food and Nutrition Board was established. The Division of Nutrition had the responsibility of advising the Commissioner, or principal officer of the Administration, on all matters pertaining to nutrition. Doctor Nelson fulfilled this obligation with distinction. He tried to get the best advice he could, as has been stated, but one well-known nutrition expert who was a consultant to the Food and Drug Administration on several occasions commented, after a conference with Doctor Nelson, that it seemed to him it was the consultants who got educated, and not the men of the Food and Drug Administration.

During discussions of questions at meetings of the Food and Nutrition Board Doctor Nelson was unusually adept at pointing out how animal feeding experiments, if properly planned, could provide the kind of information needed in order to resolve questions that sometimes arose regarding human nutrition. The question came up on one occasion about what the thiamine allowance for men in the tropics should be; some then recent evidence indicated that it ought to be greater than it is in the temperate zones. Doctor Nelson

took this problem to the laboratory and, in due course, there appeared a paper by O. L. Kline, Leo Friedman and E. M. Nelson on the effect of environmental temperature on the thiamine requirement of the rat. It was shown that the thiamine requirement, contrary to what had been reported, is decreased rather than increased in a hot environment, but that the caloric requirement is at the same time decreased, in parallel fashion. "It is to be expected," they concluded, "that a diet, adequate in respect to thiamine, when consumed in a temperate climate, would also be satisfactory under tropical conditions, even though a reduced food intake resulted from the higher environmental temperature." They also concluded that the maintenance of a uniform environmental temperature is essential for precision in performing the rat-curative assay for thiamine. The animal laboratories of the Division of Nutrition were air-conditioned long before other parts of their divided quarters in the South Building of the Department of Agriculture.

Doctor Nelson knew how to get things done. Many years ago, when informal discussions of some loopholes in the original Act of 1938 were being discussed, he pointed out that a simple solution to the problem of affording the public more adequate protection from inadequately tested food additives was available. All that would be necessary, he thought, was for the law to be revised along the lines indicated by existing requirements for new drugs, the safety of which under the recommended conditions of use had to be demonstrated before they could be sold. However, it required years of effort on the part of many persons before the law required the pre-testing and approval prior to use of food additives. The Food Additives Amendment of 1958 embodies the simple solution that Doctor Nelson had informally offered as a suggestion almost 12 years earlier.

At one of the meetings of the Food and Nutrition Board during World War II, representatives of the Army told of their need for detailed and accurate information about the nutritive values of many foods. Many of the data in the literature, it seemed, were conflicting, and some of the vitamin values were expressed in terms of units no longer used, and there were doubts about the validity of converting figures into modern units of weight. Moreover, there were many processed foods for which no data were available. A committee was formed by the Board to study the problem and to instigate at once means for obtaining whatever data might be needed. Doctor Nelson, with the approval of his superiors and the acquiescence of his staff, made available for the purpose the facilities of the Division of Nutrition. Many foods were analyzed, and the values in the older tables were subjected to critical review. The committee, of course, received help from others, and the data from all sources were included, in time, in Handbook no. 8 of the Department of Agriculture on the composition of foods.

Other committees

Among the committees on which Doctor Nelson served, and to which he gave unselfish service for many years, the following may be mentioned, in addition to those already described: the National Research Council's Committee on Food for the Quartermaster (and chairman of its subcommittee on nutrition); the Scientific Advisory Committee of the Nutrition Foundation, Incorporated; the Expert Committee on Vitamin Standardization, World Health Organization; and the Agricultural Research Institute (and its Board of Governors). Most of these committees were important working committees, and participation in their activities required the expenditure of appreciable time and effort.

Part of Doctor Nelson's success in working with and on committees was his complete lack of regard for personal glorification. He had a job to do. That job was to apply nutritional knowledge to human health and welfare. He would gladly give his time and attention to worthwhile activities directed to the same goal. Every one of the organizations of which he was a member will attest to the value of his participation. Wherever he went, respect and even admiration for the work of the federal agency that he represented were gained. He could justify, no doubt, the effort he devoted to committee activities on the grounds that the Federal Food and

Drug Administration was an organization which had been established by the Congress, representing the people, to serve the public, and his work on worthwhile committees was simply an extension of his functions in the government service. The tasks of setting up certain rules of procedure on an orderly basis, and of applying newer knowledge in a way that would assure the maximum of public benefit therefrom, were common objectives of these other organizations and of the Food and Drug Administration. The demands on him personally might be great, but the important thing was to try to attain the common goal.

Other personal characteristics

As may be gathered from the present brief account of the life of this truly great man, Doctor Nelson's work was his life. He had the ability to gain pleasure from little things. He was pleased, for example, when the U. S. Pharmacopeia adopted an assay procedure in which he had had a hand. He expressed his pleasure when for the first time the word "vitamin" appeared in a government regulation. He rejoiced as much over the successful outcome of experiments by others as if he had done the work himself. He would have been a great teacher. In a sense, he was a teacher, to all with whom he came in contact.

When he had the time, he liked to work with his hands. He took pride in laying a brick wall without help. He took pride also in a basement which he had made over, and he would display it to friends as evidence of his skill as a carpenter and a painter. He enjoyed playing golf, though in his later years he had little time for the game, and besides, he had resumed his interest in gardening. He became an amateur ornithologist, and planned elaborate schemes to outwit the squirrels that came to devour the feed in the bird-feeding station in his garden, but never put any of the plans into effect.

Above all his other traits was his desire to be helpful; mistrust of others was foreign to his nature. He accepted people at face value. When occurrences took place that showed his trust to be unfounded, his subsequent distrust was strong and lasting. Yet he never allowed his feelings to develop into either bitterness or vindictiveness. There was always a kind of serenity or equanimity about him.

His work habits were exemplary, for he was orderly, conscientious and seemingly tireless. There were few tasks left undone when he died. As the year 1958 drew to a close, he had taken a half-day off from his work, in order to go home and address Christmas cards. Just before Christmas he took off two whole days, from his accumulated leave, to take care of various personal chores. On one of these days he had visited his physician, and he had returned in high spirits because of the favorable report about his health. The next day, which was the day before Christmas, he had spent some time raking leaves in his garden, and fixing up some plants for the winter. Then he had rested. He and Mrs. Nelson planned to drive to a section of Washington where they had lived for many years, and deliver some little gifts to old friends there. When the time came to leave, Mariana Sell Nelson called her husband, but found that he could not be wakened. The labors of the one-time farm boy were over. What he had accomplished remains.

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Endogenous Hypercholesterosis in Rabbits Fed a Fat-free Purified Diet and the Effect of Unsaturated Lipid

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Quantitative and qualitative differences in dietary fat are factors in cholesterol metabolism. When male rats were fed a fat-free diet, the cholesterol content of the liver and adrenals increased, whereas the concentration in the plasma decreased; addition of appropriate amounts of fat to the diet caused these values to return to normal levels (Alfin-Slater et al., '54; Deuel et al., '55). Rats fed a diet high in unsaturated lipid had elevated hepatic cholesterol levels (Whitney and Roberts, '55) and rabbits fed diets enriched with vegetable fats became hypercholesterolemic (Steiner and Dayton, '56; Lambert et al., '58). Recently it has been shown that the amount, as well as the degree of unsaturation of dietary fat, influenced the concentration of cholesterol in the liver and blood of the rat (Klein, '58).

This report describes the effect of a fat-free diet, as well as diets with various levels of an unsaturated fat, upon the cholesterol concentration in the liver and plasma of rabbits.

EXPERIMENTAL

Forty adult male albino rabbits weighing about 2.5 kg were housed in individual cages, and were allowed diet and water ad libitum. Laboratory rabbit chow[1] was fed during a two-week control period. The animals were then divided into 4 groups of 10 rabbits each and fed purified diets (Thacker, '56), which were based on the fat-free diet (A) outlined in table 1. Diets B, C, and D, which contained 5, 10 and 20% of corn oil, respectively, were prepared by substituting corn oil for the starch on a weight per weight basis. The diets were prepared in pellet form and stored under refrigeration until used. The daily dietary consumption was noted. In some cases it was necessary to add to the diet a few pellets of rabbit chow during the first week, but this was discontinued when the diet was accepted. The animals were weighed and a sample of blood was drawn from the ear vein at biweekly intervals. After 12 weeks the final sample of blood was taken, the animals sacrificed, and livers removed. Cholesterol (Sperry and Webb, '50) was determined on the blood and liver.

TABLE 1
Composition of fat-free diet

	%
Corn oil[1]	0
Starch, potato	60
Casein, vitamin-free[2]	25
Cellulose, powdered	10
Salt mixture, major[3]	4.9
Salt mixture, minor[4]	0.1
Vitamin mixture[5]	+

[1] Mazola, Corn Products Company, Argo, Illinois.
[2] The Borden Company, Chicago.
[3] Hawk-Oser salt mixture ('31).
[4] $FeC_6H_5O_4 \cdot 1\frac{1}{2}H_2O$, 453.85 gm; $CuSO_4 \cdot 5H_2O$, 28.15 gm; $MnSO_4$, 16.50 gm; KI, 1.5 gm.
[5] The vitamin mixture supplied the following in mg/100 gm of diet: thiamine mononitrate, 0.07; riboflavin, 0.6; Ca pantothenate, 1.5; pyridoxine·HCl, 0.7; niacin, 20; choline, 100; inositol, 10; *p*-aminobenzoic acid, 0.2; folic acid, 0.10; biotin, 0.05; vitamin B_{12}, 5; calciferol, 0.02; α-tocopherol, 7.5; menadione, 0.075; and vitamin A palmitate, 666 I.U.

RESULTS AND DISCUSSION

Although adult animals were used, a small but steady rate of growth was observed in all groups during the 12-week

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[1] Purina Rabbit Chow, Ralston Purina Company, St. Louis.

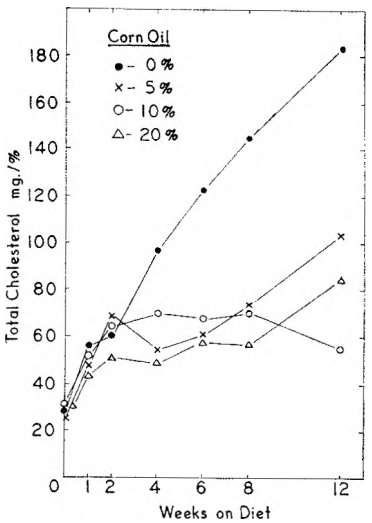

Fig. 1 Comparison of average total plasma cholesterol of rabbits fed various levels of lipid in the diet.

experimental period. No great differences in weights were seen between any of the groups despite the adjustment period to the purified diets and the lipid content of the diets.

The average total plasma cholesterol level increased over control levels in all groups during the experimental period (fig. 1). The highest level of plasma cholesterol and the greatest rate of increase occurred in those animals fed a fat-free diet (group A). In the presence of dietary fat, namely, groups B, C and D, an increased plasma cholesterol level occurred during the experimental period. The rate of increase was the same for these groups, but lower than that of group A. This increase in plasma cholesterol did not appear to be a function of the fat content of the diet. This is in contrast to the rat, wherein the cholesterol concentration in the plasma increased with the lipid content of the diet (Klein, '58).

An increased free and total hepatic cholesterol concentration was observed in all groups when compared with group B (table 2). The highest total cholesterol levels in the liver were obtained with the fat-free diet and with the diet containing 20% of fat. As determined by the decrease in free to total (F/T) ratios, the greatest increase occurred in the esterified cholesterol pool. A smaller but significant increase was observed in the free cholesterol pool. Thus, the presence of an optimal amount of fat in the diet appears to be vital for the maintenance of liver and plasma cholesterol at minimum levels in rabbits. This optimal amount of fat may be necessary to provide the concentration of unsaturated fatty acids which may be required for the normal transport and metabolism of cholesterol, as has been suggested for the rat (Alfin-Slater et al., '54; Deuel et al., '55; Aftergood et al., '57; Klein, '58). Excessive amounts of fat, although rich in unsaturated fatty acids, increased cholesterol deposition in the liver of both the rabbit, as shown by the present data, and the rat (Whitney and Roberts, '55; Avigan and Steinberg, '58; Klein, '58). The increase in hepatic cholesterol concentrations in the rat was confined to the esterified pool.

TABLE 2

Effect of fat content of the diet on hepatic cholesterol in the rabbit

Group	% Corn oil in diet	Total cholesterol	Free cholesterol	Ratio of free to total
		mg/gm	*mg/gm*	
A	0	4.81 ± 0.67[1]	3.08 ± 0.17[1]	0.679 ± 0.057[1]
B	5	2.92 ± 0.13	2.57 ± 0.10	0.885 ± 0.018
C	10	3.79 ± 0.48	2.72 ± 0.12	0.782 ± 0.059
D	20	4.46 ± 0.16	3.16 ± 0.05	0.716 ± 0.025

Significance of differences by "t" test

Groups compared	"t"	P	"t"	P	"t"	P
B—A	3.53	< 0.01	2.68	< 0.02	4.24	< 0.001
B—C	1.57	< 0.2	1.0	< 0.4	1.87	< 0.1
B—D	7.33	< 0.001	4.9	< 0.001	5.6	< 0.001

[1] Mean ± standard error.

whereas a small increase was also found in the free hepatic cholesterol of the rabbit.

Inasmuch as the purified diets were free of cholesterol, the cholesterol under consideration was of endogenous origin. It has been concluded that the accumulation of hepatic cholesterol in rats fed a fat-free diet was not the result of a redistribution of tissue cholesterol (Alfin-Slater et al., '54). Whether the increase of endogenous cholesterol in these tissues was due to greater synthesis, decreased excretion, or other factors is subject to further investigation.

It is evident from these data that the amount of lipid in the diet influences not only the level of cholesterol, but also the distribution and the ratio of esterified to free cholesterol in the blood and liver of the rabbit. The observation that unsaturated fats lead to increased plasma and hepatic cholesterol levels in the rat (Avigan and Steinberg, '58; Klein, '58) and the rabbit should be considered in any evaluation of the role of unsaturated fats as a hypocholesterolemic agent in man. Indeed, in the light of current experimental evidence in animals, the lowering of serum cholesterol in man may be the result of a shift of cholesterol from the blood pool to the hepatic or other pools within the body.

SUMMARY

Serum and hepatic cholesterol levels of male, adult rabbits were determined at the end of a 12-week period of ad libitum maintenance with cholesterol-free purified diets. The purified diets contained either no lipid or 5, 10 or 20% of corn oil.

The total cholesterol concentration of the serum increased in all groups. The group receiving no lipid exhibited a greater cholesterol rise in the serum than the groups receiving corn oil. The increase in serum cholesterol of rabbits fed corn oil did not appear to be a function of the lipid content of the diet.

The total hepatic cholesterol concentration was increased in fat deficiency, was lowest at the minimum fat concentration, and increased with increasing fat content of the diet. This increase was characterized by a greater increase in the esterified fraction than in the free fraction.

ACKNOWLEDGMENTS

The technical assistance of J. T. Ellis and R. M. Kennedy is gratefully acknowledged.

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Conjoint Effects of Dietary Vegetable Fats and Cholesterol in Rabbits[1]

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It has been shown in rats that the greatest weight gains were obtained when the dietary fat contained fatty acids which were 30% saturated (Hopkins et al., '55; Murray et al., '58), similar to those of the depot fat. In the present study, to investigate weight gain responses in rabbits, a series of dietary fats was prepared from bayberry tallow and olive oil, and added to ground commercial pellets adequate in essential fatty acids.

Bayberry tallow consists almost entirely of glycerides of myristic and palmitic acids. Mixing this fat with olive oil provided a means of raising the saturated acid content of the diet and lowering the monounsaturated acid content (oleic) while keeping the polyunsaturated acid nearly constant. The major proportion of polyunsaturated acid in these diets was in the fat of the commercial feed pellets.

It was also desired to study the effect of variation in saturated acid content of the dietary fat on serum cholesterol levels, with and without the addition of cholesterol to the diet. Lambert et al. ('58) and Miller et al. ('59) have observed that rabbits develop hypercholesterolemia and aortic plaques when fed a fully saturated fat (hydrogenated coconut oil) in a purified diet, without dietary cholesterol.

MATERIALS

The olive oil used was of a commercial edible grade. The bayberry tallow[2] (fruit coat fat of *Myrica* species) had an iodine value of less than one. Examination of its fatty acids, after saponification, showed that they were composed essentially of palmitic and myristic acids, as reported for *Myrica cordifolia* fat (Schoeman and Hawke, '48). The pelleted rabbit feed[3] contained by analysis 2.5% of fat and had an iodine value of 117. The fatty acid composition, determined by ultraviolet absorption analysis after alkali isomerization, was as follows (%): saturated acids, 19.4; oleic acid, 37.4; dienoic acids as linoleic, 37.1; trienoic acids as linolenic, 6.1.

METHODS

In the first experiment, 30 male rabbits of mixed breeding were arranged in a randomized block design in individual cages, each block representing animals of similar initial body weight. The dietary fats, listed in table 1, were incorporated into the ground rabbit pellets to provide a fat intake of 10% by weight of the diet. In the subsequent experiments in which each group consisted of 8 male albino rabbits, 0.5% of cholesterol was supplied as indicated with the 10% of fat in the diet. All animals received food and water ad libitum for 14 weeks. During this time blood samples were removed from a marginal ear vein biweekly, serum cholesterol was determined by the method of Henly ('57) and hemoglobin by the method of Rimington ('42). Red blood cell counts were made on a Spencer Brightline hemacytometer, and hematocrit determinations by an International Micro-centrifuge Model M–B. At the termination of each experiment, the rabbits were anesthetized with pentobarbital sodium,[4] decapitated and examined for gross pathology. The left adrenal and a portion of the liver of each animal were frozen immediately for analyses of cholesterol and total lipids as previously described (Graham et al., '59)

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[1] Issued as N.R.C. no. 6113.
[2] Supplied by S. B. Penick and Company.
[3] Master Feeds, Toronto Elevators Ltd.
[4] Nembutal, Abbott.

TABLE 1

Composition of dietary fat

Fat added to ground rabbit pellets[1]	Approximate composition of the fatty acids of the total dietary fat[2]		
	Saturated	Monoene	Diene[3]
	%	%	%
None			
100% Bayberry tallow	80	9	11
68% Bayberry tallow, 32% olive oil	60	29	11
36% Bayberry tallow, 64% olive oil	39	49	12
9% Bayberry tallow, 91% olive oil	20	66	14

[1] 8.5 gm of fat was added to each 100 gm of the basal diet.
[2] Including the fatty acids present in the fat of the basal diet.
[3] Includes a small proportion of triene acid.

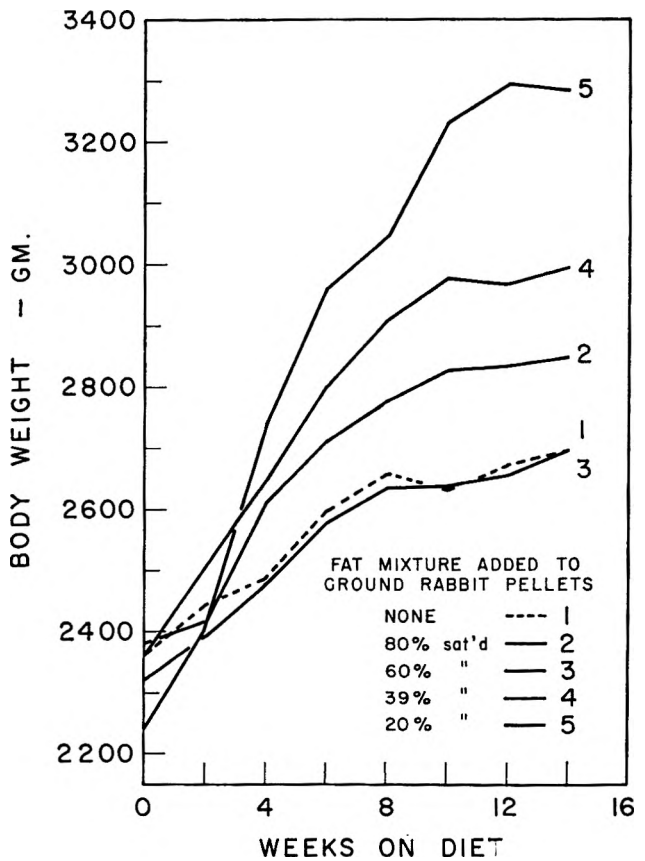

Fig. 1 Body weights of rabbits fed commercial pellets alone or supplemented to provide a 10% fat diet of 80, 60, 39 or 20% saturation.

The right adrenal, testes, heart and attached major vessels, eyes and a portion of liver, spleen, stomach, kidney, prostate and pancreas were fixed in 10% formalin in normal saline and sections of 4 μ of each tissue stained with hematoxylin and eosin. Sections of stomach were also stained with Gomori's One Step Trichrome stain and Mayer's Mucicarmine stain. Frozen stomach sections of 10 μ were

stained with Oil red O. Aortas were examined and rated by a modification of the method of Gore and Tejada ('57).

RESULTS

The weight gains of rabbits supplied with fats of varying degrees of saturation and no cholesterol, experiment 1, are shown in figure 1. With the 20% saturated fat, the weight gains were significantly greater at $P = 0.01$ than with the 60 or 80% saturated mixtures or with no additional fat, and significantly greater at $P = 0.05$ than with the 39% saturated fat. As shown in table 2, the degree of saturation of the dietary fat had no effect on organ weights nor on the total lipid concentration of the liver, but appeared to increase the cholesterol fraction in the liver of those rabbits receiving the most unsaturated fat. The addition of cholesterol to the 20% saturated fat did not affect the body weights of the albino rabbits studied in experiment 2, but significantly increased ($P = 0.01$) the liver, spleen, and adrenal weights, and the total lipid and cholesterol concentrations of the liver. In experiment 3 variation in the degree of saturation of the fat in the presence of dietary cholesterol produced no significant effects.

The values for serum cholesterol are shown in figure 2 and were found to be not significantly different for rabbits receiving the cholesterol-free diets (curves 1–6). In animals fed 0.5% of cholesterol the serum cholesterol showed a rapid increase for approximately 6 weeks followed by a more gradual elevation, the extent of which was not influenced by the degree of saturation of the fat.

In the absence of dietary cholesterol there were no significant differences in the hematological values shown in table 3 nor in the gross pathology. Rabbits fed cholesterol showed a normocytic anemia, the usual organ changes and plaque formation in the aorta which was not affected by the degree of saturation of the fat. In addition, there was an unexpected hyperplasia of the epithelium of the gastric mucosa, figure 3. This was also observed in stomachs of all rabbits receiving each degree of saturated fat with cholesterol, and appeared to be most marked in those

TABLE 2

Effect of degree of saturation of dietary fat and cholesterol on body and organ weights, and liver total lipid and cholesterol

Exp. no.	Saturation of fat	Dietary cholesterol	Body weight	Liver weight	Spleen weight	Testes weight	Left adrenal	Total liver lipid	Liver cholesterol
	%		*gm*	*gm*	*gm*	*gm/pair*	*mg*	*% by weight*	*mg/100 gm tissue*
1	—	—	2700 ± 180[1]	63 ± 4	—	5.2 ± 3.1	143 ± 30	2.18 ± 0.08	156 ± 5
	80	—	2850 ± 230	72 ± 8	—	5.8 ± 4.7	187 ± 31	2.96 ± 0.38	167 ± 9
	60	—	2700 ± 140	61 ± 3	—	5.7 ± 2.5	158 ± 29	2.71 ± 0.15	194 ± 12
	39	—	2990 ± 150	67 ± 6	—	5.6 ± 2.7	187 ± 18	2.96 ± 0.39	216 ± 23
	20	—	3280 ± 140	74 ± 8	—	6.2 ± 3.0	150 ± 17	2.93 ± 0.13	302 ± 54
2	20	—	2640 ± 130	59.2 ± 4.2	0.75 ± 0.05	4.10 ± 0.38	131 ± 15	3.07 ± 0.30	200 ± 15
	20	+	2680 ± 120	89.0 ± 4.3	1.27 ± 0.14	3.25 ± 0.30	292 ± 20	7.47 ± 0.66	1178 ± 90
3	80	+	2520 ± 253	88.9 ± 5.6	1.55 ± 0.35	2.19 ± 0.65	286 ± 50	4.83 ± 0.45	850 ± 28
	50	+	2520 ± 202	87.0 ± 11.5	1.44 ± 0.21	2.19 ± 0.45	282 ± 29	5.65 ± 0.21	1030 ± 53
	20	+	2730 ± 75	92.8 ± 4.3	1.60 ± 0.17	2.68 ± 0.24	261 ± 14	6.15 ± 0.48	1168 ± 45

[1] Mean ± standard error.

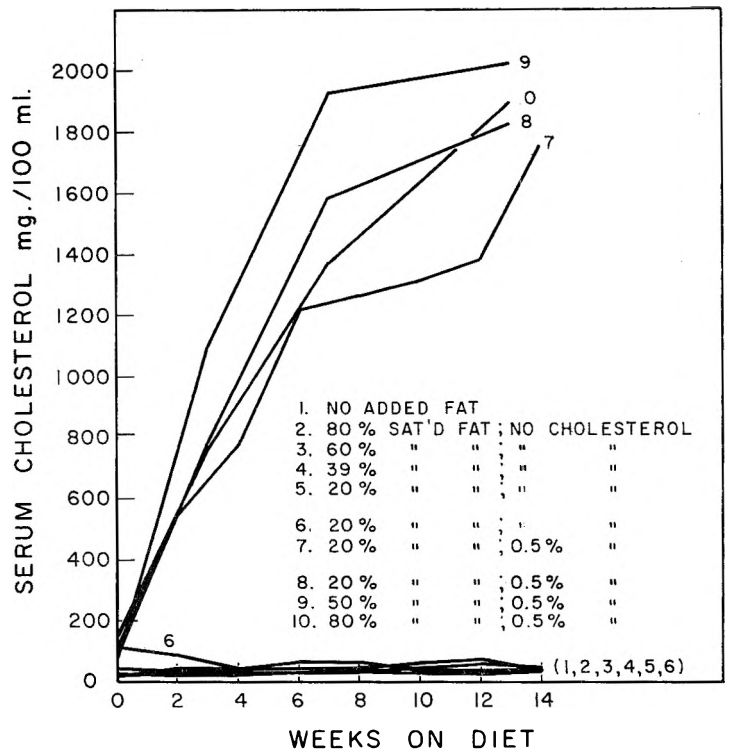

Fig. 2 Serum cholesterol levels of rabbits fed fats of varying degrees of saturation without or with 0.5% cholesterol.

TABLE 3

Hematology of rabbits fed experimental diets for 14 weeks

Exp. no.	Saturation of fat	Dietary cholesterol	R.B.C.	Hemoglobin	Hematocrit packed cells
	%		*millions/ mm³*	*gm/100 ml*	%
1	—	—	—	12.3 ± 0.5[1]	40.4 ± 1.7
	80	—	—	12.8 ± 0.5	41.9 ± 1.2
	60	—	—	13.7 ± 0.5	43.4 ± 1.2
	39	—	—	13.8 ± 0.4	44.7 ± 0.7
	20	—	—	13.3 ± 0.3	44.5 ± 0.9
2	20	—	6.7 ± 0.3	12.6 ± 0.5	41.6 ± 1.6
	20	+	5.5 ± 0.4	10.3 ± 0.5	35.4 ± 1.5
3	80	+	5.3 ± 0.2	9.5 ± 0.2	30.1 ± 0.8
	50	+	5.3 ± 0.3	8.9 ± 0.6	32.0 ± 1.5
	20	+	4.6 ± 0.2	7.8 ± 0.5	27.8 ± 1.9

[1] Mean ± standard error.

fed the most saturated fats. The hyperplasia occurred in the glandular and supporting elements with edema and infiltration of foam cells in the lamina propria, and in some cases resulted in a five- to tenfold increase in the thickness of the mucosa.

DISCUSSION

It was previously found with rats (Hopkins et al., '55; Murray et al., '58) that weight gains were greatest when the dietary fat was predominantly unsaturated. Whereas rats exhibited the best response with a fat mixture which was 30% satu-

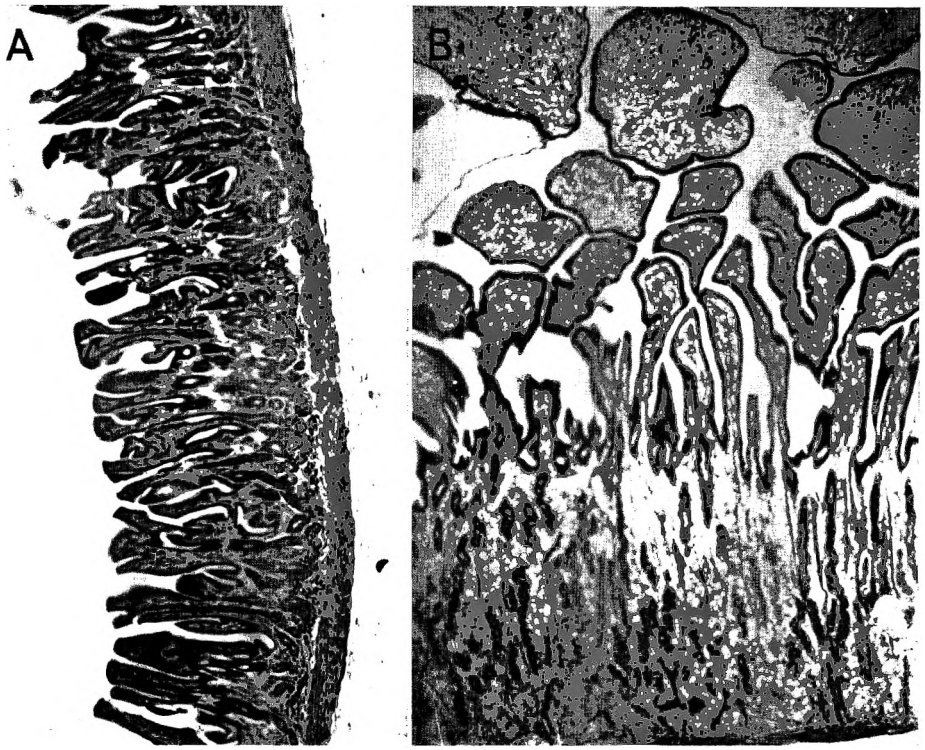

Fig. 3 (A) Normal gastric epithelium and (B) hyperplasia of the gastric epithelium with edema and infiltration of foam cells in the lamina propria. Hematoxylin and eosin; (a) × 32; (b) × 20.

rated, the rabbits gained more weight when receiving the 20% saturated fat than with the 39% saturated mixture. Early studies with rats and guinea pigs showed that cholesterol fed with fat depressed weight gains (Cook, '36). In the current experiments, the addition of cholesterol to the various fats eliminated the differences in body weights, and in a manner similar to that previously observed (Graham et al., '59), increased the liver and adrenal weights.

As shown in experiment 1 the greatest increase in the cholesterol content of the liver, as determined by a ferric chloride method, occurred with fat having the highest proportion of monounsaturated fatty acids. Using 10% of corn oil and 2% of cholesterol in a laboratory chow, Lee and Herrmann ('59) detected an increased cholesterol deposition in the liver and aorta of rats.

In the absence of dietary cholesterol, varying the degree of saturation of the fat portion of the diet did not affect serum cholesterol levels in these experiments. All of the diets, however, contained adequate essential fatty acids and the degree of saturation of the total fat was within the limits of 20 to 80%. Earlier workers observed changes in serum cholesterol levels on feeding fats whose composition was outside these limits. Thus, Lambert et al., ('58) and Miller et al., ('59) fed fats of practically 100% saturation or safflower oil. The latter has only about 12% of saturated acids and a very high content of dienoic acid.

The powerful effect of dietary cholesterol in elevating serum cholesterol and producing aortic plaques in rabbits is well known. It was thought that these effects might be reduced by one or another of the fat mixtures employed in this work. However, no significant reduction was observed. It is evident that such variation of the saturated acid content of the dietary fat in a basal diet of ground rabbit pellets

does not have much, if any, influence on the results of cholesterol feeding. Van Handel and Zilversmit ('57) detected no change in rabbits fed a commercial diet to which was added 35% of liquid cottonseed oil, selectively-hydrogenated cottonseed oil or corn oil. Likewise, Steiner et al. ('59) found that safflower oil in a commercial diet failed to reduce the serum cholesterol levels or alter the aortic condition of rabbits. Using semipurified diets, other workers (Lambert et al., '58; Wigand, '59) demonstrated a definite hypercholesterolemia and the presence of aortic plaques in rabbits fed hydrogenated coconut oil.

The marked hyperplasia of the gastric mucosa, resulting from the cholesterol-fat diets, has apparently not been observed previously. It did not occur when the same fat diets were fed without cholesterol.

The anemia resulting from the cholesterol-fat diets appeared to be a normocytic type although in earlier studies with a cholesterol-low fat diet (Graham et al., '59), a macrocytic anemia was produced.

SUMMARY

Rabbits were given a commercial diet with added fats such that the total fat contained 20, 39, 60 and 80% of saturated acids (as glycerides) and the linoleic acid content was approximately 12%. The level of fat in the diet was 10%. The 20% saturated fat produced the highest weight gain. There were no significant differences in serum cholesterol but the concentration of liver cholesterol varied inversely with the saturation of the fat, as determined by a ferric chloride method.

Addition of 0.5% of cholesterol to the above diets gave the expected high levels of serum cholesterol and formation of aortic plaques, but the degree of saturation of the dietary fat did not alter the magnitude of these effects significantly, within the limits studied. The cholesterol-fat diets increased the weights of liver, spleen and adrenals and the concentration of total lipids and cholesterol in the liver, and produced a normocytic anemia and a hyperplasia of the epithelium of the gastric mucosa.

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Effect of Choline Deficiency and Ethionine Feeding on Nucleic Acid Content of Rat Livers[1,2,3]

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The induction of tumors in rats as a result of prolonged choline deficiency was first reported from this laboratory (Copeland and Salmon, '46). Numerous studies on ethionine and other factors related to choline deficiency carcinogenesis have been made in our laboratory (Schaefer et al., '50; Salmon et al., '54, '55, '58),[5] but the biochemical lesion leading to the induction of these tumors has not been defined.

In view of a possible relationship of nucleic acids to tumorigenesis, determinations of the effects of choline deficiency on the nucleic acid content of rat livers and tumors were made. The results show significant increases of deoxyribonucleic acid (DNA) in the livers and tumors of choline-deficient rats. The effects of ethionine, methionine and vitamin B_{12} and folacin are also shown.

EXPERIMENTAL

Animals and diets. Weanling rats of the Alabama Experiment Station (AES) and Sprague-Dawley (SD) strains were grouped uniformly with respect to number, weight and sex and individually caged on wire screens in an air-conditioned animal room. Water and feed were given ad libitum.

The basal diet consisted in per cent of peanut meal, 30; casein, 6; salt mix no. 5 (Salmon, '47), 5; lard, 19; cod liver oil, 1; and α-tocopherol and the folacin-free, cobalamin–free vitamin mix as previously described (Alexander and Sauberlich, '57) and sucrose to make 100%. Diet supplements tested are shown in the respective tables.

Rats that received the choline-deficient basal diet were given an initial 0.1% of choline Cl in the diet for two weeks and 0.05% for two additional weeks. Control animal diets contained 0.2% of choline Cl for the entire experimental period. Rats with the transplanted tumors were fed a complete diet used to maintain the stock colony in this laboratory.

Transplantable hepatoma and cholangiofibrosis tumors were obtained from other experiments in this laboratory; the original tumors were induced by choline-deficient diets (Salmon et al., '55) and maintained as subcutaneous transplants in AES rats.

Assay procedure

The rats were killed and the tumors and livers removed and homogenized with a virtis homogenizer and ice bath. The separation of ribonucleic acid (RNA) and DNA from associated protein material was carried out by the procedure of Schneider ('45). The RNA was determined by the orcinol reaction for pentose as described by Brown ('45). The DNA determination was by the procedure of Webb and Levy ('55) in which *p*-nitrophenylhydrazine was used to measure quantitatively the deoxyribose.

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[3] Data from dissertation of Preston T. Farish submitted in partial fulfillment for Ph.D. degree, Auburn University.

[4] Present address: U. S. Army Medical Research and Nutrition Laboratory, Fitzsimmons General Hospital, Denver 30, Colorado.

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Aliquots of fresh tumor and liver tissue were weighed, dried in an electric oven at 95°C for 24 hours, and reweighed to determine the moisture content. The dried tissue was ground in a mortar and placed in a Nolan multiple extractor where the samples were extracted with anhydrous ether for 24 hours for determination of the "fat" content.

RESULTS AND DISCUSSION

Livers of SD rats fed a choline-deficient diet had a higher content of DNA per unit of fat-free, dry weight than livers of animals supplemented with choline (tables 1 and 2). Ethionine supplementation in the diet was more effective in increasing DNA content of livers of SD rats than a choline-deficient diet. The DNA increase caused by ethionine was not prevented by 0.20% of choline Cl but was effectively prevented by 0.50% of methionine. SD rats fed a diet containing ethionine, vitamin B_{12}, and folacin had a greater amount of DNA per unit weight than animals receiving a choline-supplemented diet or choline-deficient diet, but had less than the livers of rats fed diets containing ethionine and choline or 0.3% of ethionine.

There was some increase in DNA content of livers of AES rats fed the choline-deficient diet (table 3) but considerably less increase than in the SD rats. One difference in the two strains was a higher DNA value in the AES rats receiving choline. Since AES rats have a higher choline requirement than SD rats, it is possible that 0.20% of choline Cl in the diet was not adequate to maintain a normal DNA level in the liver. Only in the AES rats receiving vitamin B_{12} and folacin or stock diet were the liver DNA levels comparable to those of the SD rats receiving choline.

DNA values were much higher in transplanted tumors than in the liver of host AES rats receiving stock diet (table 4). The increase was 40% for the hepatomas and 83% for the cholangiofibroses when values were expressed on a dry fat-free basis. RNA values (dry weight basis) were lower in the tumors than in the livers. A similarity in the DNA content of tumors from different sources was noted by Schneider ('47) who reported an increased level of DNA in tumors as compared with the normal tissue in which the tumors developed.

SUMMARY

Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) content was determined in livers and tumors of rats. The liver DNA and RNA were measured for choline-deficient animals and for those receiving dietary supplements of choline, ethionine, methionine, betaine, vitamin B_{12}, and folacin or certain combinations of these. In the livers of Sprague–Dawley (SD) rats, the DNA content was markedly increased by choline deficiency or ethionine supplementation of the diet. DNA content of the livers of choline-deficient Alabama Experiment Station (AES) rats was also high but was not at as low a level with 0.20% of dietary choline Cl in the AES rats as it was in the SD rats. Supplementation of the basal diet with vitamin B_{12} and folacin resulted in normal levels of DNA in livers of AES rats. The DNA content of transplanted hepatomas and cholangiofibrosis tumors was much higher than of livers of AES rats bearing the transplanted tumors.

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TABLE 1

Nuclcic acid contcnt of livers of Sprague-Dawley rats receiving choline-deficient basal diet with various supplements

Group no.[1]	Supplement[2]	Deoxyribonucleic acid (DNA)		Ribonucleic acid (RNA)		Total liver weight	Total body weight
		Fresh tissue	Dry, fat-free tissue	Fresh tissue	Dry, fat-free tissue		
		mg/100 gm	*mg/100 gm*	*mg/100 gm*	*mg/100 gm*	*gm*	*gm*
1	None	279 ± 3.7[3]	1789 ± 79.7[3]	788 ± 46.3[3]	5340 ± 26.5[3]	21.9 ± 0.9[3]	267 ± 26.0[3]
2	Choline Cl, 0.20%	227 ± 15.4	1060 ± 30.0	1031 ± 21.0	4753 ± 74.2	8.2 ± 0.4	340 ± 10.7
3	Ethionine, 0.30%	598 ± 38.5	2914 ± 35.0	962 ± 14.6	4656 ± 31.1	7.7 ± 1.1	171 ± 4.0
4	Ethionine, 0.30%, choline Cl, 0.20%	567 ± 83.2	3110 ± 51.8	1020 ± 14.1	5267 ± 30.8	8.5 ± 0.9	201 ± 9.1
5	Ethionine, 0.30%, methionine, 0.50%	250 ± 14.1	1092 ± 47.1	1162 ± 44.9	5208 ± 14.2	10.9 ± 1.5	250 ± 22.1
6	Ethionine, 0.30%, B_{12}, folacin	477 ± 12.2	2223 ± 46.6	878 ± 11.7	4065 ± 37.3	7.8 ± 0.6	294 ± 7.2

[1] Six animals/group.
[2] Group 1, 0.10% choline Cl, 2 weeks; 0.05% choline Cl, 2 weeks; no choline last 5 weeks; group 2, 0.20% choline Cl, 9 weeks; group 3, same as group 1 plus 0.30% ethionine last 5 weeks; group 4, same as group 2 plus 0.30% ethionine last 5 weeks; group 5, same as group 1 plus 0.50% DL-methionine last 5 weeks; group 6, same as group 1 plus 2 mg/kg diet folacin and 50 μg/kg diet B_{12} last 5 weeks.
[3] Standard deviation.

TABLE 2

Nucleic acid content of livers of Sprague-Dawley rats receiving choline-deficient basal diet with various supplements

Group no.[1]	Supplement[2]	DNA		RNA		Total liver weight	Total body weight
		Fresh tissue	Dry, fat-free tissue	Fresh tissue	Dry, fat-free tissue		
		mg/100 gm	*mg/100 gm*	*mg/100 gm*	*mg/100 gm*	*gm*	*gm*
7	None	228 ± 10.5[3]	1545 ± 42.3[3]	700 ± 14.1[3]	4635 ± 10.5[3]	36.0 ± 0.9[3]	384 ± 65.0[3]
8	Choline Cl, 0.20%	148 ± 9.9	1019 ± 15.8	679 ± 9.0	4631 ± 15.8	21.9 ± 3.8	524 ± 46.7
9	Ethionine, 0.20%, choline Cl, 0.20%	375 ± 25.9	1740 ± 34.6	1115 ± 13.8	5073 ± 32.7	9.8 ± 0.6	207 ± 30.1
10	Ethionine, 0.20%, choline Cl, 0.20%; methionine, 0.50%, B_{12}, folacin	240 ± 8.9	1033 ± 53.0	1165 ± 27.4	5018 ± 11.7	12.7 ± 1.2	515 ± 94.2

[1] Six animals/group.
[2] Group 7, 0.10% choline Cl, 2 weeks; 0.05% choline Cl, 2 weeks; no choline last 57 weeks; group 8, 0.20% choline Cl, 61 weeks; group 9, same as group 8 plus 0.20% ethionine last 57 weeks; group 10, same as group 8 plus 0.20% DL-ethionine, 0.50% DL-methionine, 2 mg/kg diet folacin, and 50 μg/kg diet B_{12} last 57 weeks.
[3] Standard deviation.

TABLE 3

Nucleic acid content of livers of Alabama Experiment Station (AES) rats receiving choline-deficient diets with various supplements

Group no.[1]	Supplement[2]	DNA		RNA		Total liver weight	Total body weight
		Fresh tissue	Dry, fat-free tissue	Fresh tissue	Dry, fat-free tissue		
		mg/100 gm	*mg/100 gm*	*mg/100 gm*	*mg/100 gm*	*gm*	*gm*
11	None	230 ± 11.8[3]	1385 ± 38.7[3]	724 ± 28.8[3]	4342 ± 24.3[3]	25.5 ± 4.8[3]	394 ± 79.8[3]
12	Choline Cl, 0.20%	248 ± 3.0	1238 ± 64.2	954 ± 14.1	4701 ± 255.7	13.1 ± 2.2	421 ± 23.4
13	Betaine·HCl, 0.70%	288 ± 21.1	1341 ± 75.6	1045 ± 38.5	4871 ± 154.6	9.3 ± 2.4	317 ± 60.1
14	B_{12}, folacin	258 ± 16.1	1043 ± 79.1	883 ± 12.0	3530 ± 98.7	12.2 ± 1.7	510 ± 30.6

[1] Six animals/group.
[2] Group 11, 0.10% choline Cl, 2 weeks; 0.05% choline Cl, 2 weeks; no choline Cl last 57 weeks; group 12, 0.20% choline Cl, 61 weeks; group 13, same as group 11 plus 0.70% betaine·HCl last 57 weeks; group 14, same as group 11 plus 2 mg/kg diet folacin and 50 μg/kg diet B_{12} last 57 weeks.
[3] Standard deviation.

TABLE 4

Nucleic acid content of transplanted cholangiofibroses and hepatomas and of livers of host rats (AES) receiving stock diet

Tissue[1]	DNA		RNA		Total tissue weight	Total body weight
	Fresh tissue	Dry, fat-free tissue	Fresh tissue	Dry, fat-free tissue		
	mg/100 gm	*mg/100 gm*	*mg/100 gm*	*mg/100 gm*	*gm*	*gm*
Cholangiofibroses	300 ± 18.1[2]	2050 ± 104.4[2]	440 ± 41.4[2]	3005 ± 208.1[2]	11.8 ± 2.5[2]	199 ± 33.5[2]
Liver	290 ± 13.6	1120 ± 118.6	1390 ± 36.6	5350 ± 107.7	9.0 ± 1.3	
Hepatomas	280 ± 13.0	1670 ± 131.5	830 ± 33.2	4940 ± 230.0	16.7 ± 7.8	143 ± 67.2
Liver	280 ± 13.5	1190 ± 116.7	1270 ± 22.0	5400 ± 163.1	5.2 ± 1.8	

[1] Seven animals/group.
[2] Standard deviation.

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Protein Levels and Survival Time of Chicks Infected with *Salmonella gallinarum*[1]

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The effect of diet on an animal's reaction to infection has been an intriguing area of investigation for many years (see reviews, Schneider, '46; Clark et al., '49; Elberg, '56; Geiman, '58). Although the existence of a relationship between diet and resistance or susceptibility to infection is generally recognized, the facts of such a relationship have not always been clear. A case in point is the effect of protein on resistance or susceptibility to infection. The studies thus far reported do not reveal any predictable correlation between protein intake and resistance to infections in general. Koerner ('49), for instance, found that rats fed a 40% protein diet lived longer and had less extensive tuberculosis than animals fed a 25 or 15% protein diet. Ratcliffe[2] also noted that the progress of tuberculosis in guinea pigs was inversely related to the protein content of the diet. The experiments of Dubos and Schaedler ('58) and Schaedler and Dubos ('59) with tuberculosis and other infections in mice indicated the same trend.

While these findings indicate the value of higher protein levels with the infections and host species studied, other evidence has been reported indicating that higher protein levels increase susceptibility. Ratcliffe,[3] for instance, found that the progress of tuberculosis in rats, unlike guinea pigs, was directly related to the protein content of the diet. Smith and Chubb ('57) presented evidence indicating that the addition of certain protein concentrates to an all-cereal diet increased the susceptibility of chicks to *Salmonella gallinarum* infection. These latter studies, however, are subject to a number of interpretations. The differences in the kinds of protein fed might have influenced the results because of a difference in the amino acid balance of the diet, or in the case of the high levels of fish meal supplement fed, because of a difference in the mineral content of the diet.

The experiments presented in this report were designed to minimize these objections and to establish and clarify the effect of protein levels on the resistance of chicks to *S. gallinarum* infection.

EXPERIMENTAL

White Plymouth Rock chicks, obtained from a commercial hatchery, were used in all the experiments. Thirty to 40 chicks were used per treatment in each trial, and housed in electrically heated battery brooders with raised wire floors. The protocol of the experimental procedures was the same as that presented previously (Hill and Garren, '55). Briefly, the chicks were fed the experimental diets on the day of hatching. At 4 weeks of age they were inoculated orally with one milliliter of a 24-hour culture of *S. gallinarum* that had been grown on proteose no. 3 agar.[4] The organisms were washed from the agar with sterile saline (0.85%) and diluted with saline so that a reading of 10% light transmission was obtained on a Bausch and Lomb Spectronic 20 spectrophotometer at 650 mμ. The chicks were continued on the experimental regimes until the disease had run its course. This was usually within two weeks after infection. Mortality was recorded daily.

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[2] Ratcliffe, H. L. 1954 Influence of protein intake on tuberculosis in guinea pigs. Federation Proc., *13*: 441 (abstract).

[3] See footnote 2.

[4] Difco.

TABLE 1

Basal diets

	Diet 1	Diet 2 (Exp. 3)
	%	%
Soybean meal (50% protein)	20, 40 or 60	
Casein		25 or 37.5
Defluorinated rock phosphate	4.0	4.0
NaCl	0.5	0.5
DL-Methionine	0.3	0.3
Vitamin mix[1]	1.0	1.0
L-Arginine·HCl		0.7
Glycine		0.5
KCl		0.2
$MgSO_4$		0.5
$MnSO_4 \cdot 4H_2O$, mg/pound	100	100
KI, mg/pound		1
$CuSO_4 \cdot 5H_2O$, ppm		32
$FeSO_4 \cdot 7H_2O$, ppm		400
Glucose[2] to	100	100

[1] Supplies per pound of diet: (in milligrams) thiamine, 0.9; riboflavin, 1.6; niacin, 8.0; pantothenic acid, 5.0; folic acid, 0.45; pyridoxine, 1.6; choline, 700; menadione, 10; vitamin E, 10; and biotin, 45 μg; vitamin B_{12}, 4 μg; vitamin A, 3000 I.U.; and vitamin D_3, 180 I.C.U.

[2] Cerelose, Corn Products Refining Co., New York.

The basal diets used in these studies are presented in table 1. In experiments one, two and 4, all the protein was supplied by soybean oil meal. The protein level of the diet was then adjusted by changing the level of this one ingredient at the expense of glucose.[5] This method allowed the amino acid ratios to remain constant, with the exception of methionine, in all the protein levels fed. In the second experiment the calculated productive energy of the various protein diets was equalized by including 25% of nonnutritive cellulose,[6] at the expense of glucose in the 10% protein diet, and 12.5% of the cellulose in the 20% protein diet. In the third experiment the casein diet at 20 and 30% protein levels was compared with the standard soybean meal-glucose diet at these protein levels. The results of these experiments were analyzed statistically by analysis of variance. The least significant differences were calculated and are indicated in the appropriate tables.

RESULTS AND DISCUSSION

In the first and subsequent experiments protein levels of 10, 20 and 30% were used. For the chick, these levels represent a deficiency, a sufficiency and an excess of protein, respectively. The results of that study are presented in table 2. As the protein level increased the mortality increased throughout the experimental period. However, the differences due to protein level were much greater in the early part of the experimental period than at the end. As the experiment progressed the chicks fed the lower levels of protein continued to die. These findings indicate that the rate of mortality of chicks infected with this organism is directly proportional to dietary protein levels. The figures for total survivorship at the end of the experimental period are less impressive since even with the 10% protein diet 85% of the chicks died.

While the findings of the first experiment indicated a deleterious effect of protein during the course of the interaction between the pathogen and host, there was the possibility that the effect was not, in fact, due to protein. The protein was in-

[5] Cerelose, Corn Products Refining Company, New York.

[6] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

TABLE 2

Protein level and death of chicks from S. gallinarum

Protein	No. chicks	Av. weight at inoculation	Days past inoculation				
			5	7	9	11	13
%		*gm*	*% cumulative deaths*				
10	34	218	2.9	29.4	52.9	76.4	85.3
20	33	376	12.0	63.0	72.0	87.0	93.0
30	33	384	24.0	81.0	100.0	100.0	100.0
LSD 0.05				16.2	17.7	6.9	33.5
LSD 0.01				24.5	26.8	13.7	50.7

creased in the first experiment by increasing the soybean meal at the expense of glucose. This manipulation resulted in diets of widely differing productive energy contents. In order to determine whether the energy level of the diet played any part in the observed reaction, the experiment was repeated and two additional lots were used in which the productive energy levels of the 10 and 20% protein diets were reduced to that of the 30% protein diet. The results of that experiment are presented in table 3.

It is apparent that the energy level did not influence the results. Again the higher levels of protein resulted in a higher mortality during the early part of the experimental period. This effect, however, again lessened as the experiment progressed, and at the end of the experimental period, there was no significant difference between the treatments. This experiment, then, confirms the first one in that higher levels of protein increase the mortality rate during the course of this infection.

The possibility remained that the observed effect was, in fact, not due to protein. If the soybean meal contained some entity besides protein that enhanced susceptibility, increasing the soybean meal in order to increase the protein level would result in an increased level of the hypothetical susceptibility factor. To test this possibility an experiment was conducted in which 20 and 30% protein levels were fed with the protein supplied by either soybean meal or casein. The results of that study are presented in table 4.

The source of the protein did not affect the results. Thirty per cent protein from either soybean meal or casein resulted in greater mortality throughout the experimental period. At the end of this period the differences in mortality between the two protein levels from casein were statistically significant, whereas the differences between the two soybean meal levels were not. Although these results do not prove the nonexistence of a susceptibility factor, the occurrence of such a factor in two such

TABLE 3

Protein level, productive energy, and death of chicks from S. gallinarum

Protein	No. chicks	Av. weight at inoculation	Productive energy	Days past inoculation				
				5	7	9	11	13
%		*gm*	*Cal./pound*		*% cumulative deaths*			
10	37	219	1073	8.1	29.7	45.9	62.1	72.9
10	38	237	727	0	36.8	42.0	65.7	84.2
20	38	288	896	7.9	47.3	57.9	78.9	86.8
20	38	282	727	2.6	50.0	63.1	78.9	86.8
30	39	291	727	12.8	66.6	74.2	79.4	87.0
LSD 0.05	20% vs. 10%				16.3	15.3	22.4	30.8
LSD 0.01					25.5	24.0	35.1	48.3
LSD 0.05	30% vs. others				19.95	18.7	27.4	37.7
LSD 0.01					31.3	29.4	42.9	59.1

TABLE 4

Level of two proteins and death of chicks from S. gallinarum

Protein	Source	No. chicks	Av. weight at inoculation	Days past inoculation				
				5	7	9	11	13
%			*gm*		*% cumulative deaths*			
20	Casein	27	211	3.7	29.6	66.7	66.7	66.7
30	Casein	26	176	15.3	57.6	92.3	92.3	92.3
20	Soybean	56	288	1.8	30.3	53.5	57.1	60.7
30	Soybean	48	224	25.0	56.2	77.0	79.1	79.1
LSD 0.05					12.5	25.1	23.5	24.0
LSD 0.01					18.2	36.6	34.2	32.1

diverse materials as soybean meal and casein must be considered remote, and the results obtained must therefore be attributed to protein.

The results of these three experiments considered together clearly indicated that the effect of increasing dietary protein levels during the course of interaction between the host and pathogen was deleterious to the host. Since the organism was given orally, possibly the higher protein level in the intestinal contents might have affected the organism in some way to render it more virulent for the host. In order to eliminate this possibility the experiment was repeated and the organism given both orally and intramuscularly. The results of that experiment are presented in table 5.

The profile of mortality was unchanged regardless of the manner of inoculation. The effect of protein was not dependent upon diet and pathogen meeting in the intestine. At the end of the experimental period in this trial as in the second and third there was no significant difference in mortality between the three levels of soybean protein when the organism was given orally as in all the previous experiments. However, the differences between those groups infected by intramuscular inoculation continued to be statistically significant.

The results of this series of experiments clearly demonstrate that as the level of protein was increased the rate of mortality with this pathogen was increased. The fact that usually total survivorship was not affected by protein manipulation has some significance in considering the meaning of these experiments. Schneider ('60) has discussed the implications of rate of mortality as opposed to survivorship as a measure of natural resistance to infection. According to the concept developed by this investigator, neither the experiments presented in this report nor those presented by Dubos and Schaedler ('58, '59) and Schaedler and Dubos ('59) can be considered as evidence that dietary protein levels influence resistance to infection. In the chick dietary protein acts in a catalytic manner on the sequence of reactions leading to death as the host interacts with S. *gallinarum*. In the mouse with the pathogens studied by Dubos and Schaedler ('58, '59) and Schaedler and Dubos ('59), dietary protein evidently inhibits the rate of this sequence of reactions. In both species protein appears to act as a rate of reaction regulator. Whether this is a direct or indirect action is not known. The differences between the two species, in response to higher protein levels, deceleration in the case of mice and acceleration in the case of chicks is worthy of further study.

TABLE 5

Effect of protein level and route of administration of pathogen on death of chicks from S. gallinarum

Protein	No. chicks	Av. weight at inoculation	Days past inoculation (orally)				
			5	7	9	11	13
%		gm	% cumulative mortality				
10	44	199	4.5	31.8	52.2	72.7	88.6
20	44	292	0	54.5	88.6	88.6	88.6
30	42	232	11.9	66.7	90.4	97.5	97.6
LSD 0.05				16.2	17.7	6.9	33.5
LSD 0.01				24.5	26.8	13.7	50.7
			Days past inoculation (intramuscularly)				
			2	4	6	8	10
			% cumulative mortality				
10	41	210	2.4	26.8	48.7	60.9	73.1
20	47	293	0	23.4	61.7	74.4	85.1
30	42	249	11.9	54.7	83.3	97.6	100
LSD 0.05				11.5	8.8	16.6	5.9
LSD 0.01				21.0	16.2	30.5	10.8

SUMMARY

Increasing the protein level of diets from 10% to 20% to 30% resulted in a progressively increased rate of mortality of chicks from *Salmonella gallinarum* infection. The rate of mortality was not affected by the energy content of the diet. The acceleration of mortality was evident whether the protein was supplied by soybean meal or casein. Since the acceleration was noted when the organism was given either orally or intramuscularly, it was concluded that the effect of the increased protein level did not depend on a meeting of pathogen and diet in the intestinal tract. While total mortality was generally increased with increasing protein levels, the differences were not statistically significant in most of the comparisons.

ACKNOWLEDGMENT

The authors are indebted to Dr. W. G. Smart, Jr. for the statistical analysis of the data.

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Effect of Phosphorus Supplementation on Egg Shell Composition[1,2]

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In 1940 Wilhelm showed that a decline in thickness of the egg shell was correlated with a rise in ambient temperature. Also, thinner egg shells were obtained by Warren and Schnepel ('40) immediately after experimental increase in environmental temperature from 20 to 32.5°C. Recovery of shell thickness was observed when temperature was returned to normal.

The effect of phosphorus supplements and high environmental temperature on the mineral constituents of the shell has not been studied. Almquist and Burmester ('34) in describing a "glassy" egg shell which would normally fall in the commercial thin-shell grade, stated that the shell was slightly lower in $CaCO_3$; otherwise, there was no difference in the mineral composition of this shell and that of a normal shell. However, when the phosphorus values of the glassy shells were compared with those of normal shells, the percentage phosphate values were 0.100 and 0.127%, respectively. Common ('36) produced thin-shelled eggs experimentally by withholding calcium from the diet. His data also indicated that the absolute weight of phosphorus per gram of shell was decreased, in contrast with the work of Buckner et al. ('23), who pointed out that as the egg shell became thinner the percentage composition was not altered.

This study was initiated to determine the effect of dietary phosphorus level on the mineral constituents of the egg shell.

EXPERIMENTAL

At approximately 20 weeks of age, 250 Single Comb White Leghorn pullets of similar breeding were randomly distributed among 5 floor pens of 50 pullets each. Pens were equipped with an automatic water supply, metal feed troughs and the recommended amounts of roosting and nesting space. Straw was used for floor litter. A minimum of 14 hours of total light was provided daily throughout the study. Maximum and minimum laying house temperatures at bird level were recorded daily. Pullets were fed a basal diet and supplemented with soft phosphate, dicalcium phosphate or combinations thereof as shown in table 1. Phosphorus supplements were added at the expense of ground milo. The percentage composition of the basal diet follows:[3] ground yellow corn, 20; ground milo, 35.16; ground wheat, 10; dehydrated alfalfa meal (17%), 5; solvent soybean oil meal (44%), 17.70; fish meal (62%), 2.5; dried whole whey, 1.25; NaCl, 0.2; ground limestone, 5.65; and $MnSO_4 \cdot 2H_2O$ (70%), 0.04. In addition a vitamin premix, containing soybean oil meal as a carrier, was fed at the 2.5% level to supply the following per pound of diet: vitamin A, 4,500 I.U.; vitamin D_3, 700 I.C.U.; riboflavin, 2 mg; niacin, 12.5 mg; D-Ca pantothenate, 5 mg; choline chloride, 200 mg; vitamin B_{12}, 6 μg; *d*-α-tocopheryl acetate, 2.5 I.U.; menadione sodium

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[2] Arizona Agricultural Experiment Station Technical Paper no. 601.
[3] The B-vitamins and procaine penicillin used in this study were provided through the courtesy of Merck and Company, Rahway, New Jersey, and by Chas. Pfizer and Company, Inc., Terre Haute, Indiana. The menadione sodium bisulfite was furnished by Heterochemicals, Inc., Valley Stream, New York; chlortetracycline by American Cyanamid Company, Inc., Pearl River, New York; the DL-methionine by Dow Chemical Company, Midland, Michigan; and the *d*-α-tocopheryl acetate by Distillation Products Industries, Rochester, New York.

bisulfite, 1.0 mg; procaine penicillin, 2 mg; chlortetracycline, 10 mg; butylated hydroxytoluene, 56.6 mg; and DL-methionine, 0.01%. The basal diet contained 0.41% total phosphorus by analysis.

Feed and water were supplied ad libitum throughout the 30-week experimental period. Hens were trapnested the first two days of each week; eggs collected were weighed individually to the nearest 0.1 gm,

TABLE 1

Levels of supplemental phosphorus and analysis of diet

Lot no.	% Phosphorus from		Av. analysis[1]	
	Soft phosphate[2] with colloidal clay	Dicalcium phosphate[3]	% Calcium	% Total phosphorus
6	0.413	—	2.31 ± 0.08[4]	0.709 ± 0.021
7	0.252	0.151	2.30 ± 0.12	0.716 ± 0.028
8	0.162	0.269	2.30 ± 0.16	0.712 ± 0.028
9	—	0.388	2.31 ± 0.14	0.750 ± 0.024
18	—	—	2.30 ± 0.11	0.411 ± 0.033

[1] Diets contained 18.55% ± 0.49% protein.
[2] By analysis, 9.17% phosphorus.
[3] By analysis, 18.20% phosphorus.
[4] Standard deviation.

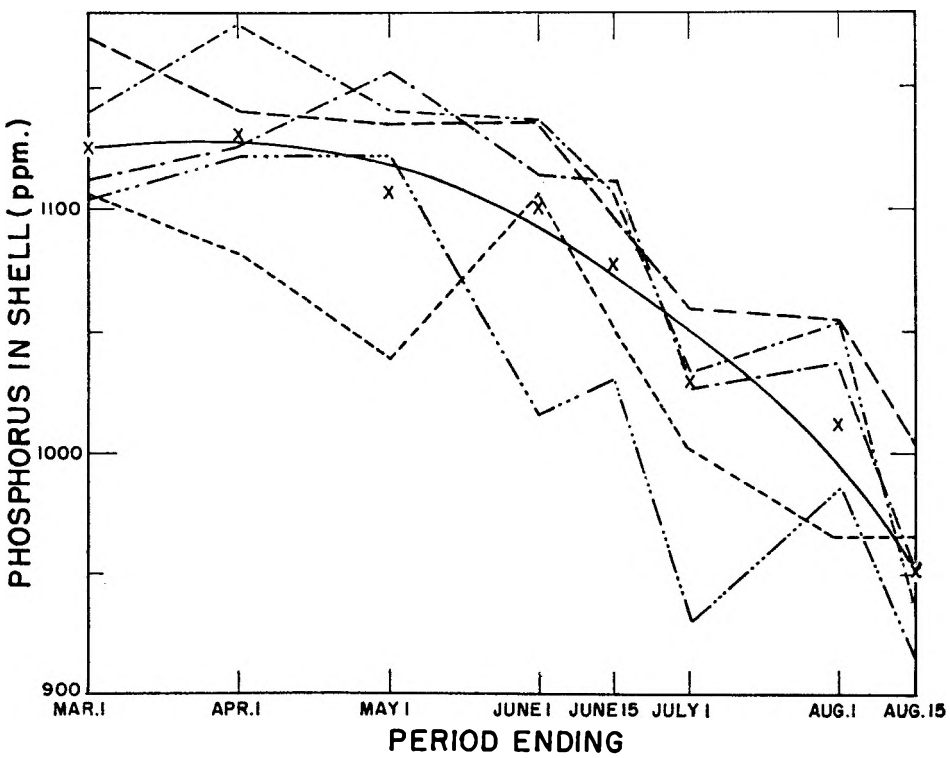

Fig. 1 Rate of decline of shell phosphorus in relation to period. Regression equations: $Y = 1073 + 10X - 0.73X^2$. Coding: ———, regression line; — — —, 0.413% added P (soft phosphate); —.—.—, 0.252% added P (soft phosphate) + 0.151% added P (dicalcium phosphate); — —..— —.., 0.162% added P (soft phosphate) + 0.269% added P (dicalcium phosphate); ·····, 0.388% added P (dicalcium phosphate); —. . .—, no supplemental phosphorus.

broken, the shells washed free of albumin, air-dried for 96 hours and weighed. At the end of each month the collected egg shells were analyzed for calcium (A.O.A.C., '50); phosphorus (Koenig and Johnson, '42); protein (Kirk, '50); and fluorine (Bellack and Schonboe, '58; Huckabay et al., '47). These data were subjected to an analysis of variance according to Snedecor ('56) and the differences were tested by the multiple range test (Duncan, '55).

RESULTS AND DISCUSSION

The monthly phosphorus levels of the egg shells showed a definite decline in all lots as the study progressed, regardless of dietary treatment (fig. 1). It was not possible to determine whether the decline in shell phosphorus content was due to increase in the age of the bird or increase in environmental temperatures. Regression analysis indicated a significant difference in the decline of the shell phosphorus between the supplemented and unsupplemented lots (fig. 1). Statistical analysis indicated that period had a highly significant effect ($P > 0.01$) on the phosphorus concentration. The phosphorus concentration of shells from the supplemented lots decreased from an average of 1132 to 964 ppm during the study, whereas for the lot receiving no phosphorus supplement (lot 18) the phosphorus concentration decreased from 1104 to 915 ppm. The rate of decline was curvilinear being accelerated as the study progressed.

Lots receiving soft phosphate (lots 6, 7, and 8) had a significantly higher ($P > 0.01$) level of phosphorus in the shell than the lots receiving no soft phosphate (lots 9 and 18). The average phosphorus concentration for the lots receiving soft phosphate was 1090 ppm, whereas that of the lots receiving no soft phosphate was 1034 ppm.

The average percentage of shell for the total study was significantly higher ($P > 0.05$) in the lot receiving no phosphorus supplement (lot 18) and in lot 8, receiving a combination of dicalcium phosphate and soft phosphate, than with the other treatments with the exception of lot 9 (table 2). This observation lends doubt that this difference is due to dietary treatment. The birds in these two lots produced smaller eggs than the other lots, and Taylor and Lerner ('39) observed that egg weight had

TABLE 2

Effect of phosphorus supplements on the total calcium, protein, fluorine and phosphorus of egg shell of Single Comb White Leghorn pullets[1]

Lot no.	% Phosphorus from		Percentage of shell			Av. shell calcium all periods	Av. shell protein all periods	Fluorine		Shell phosphate total period
	Soft phosphate[2]	Dicalcium[3] phosphate	Winter	Summer	All periods			Dietary amount from supplement	Egg shell content	
						%	%	*ppm*	*ppm*	*ppm*
6	0.413	—	8.82	8.24_a[4]	8.46_a	36.76	4.62_a	765	177	1100_a
7	0.252	0.151	8.79	8.21_a	8.45_a	36.82	4.56_a	253	69	1080_a
8	0.162	0.269	8.92	8.37_b	8.61_b	36.84	4.64_a	478	82	1090_a
9	—	0.388	8.87	8.25_a	8.52_{ab}	36.85	4.85_b	27	38	1040_b
18	—	—	8.92	8.39_b	8.61_b	36.85	4.59_a	—	38	1028_b

[1] Total number of eggs examined and analyzed: lot 6, 1518; lot 7, 1634; lot 8, 1627; lot 9, 1587; lot 18, 1847.
[2] By analysis, 1.7% fluorine.
[3] By analysis, 0.126% fluorine.
[4] Means with different subscripts are significantly different.

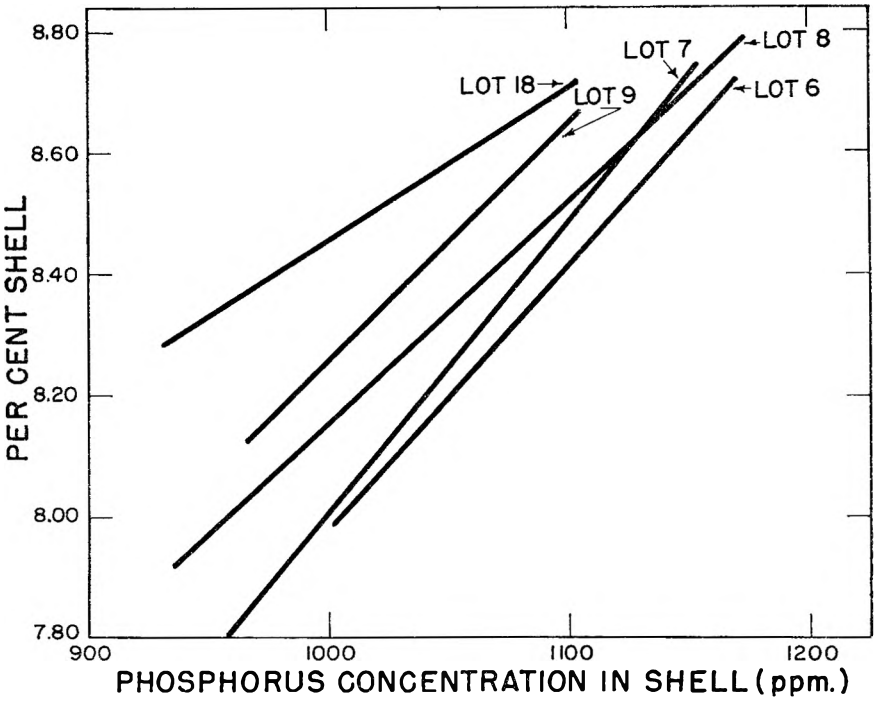

Fig. 2 Relationship of phosphorus concentration in the shell to percentage of shell. Coding: lot 18, no supplemental phosphorus; lot 9, 0.388% added P (dicalcium phosphate); lot 8, 0.162% added P (soft phosphate) + 0.269% added P (dicalcium phosphate); lot 7, 0.252% added P (soft phosphate) + 0.151% added P (dicalcium phosphate); lot 6, 0.413% added P (soft phosphate).

a greater influence on percentage of shell than the shell weight.

During the winter season (February to April, inclusive) the percentage of shell remained relatively constant. During the season of high environmental temperatures, however, there was a sharp decline in the percentage of shell in all lots. The decrease ranged from 0.53 to 0.62% and was highly significant for all lots. Percentage production ranged from 59.19% for lot 6 to 69.17% for lot 18; the unsupplemented diet (lot 18) produced a significantly higher rate of production than the other treatments used.

Since the percentage of shell declined consistently as the study progressed, an attempt was made to correlate the decline with phosphorus concentration. Regression lines relating the phosphorus content of the shell with the relative weight of the shell are given in figure 2. Regression analysis showed correlation coefficients which ranged from 0.77 (lot 8) to 0.90 (lot 7); all were significant ($P > 0.05$).

An effect of shell phosphorus concentration on percentage of egg shell is questioned, however, since the shell phosphorus content was significantly lower in the lot receiving no supplemental phosphorus where no abnormal decline in percentage of shell was noted. Smith et al. ('54) have shown that 90 to 95% of incorporated radioactive phosphorus can be removed from the egg shell by HCl before the shell is perceptibly thinned, indicating a possibility that most of the phosphorus was deposited on the outer portion of the shell late in shell formation.

The average calcium, protein and fluorine concentrations of egg shells for the entire study are shown in table 2. The average shell calcium concentration among treatments remained relatively constant, ranging from 36.76 (lot 6) to 36.85 (lots 9 and 18).

The amount of fluorine added to the diets by the phosphorus supplement is shown in table 2. In lot 6, containing all soft phosphate, the phosphorus supplement

supplied 765 ppm of fluorine, whereas in the diet containing all dicalcium phosphate the phosphorus supplement supplied only 27 ppm. Only a small percentage of the fluorine was transferred to the egg shell (table 2); however, diets with the highest fluorine concentrations produced the highest fluorine levels in the shell. The shells from the birds receiving only the dicalcium phosphate supplement contained no more fluorine than shells from hens fed no phosphorus supplement.

SUMMARY

Calcium, phosphorus, nitrogen and fluorine determinations were carried out at monthly intervals on pooled samples of 8,213 air-dried shells. Egg shells were obtained from each of 5 lots of 50 White Leghorn pullets fed a practical-type basal diet containing 0.41% total phosphorus. The basal diet was supplemented with either soft phosphate, USP grade dicalcium phosphate or combinations thereof. The control group received no supplemental phosphorus.

Percentage of shell and shell phosphorus content declined in all lots as the study progressed. The decline was more rapid in birds fed no supplemental phosphorus than in hens receiving phosphorus supplementation. Regression analysis indicated that the rate of decline of phosphorus in the shell was curvilinear, being accelerated as the study progressed. Calcium content of the shells remained relatively constant throughout the study. Shell fluorine content remained relatively constant within treatments, but varied between treatments in relation to the dietary amount contributed by the phosphorus supplement.

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The Amino Acid Composition and Nutritive Value of Proteins

IV. PHENYLALANINE, TYROSINE, METHIONINE AND CYSTINE REQUIREMENTS OF THE GROWING RAT[1]

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The minimal requirements of the essential amino acids for maximal growth and nitrogen retention in the growing rat have been determined in our laboratory (Rama Rao et al., '59). The requirements of phenylalanine plus tyrosine (supplied as phenylalanine) and of methionine plus cystine (supplied as methionine) were 0.72 and 0.5% of the diet, respectively. In these studies, diets were used containing 5% of casein and made up to the complete nitrogen requirements of the animal by the addition of L-amino acids. However, a pure amino acid diet providing all the essential amino acids at the determined requirement levels, with a mixture of nonessential amino acids as the source of nonspecific nitrogen, has been shown to support a growth rate of about 4.5 gm per day in the male weanling rat (Rama Rao et al., '60). This amino acid diet was used in these studies to determine the individual requirements of phenylalanine and of methionine.

EXPERIMENTAL

Weanling male rats of the Sprague-Dawley strain, 50 to 60 gm in weight, were used. They were placed in individual wire-screen cages and fed the amino acid diets ad libitum for 21 days. Daily weight gains and food consumption records were kept. The basal amino acid diet used is given in table 1. All the essential amino acids except phenylalanine, tyrosine, methionine and cystine were incorporated into the diets at their minimal requirement levels as the L-amino acids (Rama Rao et al., '57).[2] For the studies on phenylalanine requirements, the diet contained 0.70% of tyrosine, and phenylalanine was added at 0.0, 0.1, 0.2, 0.4 and 0.5% levels. Similarly, L-methionine at 0.0, 0.1, 0.2, 0.3 and 0.4% levels was tested in the presence of 0.5% of L-cystine in the diet. Additions of phenylalanine and methio-

TABLE 1
Amino acid diet

	gm
L-Arginine·HCl	0.28
L-Histidine HCl·H_2O	0.31
L-Lysine·HCl	1.13
L-Tryptophan	0.15
L-Isoleucine	0.55
L-Valine	0.55
L-Threonine	0.50
L-Leucine	0.70
Nonessential amino acid mixture[1]	8.00
Vitaminized sucrose[2]	15.00
Salts 446[3]	4.00
Roughage[4]	2.00
Corn oil[5]	2.00
Dextrin	64.83
Total	100.0

[1] Percentage composition of nonessential amino acid mixture: L-glutamic, 41.6; L-aspartic, 12.7; DL-serine, 12.3; DL-alanine, 5.9; L-proline, 23.7; glycine, 3.8.

[2] Rama Rao et al. ('60).

[3] Mameesh and Johnson ('58).

[4] Wood Flock, Brown Company, Chicago.

[5] Fortified with vitamin A, 400 I.U.; vitamin D, 200 I.U.; α-tocopherol, 10.0 mg.

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[1] These studies were supported in part under contract no. DA-49-007 MD-544, with the office of the Surgeon General, Department of the Army. The opinions expressed in this publication are those of the authors and not necessarily those of the Department of the Army.

[2] Rama Rao, P. B., V. C. Metta and B. C. Johnson 1957 The essential amino acids and protein requirements of the growing rat. Federation Proc., *16*: 397 (abstract).

TABLE 2

Growth of rats when fed dietary L-*phenylalanine in presence of excess tyrosine*[1]

L-Phenylalanine		Av. weight gain[2]	Av. food intake	PER.[3]
Group	% diet			
		gm	*gm*	
F	0.0	−8.0 ± 0.7[4]	82	—
G	0.1	6.2 ± 1.6	120	0.5 ± 0.14
H	0.2	37.4 ± 3.6	177	2.1 ± 0.14
J	0.4	99.4 ± 7.7	281	3.6 ± 0.03
K	0.5	101.0 ± 6.7	281	3.6 ± 0.03

[1] 0.7% of L-tyrosine in diet.
[2] Average of 5 male rats in each group for 21 days.
[3] Protein efficiency ratio: grams of weight gain/gram of conventional protein (N × 6.25) intake.
[4] Standard deviation of the mean.

nine were made at the expense of dextrin in the diet.

RESULTS AND DISCUSSION

Phenylalanine and tyrosine requirements. The data in table 2 show that a maximal growth of 4.7 gm per day was obtained at the 0.4% level of L-phenylalanine in the diet. A significant drop in weight gain and conventional protein (N × 6.25) utilization results when the phenylalanine content is decreased to 0.2% of the diet ($P < 0.01$). No significant increase in growth rate or conventional protein utilization is obtained at the 0.5% level. The requirement of phenylalanine plus tyrosine has been shown to be 0.72% of the diet (Rama Rao et al., '59). Womack and Rose ('46) have reported a phenylalanine requirement of 0.9% of the diet. Armstrong ('55) reported the optimal dietary requirement for L-phenylalanine in the presence of excess tyrosine and the absence of tyrosine to be 0.6 and 1.2%, respectively. In his study several of the essential amino acids were provided in the DL-form in a diet in which amino acids constituted 20% of the total ingredients. In our studies, all the essential amino acids were in the L-form and the total conventional protein (N × 6.25) level was maintained at 10% of the diet. Moreover, it has been shown (Borman et al., '46; Rama Rao et al., '60) that the fat content of the amino acid diet influences adversely food consumption and rat growth. The lower fat content of the diets used in these studies (2% of fat as corn oil) as compared with 31% in the diet (Armstrong and Lewis, '50) and a better balance of amino acids could explain the lower requirement of L-phenylalanine obtained in the present studies.

In figure 1 the data on weight gain are plotted against the percentage of L-phenylalanine tested. A line rising linearly and then becoming horizontal was fitted by the method of least squares. It was found to rise at the rate of 27.5 gm for each 0.1% of phenylalanine up to 0.42%, above which the weight gain was constant at about 101 gm in the 21 days, a growth rate of 4.8 gm per day. It has been shown (Rama Rao et al., '59) that 0.72% of L-phenylalanine meets the requirements of phenylalanine and tyrosine. The requirement for L-tyrosine *per se*, then, is 0.30%

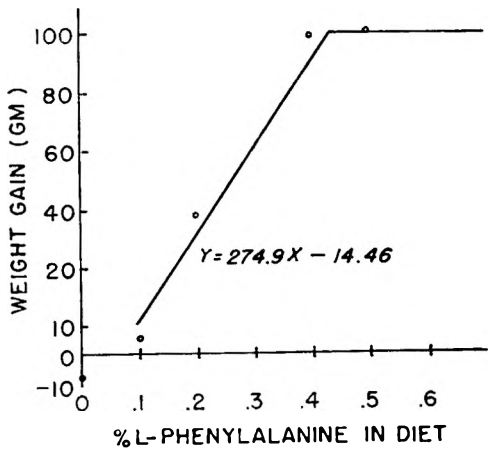

Fig. 1 Percentage of L-phenylalanine vs. weight gain. Y = 274.9X − 14.46, rising linearly to a maximum of 101 gm of weight gain at 0.42% L-phenylalanine.

TABLE 3

Growth of rats when fed dietary L-methionine in presence of excess L-cystine[1]

L-Methionine		Av. weight gain[2]	Av. food intake/rat	PER[3]
Group	% diet			
		gm	*gm*	
A	0.0	13.0 ± 0.41[4]	132	0.86 ± 0.1
B	0.1	52.6 ± 3.8	201	2.6 ± 0.16
C	0.2	77.8 ± 3.4	229	3.1 ± 0.17
D	0.3	82.4 ± 3.1	248	3.3 ± 0.17
E	0.4	76.3 ± 3.4	232	3.0 ± 0.24

[1] 0.5% of L-cystine in diet.
[2] Average of 5 male rats in group B, C, D; 4 in groups A and E; all groups fed for 21 days.
[3] Protein efficiency ratio: grams of weight gain/gram of conventional protein (N × 6.25) intake.
[4] Standard deviation of the mean.

of the diet, which is about 42% of the total phenylalanine plus tyrosine requirement.

Methionine and cystine requirements. Data in table 3 show that a maximal growth rate of about 4 gm per day is obtained at 0.2% of L-methionine in the presence of excess cystine. At 0.1% a decrease in growth rate to 2.4 gm per day is observed ($P < 0.01$). No significant improvement in growth rate is seen at the 0.3 or 0.4% methionine levels. Rose et al. ('48) reported the methionine requirement in the absence of cystine to be 0.6% of the diet. Also, Womack and Rose ('41) stated earlier that approximately 1/6th of the methionine requirement could be satisfied by cystine. On this basis, methionine and cystine requirements of the growing rat are 0.5 and 0.1%.

The weight gains were plotted against percentage of L-methionine tested (fig. 2), and a line rising linearly and then becoming horizontal was fitted by the method of least squares. It was found to rise at the rate of 41.4 gm for each 0.1% of L-methionine up to 0.16%, above which the weight gain was constant at about 79 gm for 21 days, a maximal growth rate of about 3.8 gm per day. Studies in our laboratory (Rama Rao et al., '59) have shown that L-methionine at 0.5% in the diet meets the total requirement for the sulfur amino acids, methionine and cystine. Thus the requirement for L-cystine *per se* can be taken as 0.34% of the diet, which is about 68% of the total methionine plus cystine requirement. The maximum growth rate obtained (about 3.8 gm per day) in the methionine experiment is less than that obtained in the phenylalanine study (4.7 gm per day), paralleled by a difference in food intake of about 11 gm per day in the methionine experiment (group C) as compared with 13 gm per day on the phenylalanine experiment (group J). Possibly the 0.5% of L-cystine in the diets used in the methionine study caused the reduced food intake.

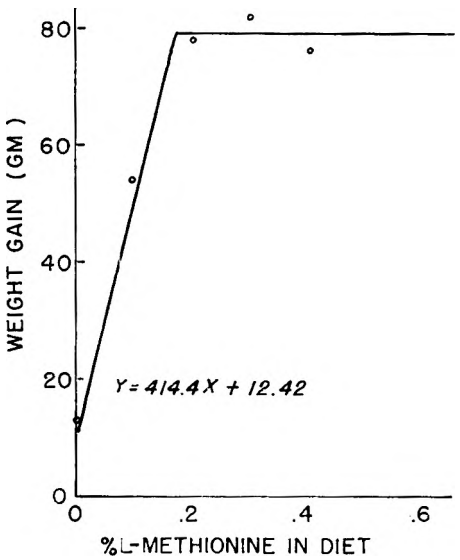

Fig. 2 Percentage of L-methionine vs. weight gain. $Y = 414.4X + 12.42$, rising linearly to a maximum of 79 gm of weight gain at 0.16% of L-methionine.

Rat growth using the minimal amino acid diet. It was considered essential to obtain growth data using an amino acid diet providing all the essential amino acids

TABLE 4

Growth of rats when fed the minimal amino acid diet

Diet	Av. weight gain[1]	Av. food intake	PER[2]
	gm	*gm*	
Whole egg protein[3]	62.6 ± 2.4[4]	162	3.9 ± 1.9
Basal amino acid diet[5] plus: 0.42% L-phenylalanine 0.30% L-tyrosine 0.16% L-methionine 0.34% L-cystine	59.5 ± 2.4	152	3.9 ± 2.6

[1] Average of 6 male rats in each group; average initial weight 56 gm; fed ad libitum for 12 days.

[2] Protein efficiency ratio: grams of weight gain/gram of conventional protein (N × 6.25) intake.

[3] 12 gm of laboratory-prepared whole egg protein replaced the essential amino acids and the nonessential amino acid mixture in the basal diet to provide a protein level of 10% (N × 6.25); see table 1.

[4] Standard deviation of the mean.

[5] Additions of methionine, cystine, phenylalanine, and tyrosine were made at the expense of dextrin.

at the minimal requirement levels arrived at in these studies. For this purpose, methionine, cystine, phenylalanine and tyrosine were incorporated in the basal amino acid diet (table 1) at 0.16, 0.34, 0.42 and 0.30%, respectively, and the growth rate was compared with that when using a whole-egg protein diet. Both diets contained N equal to 10% of conventional protein (N × 6.25). The data in table 4 show that maximal weight gains of 5 gm per day were obtained when using the egg protein diet as well as the amino acid diet. Also, the weight gain per gram of protein (N × 6.25) intake was the same in both groups.

Thus it seems quite evident that the total amino acid pattern (table 5) based on individually determined amino acid requirements is also entirely adequate.

TABLE 5

Pattern of amino acids required by the growing rat

	% of diet containing 2% fat	
L-Arginine	0.21	
L-Histidine	0.25	
L-Lysine	0.90	
L-Tryptophan	0.15	
L-Isoleucine	0.55	
L-Valine	0.55	
L-Leucine	0.70	
L-Threonine	0.50	
L-Methionine	0.16	0.50
L-Cystine	0.34	
L-Phenylalanine	0.42	0.72
L-Tyrosine	0.30	

Plus nonessential amino acid mixture to make a total conventional protein content of 10%.

SUMMARY

Male weanling rats were fed amino acid diets ad libitum in three-week growth experiments.

The requirements of L-phenylalanine in the presence of excess L-tyrosine (0.7%) and of L-methionine in the presence of excess L-cystine (0.5%) were found to be 0.4 and 0.16% of the diet, respectively, which can be supplied as tyrosine or phenylalanine, and as cystine or methionine.

Rats fed a complete L-amino acid diet containing all the amino acids at their minimal requirement levels in a 10% conventional protein (N × 6.25) ration grew normally.

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Effect of Dietary Fat and Biotin on the Oxalacetic Carboxylase Activity of Several Rat Tissues[1,2]

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Krebs et al. ('40) demonstrated that carbon dioxide was produced when oxalacetate was added to pigeon breast muscle, sheep heart and guinea pig kidney. Lichstein ('55) observed a correlation between purity of oxalacetic carboxylase and biotin present. The livers of turkeys deficient in biotin were observed to show a decrease in rate of oxalacetate decarboxylation (Ochoa et al., '47). Biotin has been demonstrated to be a factor in fat synthesis in the liver and body of rats by Gavin and McHenry ('41). Rossi et al. ('57) found that oxidation of fatty acids in the liver of rats was decreased in biotin deficiency.

The present study was made to determine the effect of varying levels of dietary fat, with and without biotin, on the oxalacetate carboxylase activity of the liver, heart and kidney of the rat.

EXPERIMENTAL

Seventy-eight Long-Evans strain, hooded rats, at 21 days of age, were divided according to sex, litter and weight into 6 dietary groups. They were fed diets that contained zero, 2 and 20% of fat; half of each dietary fat group received no biotin and the others were given biotin at the rate of 3 μg per day by subcutaneous injection of an aqueous solution at twice weekly intervals. The 2% fat diet was the no. 2 dried egg-white ration used by Axelrod et al. ('46) in biotin deficiency studies. The other fat levels were obtained by varying corn oil and sucrose in this diet. After receiving the diets for about 5 weeks, when the biotin-deprived rats were showing severe symptoms of biotin deficiency, rats of the various dietary groups were sacrificed and the liver, heart and kidney were evaluated manometrically for oxalacetate carboxylase activity at 38°C using 10% crude homogenates of the tissues with the reagents used by Herbert ('55).

The large amount of CO_2 evolved in the spontaneous decomposition of oxalacetate was determined each time manometric assays were made, and subtracted from the values obtained with the enzyme homogenate. Endogenous CO_2 obtained from the homogenates and all reagents except the oxalacetate was negligible during the 10 minutes of reaction used in the procedure. The samples were placed in ice-cold distilled water immediately after the rats were decapitated, and frozen at minus 8°C, and analyzed within a few days. The activity was calculated on the basis of microliters of CO_2 evolved per mg of nitrogen present per 10 minutes. The N was determined by the microKjeldahl method. Analysis of variance of the data was made according to Snedecor ('56) for disproportionate subclass numbers, multiple classification.[3]

RESULTS AND DISCUSSION

Data are presented graphically in figure 1 showing the effect of biotin, zero, 2 and 20% dietary fat, and sex, on the gains in weight of the rats from 21 to 49 days old. Growth was increased ($P < 0.01$) by the biotin and greater percentages of fat. The male rats gained more than the females ($P < 0.01$). Gavin and McHenry ('41) and Axelrod et al. ('46) showed that biotin stimulated growth in rats fed zero and 2% fat diets, respectively.

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[2] Investigation supported in part by a grant-in-aid by the National Heart Institute, U. S. Public Health Service.

[3] Analysis of the data was made by Dr. A. E. Brandt.

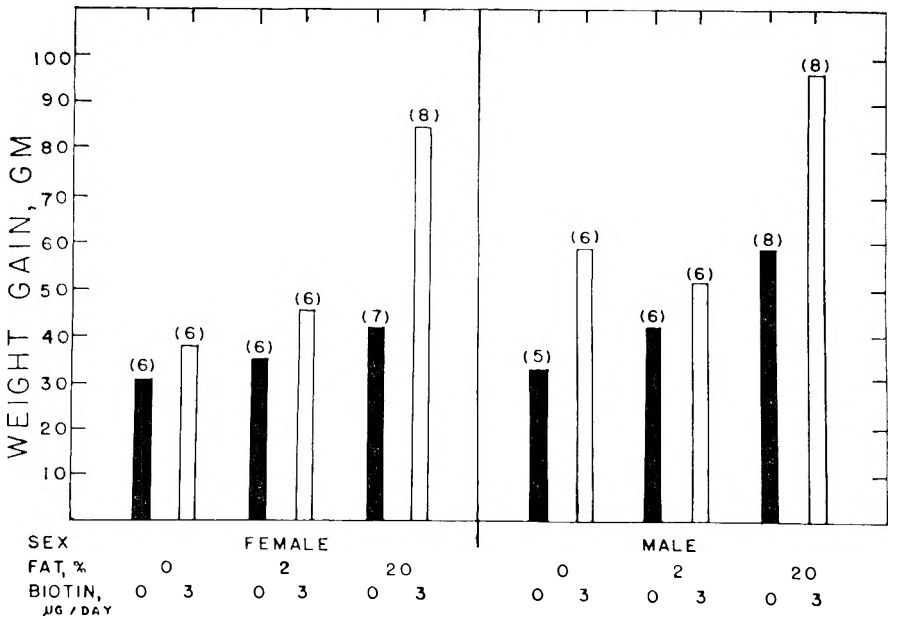

Fig. 1 Effect of zero, 2 and 20% dietary fat, with and without biotin, and sex, on the gain in weight of rats between 21 and 49 days old. The height of each bar is the average of the number of rats shown in parentheses.

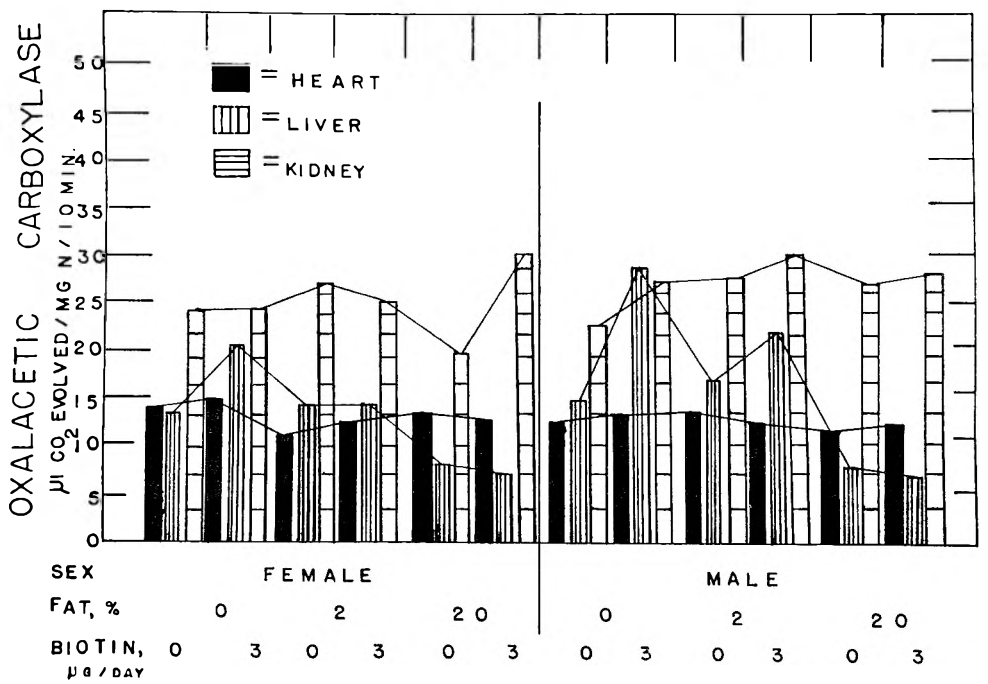

Fig. 2 Effect of dietary fat level, biotin and sex on the oxalacetic carboxylase activity of the heart, liver and kidney of rats after being fed the diets for approximately 5 weeks after weaning.

In figure 2 are presented the data obtained for the oxalacetic carboxylase activity of the liver, heart and kidney of the rats as affected by biotin, zero, 2 and 20% dietary fat levels, and sex. Overall, the oxalacetic carboxylase activity was greater in the tissues of the rats that received biotin ($P < 0.01$). Stimulation of enzyme activity by biotin was observed in the liver of rats of both sexes fed the zero % and males fed the 2% fat diets; no effect of the biotin was observed in the liver of those fed the 20% fat diets or the females fed the 2% fat diets. Biotin stimulated activity in the kidney of male rats fed the three fat levels, but only in the females fed the diets containing 20% of fat. Activity in the heart was not affected by the biotin.

The rats fed the 20% fat diet had less enzyme activity in the liver ($P < 0.01$); the activity in the heart and kidney was not affected by the different fat levels. The reason is not apparent for the 20% fat diet with biotin giving the best growth in the rats (fig. 1), yet in the liver (fig. 2) the enzyme activity was least at this dietary fat level regardless of the presence of biotin.

When the data were expressed on the basis of microliters of CO_2 evolved per gram of wet weight, the same dietary effects were observed in the tissues of both sexes. Average percentages of N in the fresh liver of the rats fed the zero, 2 and 20% fat diets were 2.84, 2.76 and 2.86, respectively. Corresponding values in the heart were 2.43, 2.40 and 2.75%; and in the kidney, values of 2.43, 2.43 and 2.25% were obtained, respectively. These variations in the nitrogen of the tissues of the dietary fat groups are not sufficient to account for the differences in specific enzyme activity observed, especially in case of the liver.

Since a function of biotin is to synthesize fat in the liver as demonstrated by Gavin and McHenry ('41) with zero % fat diets, the 20% fat diets of the present study may have been sufficient to decrease the need for fat synthesis in the liver, and therefore the need for biotin in this respect.

Biotin has been specifically implicated as a coenzyme in oleic acid synthesis, carbon dioxide fixation and decarboxylation, deamination and dehydrogenation (György, '54). In general the oxalacetic carboxylase activity in the liver was more sensitive to biotin and dietary fat than it was in the kidney and the heart. The kidney had more enzyme activity than the liver and heart ($P < 0.01$) except in the case of the male rats that received biotin with the zero % fat diet.

Using the spectrophotometric-TPN-reduction method of Ochoa et al. ('48) it was found that the heart, liver and kidney of several of the rats in the present study had considerable "malic" enzyme activity. Since Ochoa demonstrated that the "malic" enzyme would decarboxylate oxalacetate in the presence of reagents quite similar to the ones used in the present study, the values graphed in figure 2 could be high due to the presence of the "malic" enzyme. Utter and Kurahashi ('54) demonstrated that phosphoenolpyruvate would combine with CO_2 to form oxalacetate and this enzyme may also account for some liberation of CO_2 from oxalacetate in the present study. As knowledge of the number of substrates of various enzymes are discovered, recognition that several enzymes may contribute to a given reaction in homogenates of the present type will be necessary.

SUMMARY

A study has been made of the effect of zero, 2 and 20% fat diets, with and without 3 μg of biotin per day, on growth and the oxalacetic carboxylase activity of the heart, liver and kidney of rats.

Growth was stimulated ($P < 0.01$) by the biotin and increasing levels of dietary fat.

The oxalacetic carboxylase activity was greater ($P < 0.01$) overall in the tissues of the rats that received biotin. Biotin stimulation was greatest in the liver of female rats fed the zero % and in males fed the zero and 2% fat diets. Increased activity due to biotin occurred in the kidney of all male rats, but only in the kidney of females fed the 20% fat diets. Biotin had no effect on the activity in the heart.

The rats fed the 20% fat diets had less ($P < 0.01$) oxalacetic carboxylase activity in the liver; fat level had no effect on activity in the heart and kidney.

The kidney had more ($P < 0.01$) overall activity than the heart and liver.

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The Use of Nitrogen to Creatinine Ratios in Random Urine Specimens to Estimate Dietary Protein[1]

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Proper evaluation of dietary protein is one of the important problems confronting nutrition survey teams today. A biochemical test which would aid in judging the adequacy of protein intake, then, would be helpful. Such a test would have to be easily and rapidly performed for use when surveying large populations. For many years it has been known that daily urinary nitrogen excretion varies with nitrogen intake (Folin, '05). This assumes, of course, nitrogen balance. Equally well-established is a diurnal variation of nitrogen excretion such that when the daily diet is divided into three equal meals, maximum excretion occurs in the late afternoon and early evening hours (Forsgren and Schnell, '34). Thus, while we would expect 24-hour urinary nitrogen excretion to reflect protein intake, the determination of 4- and 6-hour urinary nitrogens would add the problem of diurnal variation.

Experience with nutrition surveys has taught us that timed urine collections are difficult to obtain, and the accuracy of these collections has been questioned. For this reason certain vitamin excretions have been measured in random urine samples, the results being expressed per gram of creatinine contained in the sample (Adamson et al., '45; Aykroyd et al., '49; Interdepartmental Committee on Nutrition for National Defense [ICNND], '57, '58; Lowry, '52; Plough and Consolazio, '59). Following the same line of reasoning, the present study was designed to evaluate the use of nitrogen/creatinine ratios (grams of nitrogen per gram of creatinine) in random urine samples to estimate dietary protein. Partial instead of random urine samples were collected, however, so that the ratios could be compared with timed urinary nitrogen excretion. In addition, the diurnal variation of nitrogen and creatinine excretion was examined. Because methods for measuring total nitrogen and creatinine in the urine are rapidly and easily performed, this technique can be readily adapted to field survey work. Although the accuracy may not be satisfactory for studying individuals, it is hoped this method of estimating dietary protein will be useful when applied to large populations.

METHODS

The subjects were 6 healthy young men, 18 to 23 years old, whose average weight was 74.8 kg (range 62.0 to 92.1 kg). They were housed in a metabolic ward, but were permitted off the ward for supervised activities. During the study they completely consumed weighed test diets as the only source of food. The daily water intake which exceeded two liters for each subject, provided adequate urine specimens. Timed urine samples were collected throughout the study at 3-, 4-, and 5-hour intervals (5 to 8 A.M., 8 to 12 A.M., 12 to 5 P.M., 5 to 9 P.M., 9 to 1 A.M., and 1 to 5 A.M.). The volume of each urine sample was recorded, and each specimen was analyzed for total nitrogen using a microKjeldahl method (Hawk and Bergeim, '37) and for creatinine using the Jaffe reaction (Knowlton et al., '55). This permitted us to express urinary nitrogen excretion both as grams of nitrogen per collection period and as grams of nitrogen per gram creatinine.

The study was divided into three periods differing in dietary protein (17, 13 and 13 days respectively). In the first period all 6 subjects consumed a daily diet containing by analysis: 84 gm of protein, 135 gm of fat, and 359 gm of carbohydrate. In the

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second and third periods the subjects were divided into two groups of three men. In the first group a protein supplement of wheat gluten was added to the previous diet and served as gluten bread; in the second group a protein supplement of meat was added. Macronutrients were adjusted to keep the total calories relatively constant at 3000 per man per day. The gluten group in the second period consumed a diet containing by analysis: 100 gm of protein, 122 gm of fat, and 339 gm of carbohydrate; in the third period: 113 gm of protein, 111 gm of fat, and 340 gm of carbohydrate. The meat group in the second period consumed a diet containing by analysis: 100 gm of protein, 120 gm of fat, and 354 gm of carbohydrate; in the third period: 128 gm of protein, 124 gm of fat, and 351 gm of carbohydrate. The diet in the first period contained approximately 25 gm of protein as meat. Diet composites were also analyzed for preformed creatinine and creatine.

The daily ration was divided into three equal parts and served at 8 A.M., 12 noon, and 5 P.M. The following exceptions are noted. On the 11th day of each period 4 meals (divided equally) were served at 8 A.M., 12 noon, 5 P.M. and 9 P.M. On the 12th and 13th days of each period two meals were served at 12 noon and 5 P.M. On the 4th day of the study 1.8 gm of creatinine was given orally before breakfast to each subject in order to evaluate its effect on urinary creatinine excretion.

RESULTS

The diets were tolerated well by all subjects. Each man in the gluten group (average weight 64 kg) gained approximately 0.5 kg during the study. The subjects in the meat group (average weight 86 kg), on the other hand, each lost approximately 0.7 kg. Daily urinary nitrogen and creatinine excretions were obtained by adding the results of the 6 collection periods (5 A.M. to 5 A.M.). The 24-hour urine excretions of all subjects in each group were averaged using the last 5 days of each period. These results, together with nitrogen intake, are shown in figure 1.

Fig. 1 Nitrogen intake, and average 24-hour urinary nitrogen and creatinine excretions in each period with one standard deviation. Three subjects in each group were averaged using the last 5 days of each diet period.

When 1.8 gm of creatinine was given orally to each subject before breakfast, an additional 1.0 gm of creatinine on the average was recovered in the urine over the next 24 hours. Diet analyses for preformed creatinine averaged 0.16 gm per day in the control and gluten diets, and averaged 0.24 gm per day in both meat-supplemented periods. Analyses for dietary creatine averaged 0.21 gm per day in the control and gluten diets, and 0.36 gm per day in both meat-supplemented periods.

The similarity of the diurnal variations of nitrogen and creatinine excretion is well demonstrated in figure 2. The average of all subjects for the last 5 days in the first period is graphed. Note also the diurnal variation of nitrogen/creatinine ratios (fig. 2). Similar results were obtained in the other periods. Changing mealtimes did not affect the diurnal variation of creatinine excretion. Administration of ¼th of the daily ration at 9 P.M. did not change nitrogen excretion the following morning. When breakfast was omitted on two consecutive mornings (the total daily ration remaining constant) there was a tendency for nitrogen excretion to reach a peak later in the day. This was noticeable only in the third period when nitrogen intake was at its highest level and was statistically significant only during collection periods from 9 P.M. to 5 A.M. ($P < 0.025$). The nitrogen/creatinine ratios were not significantly altered by changing mealtimes under the conditions of this experiment.

Linear regression equations were calculated for nitrogen intake as a function of urinary nitrogen excretion (table 1). These equations and correlation coefficients are shown, first expressing urinary nitrogen as grams of nitrogen per collection period, and then as grams of nitrogen per gram creatinine. Only the last 5 days of each period were included. Thus, each equation was calculated from 5 urine specimens collected at the appropriate time of day from each subject in each period (a total of 90 specimens). Nitrogen/creatinine ratios were calculated for each of the 90 specimens and these data were used to obtain the second set of equations. The 24-hour urinary excretions were obtained by adding the results of the 6 collection periods (5 A.M. to 5 A.M.). Prediction of daily nitrogen intake from nitrogen/creatinine ratios is shown graph-

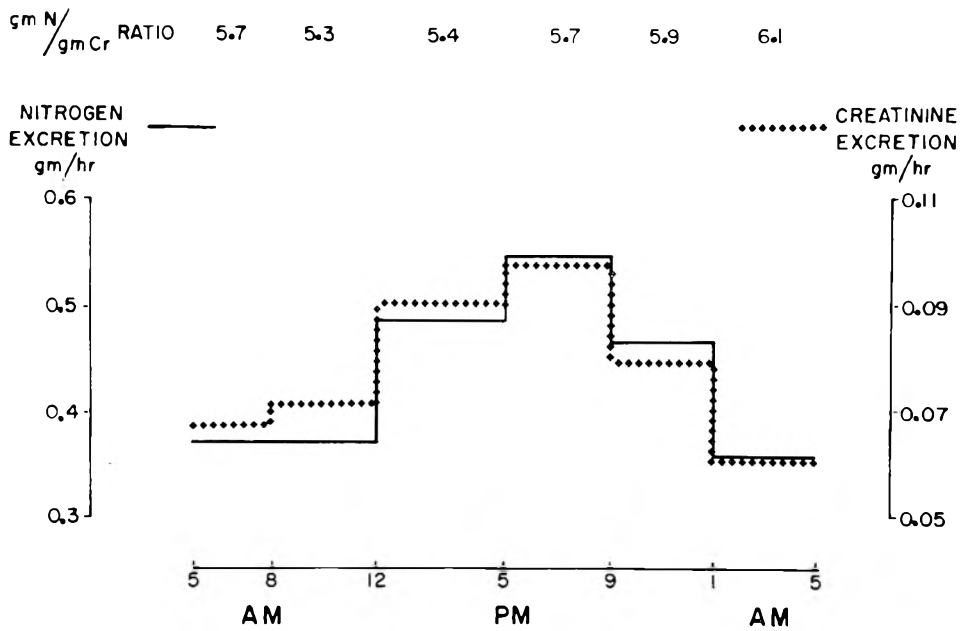

Fig. 2 Diurnal variation of nitrogen and creatinine excretion. Variation of the nitrogen/creatinine ratios is shown numerically at the top. The values for 6 subjects were averaged using the last 5 days of the first period.

TABLE 1

Prediction of daily nitrogen intake (Y) from timed urinary nitrogen excretion (X)

Collection time	Regression equation	Correlation coefficient
5– 8 A.M.	Y=8.4+6.0X	0.68
8–12 A.M.	Y=8.3+4.1X	0.74
12– 5 P.M.	Y=6.3+3.3X	0.79
5– 9 P.M.	Y=7.6+3.0X	0.80
9– 1 A.M.	Y=9.6+2.8X	0.70
1– 5 A.M.	Y=8.7+4.0X	0.76
24-hour[1]	Y=4.3+0.89X	0.92

Prediction of daily nitrogen intake (Y) from nitrogen creatinine ratios (X)

Collection time	Regression equation	Correlation coefficient
5– 8 A.M.	Y= 6.6+1.4X	0.65
8–12 A.M.	Y= 6.7+1.5X	0.69
12– 5 P.M.	Y= 8.3+1.2X	0.63
5– 9 P.M.	Y= 9.2+1.0X	0.62
9– 1 A.M.	Y=10.6+0.76X	0.59
1– 5 A.M.	Y= 8.2+1.1X	0.62
24-hour[1]	Y= 4.2+1.8X	0.80

[1] The 24-hour excretion was obtained by adding the results of the 6 collections (5 A.M. to 5 A.M.).

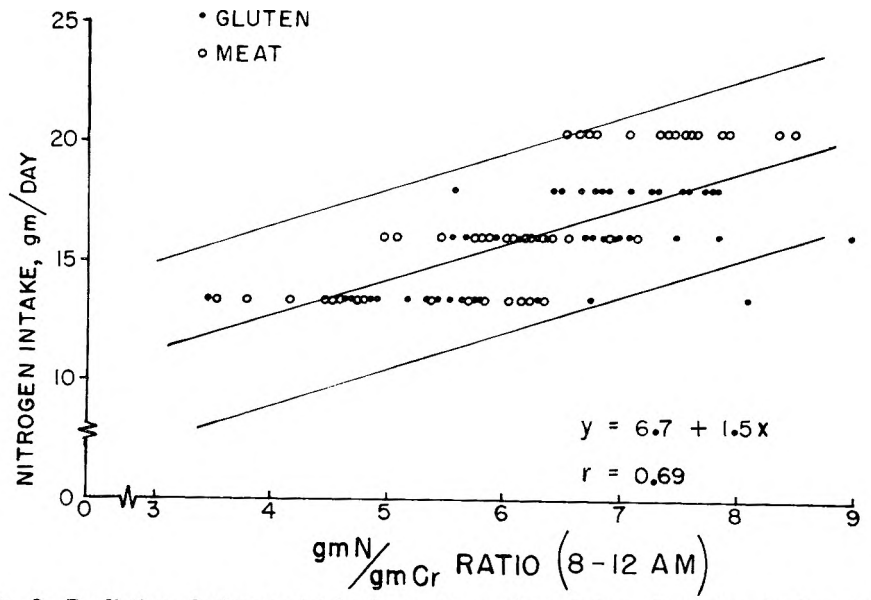

Fig. 3 Prediction of nitrogen intake from nitrogen/creatinine ratios using the 8 to 12 A.M. collection period. The equation, correlation coefficient, and 95% confidence limits are shown.

ically in figure 3. The ratios obtained from the 8 to 12 A.M. collection period are plotted, and are representative of our data. The 95% confidence limits are also shown.

DISCUSSION

Even under the ideal conditions of our experiment we did not, of course, find a perfect correlation between nitrogen intake and nitrogen excretion. This is readily seen in figure 1 where nitrogen excretion was identical in period 3 for the gluten group and period 2 for the meat group, although nitrogen intake differed by two grams (12.5 gm of protein). A possible difficulty is failure of the subjects to achieve nitrogen balance. We believe this was minimized by excluding the first 8

days, or more, of each period from the calculation to allow for diet adjustment. In fact, our data showed that nitrogen excretion was stabilized within 4 days after a diet change. Although greater changes in nitrogen intake would require more time for nitrogen equilibrium, such changes have rarely been noted in dietary surveys. An adequate caloric intake is also important for nitrogen balance. In this study each subject consumed approximately 3000 Cal. daily regardless of his size. There was a mimimal weight gain in the gluten group, and a slight weight loss in the meat group. These small weight changes may not be significant; however, it is possible that there may have been a greater caloric demand in the meat group, thus affecting nitrogen utilization.

Less easily explained was the variation in creatinine excretion (fig. 1). The average excretion of those in the meat group was 0.25 gm higher in the first period when all subjects were eating the same diet. This difference we feel is the result of a discrepancy in body size between groups. Endogenous creatinine excretion tends to vary with body size or lean body mass (Miller and Blyth, '52), and the subjects in the meat group weighed an average of 20 kg more than those in the gluten group. The increased creatinine excretion in the meat group during periods 2 and 3, we feel, is a result of a greater meat intake and therefore more preformed creatinine in the daily ration. Note the diet analyses for preformed creatinine. When 1.8 gm of creatinine was consumed before breakfast, we recovered approximately 56% in the urine within 24 hours. This recovery for oral creatinine agrees with the results of Dominguez and Pomerene ('45).

Variation in creatinine excretion, as would be expected, caused changes in the nitrogen/creatinine ratios independent of the protein intake. For example, during the second period when nitrogen intake was the same for all subjects, the average ratio using 24-hour urine specimens for the gluten group was 7.32, and for the meat group, 6.41. In period three, the ratios were 8.21 and 7.81, respectively, even though the nitrogen intake was 2.5 gm higher by those in the meat group. This is also shown in figure 3. Note that the meat group (open circles) tends to fall to the left (smaller ratios) of the regression line. We feel this tendency for smaller nitrogen/creatinine ratios in the meat group can be explained by the greater creatinine excretion. In an attempt to partially correct for this, we studied our data by expressing creatinine excretion per kilogram of body weight. This manipulation, however, seemed to overcorrect and did not improve the results. It would probably be more satisfactory if creatinine was expressed per unit of lean body mass, although even this would not account for changes in dietary creatinine.

The diurnal variation of creatinine and nitrogen excretion was similar (fig. 2). This tended to cause less variation in the nitrogen/creatinine ratios with respect to time of day, a possible advantage in the use of ratios. With our data it was possible to combine the results on specimens collected from 5 A.M. to 9 P.M. in predicting nitrogen intake without loss of accuracy. Although admittedly we have not tested large shifts in nitrogen intake, changes in mealtimes under conditions of this experiment did not significantly alter our results.

The linear regression equations (table 1) allow a comparison of timed urinary nitrogen and nitrogen/creatinine ratios (X) when used to predict nitrogen intake (Y). As we might expect, 24-hour nitrogen excretion yields the best correlation coefficient. However, it is our feeling that there is little to choose from when comparing 4-hour urinary nitrogen excretion with nitrogen/creatinine ratios under these conditions. The use of ratios to predict nitrogen intake is shown graphically in figure 3. The 90 specimens collected from 8 to 12 A.M. are plotted. Note also the 95% confidence limits. Over our range of data these limits expressed as grams of nitrogen intake are $\pm$ 3.66 gm. Using 4-hour urinary nitrogen collected from 8 to 12 A.M. to predict nitrogen intake, the confidence limits are $\pm$ 3.37 gm. These limits define the predictability of dietary nitrogen from one urine specimen in one man. When more than one individual is sampled, the average nitrogen intake for the group can be predicted more accurately.

The greatest drawback in the use of nitrogen/creatinine ratios to estimate the protein intake of a population does not

appear to be variation in the ratio *per se*, but variation in the actual output of creatinine. The equations in table 1 were calculated from men excreting about 2.0 gm per day of creatinine. Using the equation for the 8 to 12 A.M. collection period, an observed ratio of 5 gm nitrogen per gram creatinine indicates a daily intake of 14.2 gm nitrogen. If an individual is consuming this amount of nitrogen but excreting only 1.0 gm of creatinine (to use an extreme example), the ratio would be 10, and the estimated dietary intake 21.7 gm. It may be possible in surveys to obtain some 24-hour urine collections for creatinine, or perhaps make a rough estimate of creatinine excretion from body size, and use this to adjust the equation. For a daily excretion of 1.0 gm creatinine, the 8 A.M. to 12 A.M. equation becomes $Y = 6.7 + 0.75X$. Another limitation should be mentioned. This method for estimating protein intake would be useful only in evaluating adult populations where nitrogen retention is not a large factor. It is our feeling, however, that the use of nitrogen/creatinine ratios merits further consideration. It would be helpful to know whether the same correlation exists for individuals consuming very low and very high protein diets. The effects of changing mealtimes should be more thoroughly evaluated and truly random samples should be examined.

SUMMARY

In this study, we have considered the use of nitrogen/creatinine ratios (grams of nitrogen per gram of creatinine) in random urine samples to estimate dietary protein. With diets varying from 80 to 125 grams of protein, we compared the accuracy of 3-, 4-, and 5-hour timed urinary nitrogen excretion with nitrogen/creatinine ratios determined from the same samples in the prediction of nitrogen intake. Although 24-hour urinary nitrogen gave the best correlation, we found little difference in results when comparing 4-hour urinary nitrogen with the ratios. Difficulties from diurnal variation of nitrogen excretion were reduced by the use of ratios, but the variability in creatinine excretion among individuals raised other problems. It is concluded that for surveys of large groups of individuals the use of nitrogen/creatinine ratios in random urine samples to estimate protein intake merits further consideration.

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The Effect of a Pyridoxine Deficiency on Cold-Adjusted Rats[1]

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Earlier reports (Vaughan and Vaughan, '59, '60) have indicated that rats previously adjusted to a temperature of 5°C may tolerate a vitamin deficiency as well as rats maintained at 25°C. In the case of pyridoxine, Ershoff ('51) has reported that rats already adjusted to cold survive as long as warm rats when both are fed pyridoxine-deficient diets—pyridoxine-deficient rats, however, were unable to adjust to the initial stress of cold. Mills ('43) could find no difference in pyridoxine requirement between rats maintained at 68° and 90°F.

In the experiment herein reported, the responses of cold-adjusted rats to various levels of dietary pyridoxine were studied.

EXPERIMENTAL

Ninety-one male, Sprague-Dawley rats, ranging in weight from 150 to 206 gm were divided into two groups. One group, consisting of 42 animals, was placed in a cold room at 5°C; the other group of 49 animals, was kept at 25°C. Individual, wire-bottom cages were used throughout the experiment. Three days after being so divided, all rats were fed a pyridoxine-deficient basal diet[2] of the following percentage composition: vitamin-free casein, 22.0; sucrose, 69.4; cottonseed oil, 2.0; salts, 4.0; wheat germ oil, 0.5; choline chloride, 0.1; and cod liver oil, 2.0; also, in milligrams per kilogram of diet: thiamine, 5.0; riboflavin, 8.0; niacin, 40.3; Ca pantothenate, 40.3; inositol, 100.0; and menadione, 5.0.

At the end of 60 days, namely, when growth had ceased, the animals were subdivided into 6 groups in the cold room and 7 groups in the warm—each group consisting of 7 rats. In the warm room 6 groups were fed ad libitum while receiving an oral supplement of pyridoxine hydrochloride to give the following levels: 0.2, 0.4, 0.6, 0.8, 1.0 and 5.0 μg per gm of food. The 7th group also received 5.0 μg per gm of food, but was pair-fed with the group receiving 0.2 μg per gm. In the cold room, the 6 groups were given the same levels of pyridoxine as those in the warm room and were allowed to eat ad libitum. The pyridoxine supplement was mixed into the food slurry every time fresh food was offered. This was done every other day. Food refusals were also weighed every other day. The repletion experiment was continued for 28 days. As in previous reports, weight changes were statistically evaluated by analyses of variance; food intake by a covariance analysis, in which the observed intakes were adjusted in terms of their regression on the initial weights of the rats; and weight and food intake by a covariance analysis in which final weight was adjusted for food intake and initial weight.

It is realized that the rats used in this experiment were older than those usually used. However, in order to avoid death losses, both from the deficiency and the cold stress, it was necessary to use rats with initial weights indicated in table 1.

RESULTS AND DISCUSSION

Growth. Mean weight changes, adjusted final weights, initial weights and final weights are summarized in table 1.

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[1] The views expressed are those of the authors and do not necessarily represent official Air Force policy. The experiments were conducted according to the "Rules Regarding Animal Care" as established by the American Medical Association. (Air Force Regulation: 160–94).

[2] Purchased from General Biochemicals, Inc., Chagrin Falls, Ohio.

An analysis of variance of the weight changes (ΔW) showed that growth was slightly but significantly depressed by cold ($P < 0.05$). Growth, of course, was significantly affected by the level of pyridoxine in the diet ($P < 0.05$).

In order to estimate the pyridoxine requirement for maximum growth, the graphs shown in figure 1 were prepared. Weight changes were plotted against the logarithms of the pyridoxine levels in the diet. The lines representing the growth

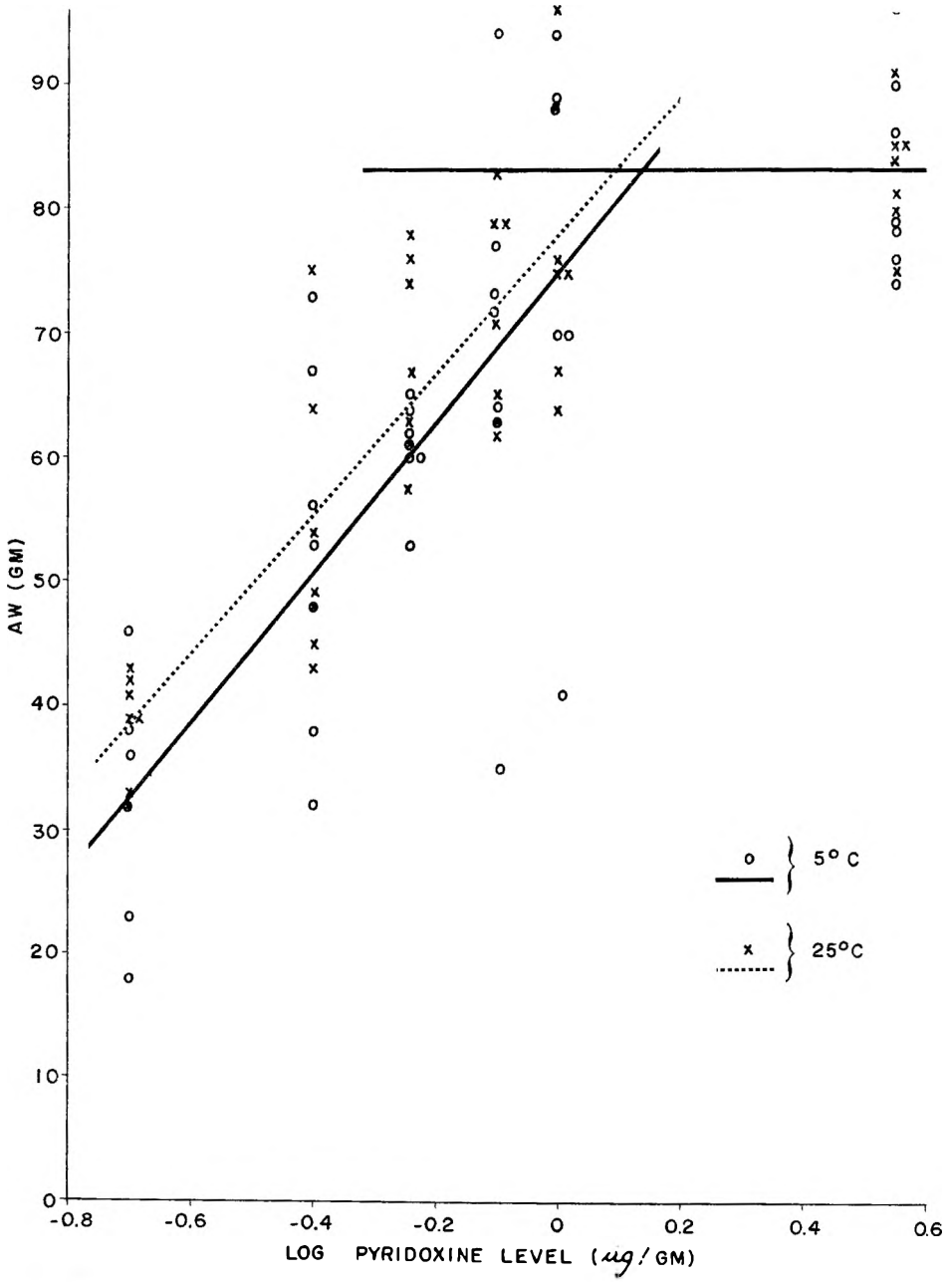

Fig. 1 Relation between pyridoxine levels and weight changes. At 5°: $Y = 74.8 + 60.1X$; 25°: $Y = 77.8 + 56.0X$. (Supplemental levels of pyridoxine in micrograms per gram of food are given in the text.)

TABLE 1

Weight responses of experimental animals[1] *(28 days)*

Pyridoxine·HCl	Initial weight		Final weight		ΔW		Adjusted final weight[2]	
	25°	5°	25°	5°	25°	5°	25°	5°
μg/gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
0.2	273	248	312	274	39	26	356	265
0.4	268	250	322	302	54	52	361	273
0.6	267	246	335	307	68	61	364	265
0.8	273	240	345	308	72	68	371	281
1.0	272	237	349	307	77	70	374	282
5.0	272	255	355	338	83	83	372	284
5.0[3]	262		316		54			

[1] Each mean represents the average of 7 animals.
[2] $b_1=0.679$; $b_2=0.288$; $Ry\cdot xz=0.911$; where x=initial weight, y=final weight and z=food intake.
[3] Pair-fed animals.

responses to the pyridoxine increments were calculated by the method of least squares. If the intersections of these lines with the line of maximum growth (83 gm) are taken to be the minimum requirements of pyridoxine for maximum growth, the requirement for the warm rat becomes approximately 1.3 μg per gm of food and that of the cold rats 1.4 μg per gm of food.

It would thus appear that a somewhat higher pyridoxine requirement is evident during cold exposure. A possible reason for this might be that, at 5°C, more food is eaten—and, since the food is premixed to standard levels of protein, carbohydrate, and fat, a larger proportion of the total protein intake is used for purposes other than growth. Since catabolism of amino acids involves pyridoxal-dependent enzyme systems, a larger dietary requirement might be indicated. This situation may be somewhat similar to experiments of DeBey et al. ('52) who observed that increasing the percentage of methionine in the diet increased the requirement for pyridoxine. In our experiment, the proportion of methionine not used for growth was probably increased by virtue of the increased casein intake, although the percentage of methionine in the diet was not increased. This increase, however, was quite small, and the consequent slight growth depression was comparable to that found by DeBey et al. ('52) when the methionine content of their diet was raised from 0.6 to 1.0%. The rather small difference in requirements between warm and cold rats, therefore, would seem to indicate that the pyridoxine requirement is close to being proportional to food intake or possibly protein intake, within the limits imposed by the cold-induced extra food intake.

TABLE 2

Food intake of experimental animals[1] *(28 days)*

Pyridoxine·HCl	Observed food intake		Adjusted food intake[2]		Δ (Cold-warm)
	25°	5°	25°	5°	
μg/gm	*gm*	*gm*	*gm*	*gm*	*gm*
0.2	386	632	371	642	271
0.4	415	694	405	702	297
0.6	452	748	443	760	317
0.8	451	709	436	726	290
1.0	453	711	439	731	292
5.0	480	771	466	774	308
				Mean:	296 ± 6.5[3]

[1] Each mean represents the average of 7 animals.
[2] $b=0.970$; $r_{xz}=0.482$, where x=initial weight and z=food intake.
[3] Standard error of the mean.

Food intake. The observed food intakes (table 2) of the various groups were adjusted for the regression of food intake on initial weight. It is apparent that food intake, in both warm and cold rats, fell off quite steeply at levels below 0.6 μg per gm of pyridoxine. This confirms reports of Voris et al. ('42) who have observed appetite depression in pyridoxine-deficient rats. In order to estimate the relative importance of appetite and efficiency of food utilization, the adjusted final weight calculation was made, based on the multiple regression of final weight upon food intake and initial weight. This calculation simulates a paired feeding situation and the increment in adjusted weights so obtained should be indicative of changes in efficiency of food utilization. It can be seen (table 1) that a pyridoxine deficiency does affect food utilization to some extent —interestingly enough, there appears to be a break in the 5°C adjusted final weights between the 0.6 and 0.8 μg per gm levels, with little or no graded effect above or below these levels.

In order to compare this mathematical technique with an actual paired feeding episode, one group of animals receiving 5.0 μg per gm in the warm room were pair fed with the group receiving only 0.2 μg per gm. These results are also shown in table 1. The increment in ΔW's between the two levels of vitamin is 15 gm. This compares quite well with the increment of 16 gm between the 0.2 and 5.0 μg per gm levels obtained in the adjusted final weights.

Appetite, however, appears to be the more important factor contributing to weight gains, for at least two thirds of the increment in weight gain between the highest and lowest level of vitamin intake is unaccounted for by the paired-feeding technique, either actual or calculated. The increment in food intake (table 2) between warm and cold rats followed the pattern observed in previous experiments (Vaughan and Vaughan '59, '60), namely, remaining fairly constant while, at the same time, food intake in each group was depressed by the pyridoxine deficiency.

SUMMARY

Ninety-one rats of which 49 were kept at 25°C and 42 at 5°C were fed a pyridoxine-free diet until growth ceased. Following this, graded levels of pyridoxine were fed for 28 days at both temperatures. Food intake and growth were measured. It was found that the pyridoxine requirement, expressed as micrograms per gram of food, was slightly greater in the cold. Growth rate appeared to be more dependent upon appetite than on efficiency of food utilization. Food intake in the cold rats was characteristically depressed by the vitamin deficiency while being simultaneously stimulated by the low environmental temperature.

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Tryptophan Metabolism in Human Subjects[1,2]

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Although the urinary excretion of tryptophan and its metabolites under different conditions of dietary intake has been the subject of extensive investigation, information concerning plasma levels or renal clearances of tryptophan after its ingestion by human subjects is limited. It has been reported that plasma levels of tryptophan were reduced by a 48-hour fast (Charkey et al., '55); were not significantly altered by the ingestion of a high protein meal (Wynne and Cott, '56); and were only slightly increased by relatively large increases in protein intake (Steele et al., '50). The data obtained by Steele and associates ('50), Frazier ('54) and Leverton et al. ('59) demonstrated that the urinary excretion of tryptophan was also only slightly affected by changes in intake. The results of studies by Doolan et al. ('55, '56) in which tryptophan, in solution with other amino acids, was administered by intravenous infusion at different rates, showed that the amounts of tryptophan reabsorbed by the renal tubles continued to increase as the filtered load was increased.

The present paper reports data showing the effect of the ingestion of different amounts of tryptophan on the concentration of this amino acid in the plasma and blood cells, and on the rate of its excretion in the urine. Renal clearances of tryptophan were determined for three of the experimental subjects.

EXPERIMENTAL

Subjects. Eight subjects, 4 men and 4 women, ranging in age from 24 to 48 years and in weight from 65 to 83 kg cooperated in this study. All were healthy, active graduate students or staff members with no known metabolic defects, who were carrying out their usual activities. Three of the subjects took part in more than one phase of the experiment.

Experimental plan. The major part of the study was concerned with the effect of the ingestion of 1 gm of crystalline L-tryptophan on the tryptophan levels in the plasma, blood cells and urine of 8 subjects in the fasting state. Although it was possible to handle only one subject at a time, all subjects followed the same experimental routine. Each subject fasted from 10 P.M. on the night preceding the test day. The test period began with normal micturition and withdrawal of approximately 20 ml of blood in an heparinized syringe from the cubital vein at about 8:30 A.M. Immediately following the withdrawal of blood each subject ingested one gm of crystalline L-tryptophan in 500 ml of water and drank two glasses of water every hour thereafter for 4 hours. Blood samples were withdrawn at intervals of 30, 60, 90 and 240 minutes after the ingestion of tryptophan. At the end of the 4-hour test period each subject resumed eating ad libitum.

All voidings of urine were collected and pooled for each subject between 10 P.M. and the withdrawal of the first blood sample; urine samples were obtained by normal micturition at hourly intervals for 4 hours thereafter. Throughout the next 20 hours all voidings of urine were collected and pooled.

Upon completion of the above experiment, additional experiments were carried out with fewer subjects. Three of the female subjects who had cooperated on the previous experiment ingested larger doses of tryptophan; one subject ingested 2 gm and two subjects ingested 3 gm of crystal-

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[1] Contribution no. 1258, University of Massachusetts Agricultural Experiment Station.

[2] Portions of the data were taken from a thesis submitted by Georgia P. French to the Graduate School of the University of Massachusetts in partial fulfillment of the requirements for the degree of Master of Science.

line L-tryptophan. The experimental routine described above was followed throughout this experiment.

In order to determine whether normal activity or other conditions imposed by the experimental routine would result in any changes in plasma levels and excretion of tryptophan throughout the test period, the same three subjects, without ingesting any tryptophan, carried through the experimental procedures of fasting, withdrawal of blood specimens and collection of urine samples.

The tryptophan levels in the urine, plasma, and blood cells were determined for two subjects after the ingestion of a test meal containing approximately 0.35 mg of tryptophan on one day, and after the ingestion of a similar meal plus 1 gm of crystalline L-tryptophan on a subsequent day. The experimental routine was followed as described above with additional blood specimens withdrawn at 120 and 180 minutes after the ingestion of the meal.

Preparation of samples and analytical methods. Immediately after the withdrawal of blood the plasma was separated from the cells by centrifugation. The plasma was removed and the cells washed twice with an equal volume of normal saline. The white cell layer was not removed. Tungstic acid filtrates were prepared from the plasma (Hier and Bergeim, '45) and from the cells (Dunn et al., '45). The filtrates were collected under refrigeration, adjusted to pH 6.8 and stored at −20°C until analyzed. Urine samples, collected in brown bottles to which toluene had been added, were refrigerated until the samples could be measured and adjusted to pH 6.8, and were then stored at −20°C until analyzed.

The microbiological method described by Steele et al. ('49) with *Leuconostoc mesenteroides* as the test organism and L-tryptophan as the reference standard was used for the determinations of unbound tryptophan in the protein-free filtrates of the plasma and blood cells, and in the urine samples. By the use of this method, repeated assays of the same samples have been found to give results which are reproducible within ±5%. Creatinine was determined in the plasma filtrates and urine samples of three subjects. The alkaline picrate method described by Folin and Wu ('19) was adapted for use with a Coleman Junior Model Spectrophotometer Creatinine solutions containing zero to 30 μg/5 ml were used for preparation of a standard curve along with each set of unknowns; within the zero to 20 μg/5 ml range a rectilinear relationship between concentration and density was obtained. Creatinine clearances were calculated by the formula $C = UV/P$, in which C = clearance; U = concentration of creatinine in each cm^3 of urine; V = the rate of urine production in cm^3/minute; and P = concentration of creatinine in each cm^3 of plasma (Smith, '51).

The data were statistically analyzed by use of the "t" test described by Snedecor ('56).

RESULTS AND DISCUSSION

Urinary tryptophan excretion. Data on the urinary excretion of tryptophan by the experimental subjects under different conditions of tryptophan intake are presented in table 1. During the course of the study, the question arose concerning whether the rate of excretion of tryptophan would be altered throughout the experimental period if the subjects were ingesting neither food nor tryptophan; it was suggested that physical activity or other conditions imposed by the experiment might increase the excretion. The first row of figures in table 1 illustrates the gradual decline in the hourly rate of tryptophan excretion for three subjects who ingested neither food nor tryptophan during the 4-hour experimental period. The difference between the overnight rate and the rate during the 4th hour was significant at the 5% level of probability. During the following 20-hour period, when the subjects were eating ad libitum, the rate of excretion was approximately that of the overnight period. These findings suggested that any increment in the rate of tryptophan excretion found during the test periods would be the result of tryptophan ingestion rather than of any other conditions imposed by the experiment.

Considerable variation was found in the amounts of tryptophan excreted by the different individuals, during the overnight period and during the test day following the ingestion of 1 gm of tryptophan. How-

TABLE 1

Average amounts of tryptophan excreted in urine of experimental subjects under different conditions of tryptophan intake

No. of subjects	Trypto-phan intake	Urine tryptophan levels								
		Over-night period	Minutes after ingestion of tryptophan					Amt. excreted		Amount dose recovered
			0–60	61–120	121–180	181–240	241–1440	Test day	Control day	
	gm	*µg/min*	*µg/min*	*µg/min*	*µg/min*	*µg/min*	*µg/min*	*mg/day*	*mg/day*	*%*
3	0	7.8 ± 5.4[1]	6.5 ± 4.4	6.2 ± 2.9	5.0 ± 3.9	4.6 ± 3.3	7.3 ± 2.6		10.1 ± 4.5	
8	1	11.6 ± 6.3	39.1 ± 15.5	29.8 ± 10.4	24.5 ± 8.7	14.6 ± 7.1	12.3 ± 5.8	21.1 ± 9.1	16.7[2] ± 8.6	0.5 ± 0.3
1	2	10.0	38.9	45.6	36.3	23.4	12.0	23.2	14.6	0.4
2	3	5.0	19.6	31.0	19.6	17.5	6.8	14.0	7.7	0.2
		7.8	22.7	28.0	20.5	21.4	7.0	14.0	7.9	0.2
2	0.35 in meal	5.1	7.1	7.9	8.4	6.8	6.6		9.9	
		6.4	7.9	9.3	11.4	12.2	7.4		11.8	
2	1 and 0.35 in meal	6.5	9.8	14.6	10.9	10.2	5.8	10.0	9.9	0.01
		7.6	13.5	17.9	18.8	18.8	8.4	14.6	11.8	0.3

[1] Mean ± standard deviation.

[2] Calculated on basis of overnight excretion for these subjects.

ever, all subjects responded in the same manner to the test dose by showing an increase in the rate of excretion, although the increase at the time of maximum excretion varied from 132 to 693% above the overnight rates for individual subjects. During the first hour following the dose the average rate of excretion increased more than three times and was significantly higher ($P < 0.001$) than the rate during the overnight period; the rate declined thereafter and was only 26% above during the 4th hour and approximately 6% above during the subsequent 20 hours. During the last 15 hours of the test day the rate of excretion of three subjects fell below the rates each had maintained during the overnight period previous to the test day. Although the rate of tryptophan excretion increased significantly the first hour after the ingestion of the test dose, the actual amount of tryptophan recovered in the urine on the test day above that amount calculated as a normal 24-hour excretion (based on the rate of overnight excretion for these subjects) represented only 0.5% of the dose.

For three subjects who ingested larger doses of tryptophan the rates of excretion were not strikingly different from the rates following the ingestion of 1 gm; the rates for two subjects were slightly higher and for one subject somewhat lower than after the 1-gm dose. Less than 0.5% of these larger doses were recovered in the urine. These results support the findings of other workers cited above that large differences in tryptophan intake have only a slight effect on tryptophan excretion.

Plasma tryptophan. The average tryptophan levels in the plasma of the experimental subjects under different conditions of tryptophan intake are summarized in table 2. The mean fasting tryptophan level for 8 subjects was 9.6 µg/ml of plasma with a range between 6.8 and 12.0 µg/ml which is in agreement with the range reported by Hier and Bergeim ('46), and at the lower end of the range found by Johnson and Bergeim ('51), Dunn et al. ('45) and Harper and associates ('52). The average fasting level for the 4 male subjects was 10.2 µg/ml compared with the average value of 9.1 µg/ml of plasma for the 4 female subjects. This difference was

TABLE 2

Average tryptophan levels in plasma of experimental subjects under different conditions of tryptophan intake

No. of subjects	Tryptophan intake	Plasma tryptophan levels						
		Minutes after ingestion of tryptophan						
		0	30	60	90	120	180	240
	gm	*μg/ml*	*μg/ml*	*μg/ml*	*μg/ml*	*μg/ml*	*μg/ml*	*μg/ml*
3	0	10.3 ± 3.1[1]	8.3 ± 2.6	8.4 ± 2.7	8.5 ± 1.5			8.5 ± 2.6
8	1	9.6 ± 1.7	43.2 ± 1.4	41.9 ± 2.3	29.6 ± 4.6			13.4 ± 3.4
1	2	10.0	50.0	57.6	61.6			23.7
2	3	8.1	85.5	96.6	86.3			35.8
		10.8	150.6	62.4	59.2			32.0
2	0.35	10.0	11.4	11.9	11.0	12.9	12.3	11.6
	in meal	14.6	16.5	16.2	18.2	19.8	17.0	17.2
2	1 and 0.35 in meal	8.8	32.9	27.1	23.3	21.1	17.8	13.6
		11.5	33.3	33.6	28.5	27.4	26.4	18.3

[1] Mean ± standard deviation.

not significant. Neither was there any significant change throughout the 4-hour experimental period in the plasma tryptophan levels of the three subjects who did not ingest food or tryptophan.

Although it was impossible to determine the exact time when the maximum amount of tryptophan appeared in the plasma, it was apparent that the peak levels for all subjects after the ingestion of 1 gm of tryptophan occurred before 90 minutes had elapsed. At the 30-minute interval there was a highly significant increase ($P < 0.001$) in plasma tryptophan, which was more than 4 times the mean fasting level. All subjects, however, did not exhibit their maximum increase at the 30-minute interval; three subjects showed maximum levels at 60 minutes. At 90 minutes, the plasma tryptophan was markedly less than it had been at 60 minutes for each subject, and at 240 minutes the mean plasma level had decreased to only about 40% above the mean fasting level. The ingestion of larger doses of tryptophan resulted in larger increases in the plasma; the two subjects ingesting a 3-gm dose showed maximum plasma levels of 11 and 13 times the fasting levels. At 240 minutes, the mean plasma tryptophan was still more than three times the mean fasting level. These results illustrate the rapid absorption of crystalline tryptophan from the gastrointestinal tract. How great the plasma tryptophan concentration becomes and how long the plasma tryptophan levels remain abnormally high appear to be influenced by the size of the dose.

The ingestion of a meal containing about 350 mg of tryptophan resulted in only a small average increase (33% above fasting level) in the plasma tryptophan of two subjects. This finding agrees with that reported by Wynne and Cott ('56) who did not find a significant increase in plasma tryptophan at one and three hours after food intake. The ingestion of 1 gm of crystalline tryptophan along with an identical meal on a different day by the same two subjects resulted in an average plasma tryptophan level at 30 minutes which was three times the average fasting value. This increase was somewhat less than the fourfold increase in plasma levels that were obtained when the same amount of trypto-

phan was ingested without food. It is quite probable that the presence of food in the digestive tract would tend to decrease the rate of absorption of the amino acid below that rate which occurred when the dose was taken without accompanying food.

In an attempt to estimate the percentage of the dose of tryptophan that was circulating in the plasma at the time of maximum concentration, the total amounts of unbound tryptophan circulating in the fasting state and at the time of maximum concentration were calculated for all but one of the subjects. In these calculations the plasma volume of each subject was estimated from the values of 43.1 and 41.5 cm^3/kg of body weight for men and women, respectively (Ferguson, '47). The amount of tryptophan which was circulating in the plasma at the time of maximum concentration in excess of that amount circulating in the fasting state represented 12% of the dose for 7 subjects who had ingested 1 gm and 13% for two subjects ingesting a 3-gm dose.

Tryptophan in blood cells. Since the blood cells were available, changes in tryptophan concentration occurring during the experimental period were determined. Considerable variation was found in the tryptophan content of the cells of the subjects in the fasting state (table 3). Differences were found for the same subject in the fasting state on different days, as illustrated by the data for the two subjects who cooperated in the last three phases of the experiment. The tryptophan content of the blood cells of subjects in the fasting state ranged from 0.9 to 4.0 µg/ml with an average of 2.0 µg/ml. This value is lower than the mean of 2.9 µg/ml reported by Johnson and Bergeim ('51) as the tryptophan content of erythrocytes of human males.

The ingestion of 1 gm of tryptophan resulted in a statistically significant ($P < 0.01$) increase of tryptophan in the blood cells at the 30-minute interval. It was noted that the individuals having the highest plasma level did not uniformly have the highest concentrations of tryptophan in the cells. In general, however, the increases in the cells were parallel to the increases in the plasma. Although the percentage of tryptophan content of the cells increased

TABLE 3

Average tryptophan levels in blood cells of experimental subjects under different conditions of tryptophan intake

No. of subjects	Tryptophan intake	Blood cell tryptophan levels									
		Minutes after ingestion of tryptophan									
		0		30		60		90	120	180	240
	gm	*µg/ml*		*µg/ml*		*µg/ml*		*µg/ml*	*µg/ml*	*µg/ml*	*µg/ml*
3	0	1.7 ± 0.7[1]		1.5 ± 0.6		1.8 ± 1.0		1.3 ± 0.4			1.3 ± 0.3
6	1	2.2 ± 0.3		6.4 ± 2.0		5.1 ± 1.6		4.9 ± 0.2			3.7 ± 0.9
1	2	0.9		11.7		7.9		10.7			3.4
2	3	1.4		25.4		23.0		24.0			9.4
		1.0		8.5		12.6		11.8			4.4
2	0.35	2.1		2.6		2.0		1.8	2.2	2.6	2.7
	in meal	4.0		4.7		3.8		4.0	4.4	4.0	3.7
2	1 and 0.35	1.0		5.4		3.8		4.3		2.5	1.5
	in meal	2.6		8.8		9.8		7.9		7.3	6.0

[1] Mean ± standard deviation.

markedly, especially with the higher intakes, the average amount of tryptophan present in the blood cells at the period of maximum concentration as unbound tryptophan was estimated to represent only about 2% of the dose.

Renal clearance of tryptophan. In order to estimate the amount of tryptophan filtered at the glomerulus it was necessary to determine the glomerular filtration rate (GFR). Under conditions in this laboratory it was not possible to measure GFR by using the standard technique of inulin clearance. Consequently, creatinine clearance was used as an index of the GFR of the three subjects who ingested different amounts of tryptophan. Although the use of creatinine clearance as a measure of GFR is questionable (Smith, '51), the data reported by Brod and Sirota ('48) indicated that endogenous "creatinine" chromogen clearance might be used as an index of filtration rate in normal adults. Van Pilsum and Seljeskog ('58) reported that one-hour creatinine clearances were similar to 24-hour clearances. In the present study, 4 one-hour creatinine clearances were calculated on the control day and on the day of the largest tryptophan intake for each of the three individuals. Plasma creatinines were determined on 5 separate blood specimens and an average of these results was used in the calculation of the clearances. For each subject, the average of the 8 clearances was taken to represent the GFR.

The amounts of tryptophan filtered and reabsorbed under different conditions of intake are presented in table 4. It may be seen from the data that as the filtered load was increased the amount that was reabsorbed was increased, which is in agreement with the findings of Doolan et al. ('55, '56). From the results of the experiment of Doolan et al. ('56), in which tryptophan was administered in solution with other amino acids by infusion, it appeared that the increase in the amount of tryptophan reabsorbed was not equally proportionate to the increase in that filtered, which resulted in a decrease in the percentage reabsorbed. In the present study, all three subjects reabsorbed practically the same percentage (98.6 to 99.8%) of that filtered irrespective of the amount of tryptophan ingested or the size of the filtered load. It is probable that this difference in findings can be attributed to differences in experimental conditions. It would be of interest to determine at what level of tryptophan intake the capacity of the renal tubules to reabsorb tryptophan would be exceeded.

TABLE 4

Amount of tryptophan filtered and reabsorbed by three subjects under different conditions of tryptophan intake (calculated at the highest level of plasma tryptophan for each experiment)

Subject	Creatinine clearance[1]	Tryptophan intake		Plasma tryptophan	Amount tryptophan			
		In food	As dose		Filtered	Excreted	Reabsorbed	
	ml/min	*gm*	*gm*	*μg/ml*	*μg/min*	*μg/min*	*μg/min*	*%*
A, female, age 48	77.4 ± 5.5[2]	0	0	9.6	743	10	733	98.6
		0	1	57.6	4458	33	4425	99.2
		0	2	61.6	4768	46	4722	99.0
B, female, age 46	76.8 ± 6.1	0	0	9.3	714	7	707	99.0
		0.35	0	11.9	914	8	906	99.1
		0	1	43.6	3348	48	3300	98.6
		0.35	1	32.9	2527	15	2512	99.4
		0	3	96.6	7419	31	7388	99.6
C, female, age 41	90.9 ± 7.6	0	0	10.0	909	6	903	99.3
		0.35	0	16.5	1500	12	1488	99.2
		0	1	38.8	3527	15	3512	99.6
		0.35	1	33.6	3054	19	3035	99.4
		0	3	150.6	13690	28	13662	99.8

[1] Assumed in these calculations to be equal to the glomerular filtration rate (not corrected for surface area).

[2] Mean ± standard deviation.

SUMMARY

Tryptophan in the urine, plasma, and blood cells was determined for human subjects under different conditions of tryptophan intake. The concentration of tryptophan in the plasma and blood cells increased after the ingestion of crystalline L-tryptophan; at the maximum concentrations the amount of unbound tryptophan circulating in the plasma and cells in excess of that circulating immediately before ingestion of the dose was estimated to represent about 14 to 15% of the dose, regardless of the size of dose.

The amount of unbound tryptophan recovered in the urine in the 24 hours following the dose represented 0.5% or less of the dose.

The data indicated that as the load filtered at the glomerulus was increased the amount of tryptophan that was reabsorbed by the renal tubules increased; the percentage reabsorbed remained practically the same for the different filtered loads.

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On the Function and Metabolism of Vitamin E

III. VITAMIN E AND ANTIOXIDANTS IN THE NUTRITION OF THE RAT[1]

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Synthetic antioxidants N,N′-diphenyl-*p*-phenylenediamine (DPPD) and methylene blue (MB) can prevent and cure the reproduction failure of vitamin E deficiency in female rats (Markees, '53, '55; Draper et al., '56, '58; Draper and Johnson, '58).

In a further extension of these studies it has now been found that rats carried through a resorption-gestation when receiving a vitamin E-deficient diet will respond to the antioxidants N,N′-diphenyl-*p*-phenylenediamine (DPPD) and 1,2-dihydro - 6 - ethoxy-2,2,4-trimethylquinoline (ethoxyquin), but not to 2,6-di-*tert*-butyl-4-methylphenol (BHT) or ubichromanol (acid-reduced ubiquinone or coenzyme Q_{10}), by bearing live young in a second reproduction cycle. Since it was possible to maintain the fertility of female rats receiving a vitamin E-deficient diet by supplementation with DPPD or ethoxyquin through two generations, assays were run to determine quantitatively the levels of the individual Emmerie-Engel-active compounds present in the livers of these animals.

EXPERIMENTAL

Weanling female rats of the Sprague-Dawley strain were placed on the vitamin E-free synthetic diet 1 given in table 1 (Draper et al., '58). Vitamin A and D were given orally to each rat to provide 5,000 and 500 I.U. per week, respectively. Feed and water were provided ad libitum. All female rats were kept in individual wire cages in a constant-temperature animal room. The male rats were fed a stock diet[2] throughout the experiment.

The rats were maintained with the vitamin E-free diet for at least 8 weeks before mating. Mating resulted in 100% reproduction failure, thus showing that the amount of vitamin E still present in the animal body was insufficient to support reproduction. After this resorption-gestation the molecularly distilled vitamin E-free lard of diet 1 was removed from the basal vitamin E-free diet and the essential fatty acids were supplied by methyl linoleate at 1.5% of the diet given as the 25% methyl linoleate urea complex,[3] i.e., diet 2. In all later experiments (experiment 2 and all subsequent experiments) diet 3 was used and 3% of a 60% concentrate of methyl linoleate was added to this diet twice a week.

METHODS

At the end of each experiment the animals were killed and the livers removed and frozen for analysis. Known amounts of wet liver were homogenized and extracted for 10 minutes in a Waring blender with foaming solvent (10 parts of absolute ethanol to 8½ parts of Skellysolve B [Moore and Ely, '41]) in the presence of pyrogallol (1% of liver weight). The residual tissue was removed by suction and the filtrate after addition of one volume of water was extracted with 100 ml of Skellysolve B in a mechanical shaker for 10 minutes. The Skellysolve extract was removed and the aqueous ethanol layer was extracted twice with 100 ml of Skellysolve B. The Skellysolve extracts were combined, and the solvent removed in vacuo at 80°C, under nitrogen. The residue was taken up in 100 ml of absolute ethanol and the neutral fats were precipitated by cooling the solu-

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[2] Checkers, Ralston Purina Company, St. Louis.
[3] Hormel.

tion in a dry ice-acetone bath for one hour. The precipitated material was removed by filtration through a jacketed funnel containing a slurry of dry ice-acetone and the crystallized lipids were washed twice with 25 ml of cold absolute ethanol precooled to − 70°C. The ethanolic filtrate was taken to dryness in vacuo at 50°C under nitrogen and the residue was redissolved in 3 ml of cyclohexane. An aliquot was taken to dryness in vacuo under nitrogen, dissolved in 4 ml of benzene and put on a Florex XXS column (1 × 5 cm) previously treated with stannous chloride (Beckman, '52). The column was rinsed with three 3-ml portions of benzene. The eluates were combined and evaporated to dryness in vacuo under nitrogen. The residue was dissolved in absolute ethanol and an aliquot was used for determination of the total reducing substances by the Emmerie-Engel ('38) method. Another aliquot was used for reverse-phase paper chromatography of antioxidants and other reducing compounds. Non-vitamin E reducing compounds were separated from vitamin E by the paper chromatographic procedure of Eggitt and Ward ('53), the chromatograms being developed with 75% ethanol. All reagents used in the assay procedure were of reagent grade and were redistilled before use. Skellysolve B was purified according to the procedure used by Hines and Mattill ('43).

RESULTS AND DISCUSSION

The reproduction data from the first two experiments are summarized in table 2. In the first experiment, three groups of animals were used: one receiving 30 mg of α-tocopherol per rat per week; a second, DPPD at a level of 0.005% of the diet; and the third, no antioxidant. A regeneration period of 15 days was allowed before mat-

TABLE 1

Purified vitamin E-free basal diets

	Percentage composition		
	1	2	3
	%	%	%
Dextrose[1]	65.4	73.9	75.4
Casein[2]	20.0	20.0	20.0
Minerals (Salts 446)[3]	4.0	4.0	4.0
Choline chloride	0.1	0.1	0.1
Vitamin mixture[4]	0.5	0.5	0.5
Lard (vitamin E-free)	10.0	—	—
Methyl linoleate (urea complex)	—	1.5	—

[1] Cerelose, Corn Products Refining Company, New York.
[2] Labco, Vitamin-Free Casein, The Borden Company, New York.
[3] Mameesh, M. S., and B. C. Johnson ('58).
[4] Draper, H. H., S. Goodyear, K. D. Barbee and B. C. Johnson ('56).

TABLE 2

Reproduction of female rats fed DPPD[1] and BHT[2]

Treatment	No. of females	No. of litters born	No. of pups born	No. of pups born alive	Per cent born alive
		Experiment 1			
Vitamin E, 30 mg/week	25	3	7	2	28
DPPD, 0.005% of diet	24	11	60	43	70
None	25	0	0	0	0
		Experiment 2			
Vitamin E, 30 mg/week	25	12			
DPPD, 0.005% of diet	25	11			
BHT, 0.1% of diet	26	0			
None	25	0			

[1] N,N′-diphenyl-*p*-phenylenediamine.
[2] Butylated hydroxytoluene.

ing. The results show clearly that DPPD, as well as α-tocopherol, was able to restore fertility in the vitamin E-deficient female rat. The experiment was repeated (experiment 2, table 2) with the addition of a 4th group which was fed BHT at the level of 0.1% of the diet. It is clear from this experiment that BHT was biologically inactive in this respect.

The recent isolation and identification of ubiquinone and its cyclization products, ubichromenol and ubichromanol (Crane et al., '57; Morton et al., 58; Laidman et al., '60), and their structural resemblance to α-tocopherol, prompted the inclusion of the acid-reduced ubiquinone (ubichromanol) in the next experiment (experiment 3, table 3). The positive control group received 10 mg of α-tocopherol orally, and the test group 5 mg of ubichromanol injected intraperitoneally. Both compounds were given 5 days following mating as single curative doses as used by Goettsch and Pappenheimer ('41). Fertility was restored by α-tocopherol, whereas ubichromanol was found to be inactive. Three groups of female rats were used in experiment 4 (table 3). The positive and negative control groups were handled as in the previous experiment. The test group was fed the synthetic antioxidant ethoxyquin at 0.1% of the diet commencing 10 days prior to mating. The data show that ethoxyquin was also able to restore fertility.

Since the previous experiments were fairly short-term, involving only one generation of rats and two reproduction cycles, two experiments (5 and 6, table 4) were carried out through three generations. Twenty-four α-tocopherol-depleted females were fed the vitamin E-free diet containing 0.005% of DPPD 9 days prior to the second mating. Nine male pups and 8 female pups were weaned from these matings.

TABLE 3

Reproduction of female rats fed ubichromanol and ethoxyquin[1]

	No. of females	No. of litters born	No. of rats born	No. of rats born alive	Per cent born alive
		Experiment 3			
Vitamin E, 10 mg[2]	25	4	7	4	52
Ubichromanol, 5 mg[3]	27	0	0	0	
None	25				
		Experiment 4			
Vitamin E, 10 mg[2]	22	8	51	41	80
Ethoxyquin, 0.1% of diet[4]	11	5	18	10	55
None					

[1] 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline.
[2] One oral dose 5 days following mating.
[3] One intraperitoneal dose 5 days following mating.
[4] Started 10 days prior to mating.

TABLE 4

Reproduction of female rats fed DPPD[1] *over an extended period*

Treatment	No. of females	No. of litters	No. of pups born	Born alive	Weaned
				%	
		Experiment 5			
DPPD, 0.005% of diet	24	11	75	70	9 males 8 females
		Experiment 6			
1st mating:					
DPPD, 0.005% of diet	8	4	29	100	31%
2nd mating:					
DPPD, 0.005% of diet	8	3	23	96	68%

[1] N,N'-diphenyl-p-phenylenediamine.

The female pups were fed the vitamin E-free diet containing 0.005% of DPPD following weaning and these females were mated twice after reaching maturity. Pups were obtained from both matings, thus showing that DPPD can support reproduction in the female rat when fed as the sole source of antioxidant over two generations.

Since it was possible to maintain fertility of female rats through two generations receiving the vitamin E-free diet supplemented with synthetic antioxidants, liver assays were run to determine whether residual amounts of α-tocopherol could possibly be present. Extracts of liver samples from each experiment were analyzed for total apparent tocopherol content by the Emmerie-Engel reaction ('38), as described previously, and at the same time were subjected to the paper chromatographic procedure of Eggitt and Ward ('53). For quantitative assay of the individual reducing compounds, namely, α-tocopherol, DPPD and ethoxyquin, the corresponding spots were eluted and assayed again with Emmerie-Engel reagent. These quantitative data described by Crider et al. ('60) show that in liver tissue of depleted animals there was always a certain level of Emmerie-Engel reactive material (approximately 9.0 μg/gm liver) which on paper chromatography could not be identified as α-tocopherol. This observation is similar to that of Bieri et al. ('60) in vitamin E-depleted chicks. To obtain further information on all possible reducing compounds in liver tissue the Eggitt and Ward paper chromatograms were developed not only with 75% ethanol but also with 80% *n*-propanol. In addition, the samples were chromatographed on silicone-treated paper and developed with 80% *n*-propanol (Lester and Ramasarma, '59), and on zinc carbonate paper (Green et al., '55) and developed with a mixture of 90% cyclohexane and 10% benzene. The R_f values obtained for the various compounds found to be present in the livers are shown in table 5.

An Emmerie-Engel-reactive compound with R_f values of 0.00 and 0.40 on Eggitt and Ward paper developed with 75% ethanol and 80% *n*-propanol, respectively, was identified as acid-reduced ubiquinone (ubichromanol). This compound was always found to be present in extracts which were passed through the Florex XXS column, and to be formed from ubiquinone by the column. Pudelkiewicz and Matterson ('60) observed the same reduction of ubiquinone by the Florex XXS column, but described the reaction product as a semiform or hydroquinone form of ubiquinone. To distinguish between ubichromanol and ubiquinone hydroquinone we eluted the Emmerie-Engel reaction compound from the paper and oxidized it with lead tetraacetate in acetic acid. Under these conditions ubichromanol yielded a quinone analogous to tocopheryl quinone (the isoprene unit nearest the benzene ring being unsaturated), whereas ubiquinone hydroquinone

TABLE 5

R_f values of Emmerie-Engel reactive compounds present in liver extracts

Diet	Treatment	Eggitt and Ward		Silicone 80% *n*-propanol
		75% ethanol	80% *n*-propanol	
Vitamin E-free	None	0.00[1]	0.53[1]	0.77
Vitamin E-free	α-tocopherol	0.00[1]	0.53[1]	0.78
		0.25	0.85	0.84
Vitamin E-free	DPPD	0.00[1]	0.53[1]	0.77
		0.83	0.87	0.84
Vitamin E-free	BHT	0.00[1]	0.53[1]	—
		0.95	0.95	—
Vitamin E-free	Ethoxyquin	0.00[1]	0.53[1]	—
		0.84	0.90	—
Stock diet	None	0.00[1]	0.53[1]	0.74

[1] These are initial values. Final values after repeated chromatography and purification are 0.79 and 0.90 (see text).

yielded ubiquinone. When the isolated reaction product was put on Eggit and Ward and on silicone papers, R_f values of 0.05 and 0.84 were obtained. These R_f values corresponded to those of an oxidized sample of synthetic ubichromanol rather than of oxidized ubiquinone. The latter should have been found had hydroquinone been the reduction product formed on the column. Since ubichromanol was shown to be an isolation artifact, it is not indicated in table 5.

It was found, however, that one Emmerie-Engel reactive compound was present in all livers assayed. This compound was also found to be present in the muscle tissue of all rats examined. The compound from rat tissues initially stayed at the origin on Eggitt and Ward paper (developed with 75% ethanol) and had an R_f value of 0.53 on the same paper when developed with 80% *n*-propanol. However, when similar assays were carried out with chicken tissues, with pork heart or with beef mitochondria, the only Emmerie-Engel-reactive compound (after removal of α-tocopherol and vitamin A) had R_f values of 0.79 on the Eggitt and Ward-ethanol system and 0.9 on the propanol system. In the case of the rat tissue extracts, it was subsequently found that with re-chromatography two Emmerie-Engel spots in the propanol system (one at 0.5, the other at 0.9) were obtained and that with continued purification and repeated chromatography eventually all Emmerie-Engel material appeared at 0.9. Why the rat compound behaved in this manner is not obvious to us at this time, but it appears that in actual fact the same new Emmerie-Engel-reactive compound is present in all species investigated. This compound was the only one detected in the livers from animals fed the vitamin E-free diet with no anioxidant and from those fed the stock diet. Extracts of livers from animals that had received an antioxidant (vitamin E, DPPD, ethoxyquin or BHT) showed the presence of two Emmerie-Engel-reactive compounds: one, the unidentified compound; the other, the antioxidant that was fed. α-Tocopherol was detected chromatographically only when it had been administered to the animal.

In an attempt to identify this unknown compound, the R_f values of some known Emmerie- Engel-reactive compounds were examined using the 4 paper chromatographic systems shown in table 6. We were able to differentiate and separate this unknown compound (designated in table 6 as compound X) from all Emmerie-Engel-reactive compounds studied, namely, tocopherols, ubichromanol, ubichromenol, solanachromene, a new antioxidant isolated from yeast (Forbes et al., '58) and vitamin A, using the two systems given in the second and third column of table 6.

The non-vitamin E Emmerie-Engel-reactive mtaerial previously reported by Bolliger and Bolliger-Quaife ('55) appears now to have been due to the presence of ubichromanol formed on the Florex XXS column. The unknown compound X reported in the present studies was probably included in the α-tocopherol spot, since in the paper chromatographic system used (Green et al., '55) the R_f values for both compounds are quite similar (0.72 for α-tocopherol and 0.78 for the unknown). Bieri et al. ('60), using Green's system, reported finding an Emmerie-Engel-reactive compound in the livers of vitamin E depleted chicks, which migrated with the same R_f value

TABLE 6

R_f values for Emmerie-Engel-reactive compounds

Compounds	Eggitt and Ward		Silicone	Green
	75% ethanol	80% *n*-propanol	80% *n*-propanol	
α-Tocopherol	0.25	0.85	0.85	0.75
DPPD	0.83	0.95	0.84	0.82
Ethoxyquin	0.84	0.90	0.91	—
BHT	0.95	0.95	0.80	—
Ubichromenol (SC)	0.00	0.40	—	—
Ubichromanol	0.00	0.34	0.62	0.73
Q_{10} Hydroquinone	0.00	—	—	0.73
Compound X	0.00(initial) 0.79(final)	0.53(initial) 0.90(final)	0.70	0.78

as α-tocopherol. He found, however, that when the sterols in the extract were removed, the R_f value was reduced almost to zero. Part of this reducing compound was found to be reduced ubiquinone (ubichromanol), because the Florex XXS column was used during the analytical procedure. Since, in contrast with the present procedure, saponification was used by Bieri and co-workers, it is possible that another part of their reducing material is ubichromenol, which, according to Draper and Csallany ('60) and to Links ('60) could be formed from ubiquinone in boiling alkaline ethanol solution. However, on the basis of preliminary work with the chicks, we feel that part of the reducing activity found by Bieri probably is identical to our unknown compound. Work is in progress on the identification and biological importance of compound X.

SUMMARY

It has been found that rats carried through resorption-gestation when fed vitamin E-deficient diets will respond in a second reproduction cycle to the antioxidants N,N′-diphenyl-*p*-phenylenediamine (DPPD) and 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline (ethoxyquin), but not to butylated hydroxytoluene (BHT) nor acid-reduced ubiquinone (ubichromanol), by bearing live young.

In addition, following resorption-gestation due to vitamin E deficiency in the original dams, rats have been carried through two litters in the second generation when fed vitamin E-free diets containing DPPD.

The livers and carcasses of rats receiving DPPD, of control rats fed a stock diet, of vitamin E-deficient rats, of rats on ethoxyquin, and of rats receiving high levels of α-tocopherol were all found to contain a material which reacts with α,α′-dipyridyl and is usually assumed to be vitamin E.

However, it was found chromatographically that, in fact, while all animals contained a new reducing compound (compound X), only the animals given high levels of α-tocopherol contained vitamin E in addition to compound X. When DPPD and ethoxyquin were fed, these compounds, in addition to the unknown compound X, were found chromatographically in the livers of the animals.

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The Influence of Soybean Antitrypsin on the Intestinal Proteolysis of the Chick[1]

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The inhibition of chick growth by soybean antitrypsin was reported by Ham and associates ('45) who worked with crude preparations of the inhibitor. They found a drop in the intestinal proteolysis of chicks 4 weeks old. However, many contradictory results were later obtained and the mechanism of growth inhibition by raw soybean is still obscure.

Westfall et al. ('48) added a trypsin inhibitor preparation to predigested proteins, and Hill et al. ('53) added essential amino acids to a raw soybean diet. In neither investigation was the expected improvement in growth obtained.

Borchers ('58) has cited many experiments, the results of which are difficult to correlate with a decreased availability of amino acids or with the action of an antitrypsin; Borchers et al. ('48) did not obtain growth inhibition with concentrates of trypsin inhibitor, but could obtain growth improvement of rats by addition of antibiotics to raw soybean oil meal diet (Borchers et al., '57).

On the other hand, Fisher and Johnson ('58) succeeded in overcoming nearly all growth depression in chicks by supplementing a raw soybean diet with a mixture of essential amino acids. They suggested that poor results obtained by earlier workers could have been caused by an inadequate balance of amino acids, and concluded that the availability of amino acids is a major factor in the growth depressing activity of raw soybean. Another "toxic principle," soyin (Liener, '53), was probably responsible for a minor growth depression in chicks fed the amino acid-supplemented raw-soybean diet.

Other facts also indicate that soyin is not the principal growth inhibitor (Almquist and Merritt, '52). According to Borchers ('58) the principal growth inhibitor is not toxic.

Lyman and Lepkovsky ('57) observed an increase of pancreatic activity in rats fed raw-soybean diets, while Chernick et al. ('48) found in chicks, hypertrophic pancreas containing excessive amounts of proteolytic enzymes.

Most of these facts suggest that a factor concerned with intestinal proteolysis may be a major cause of growth retardation by raw soybean. The work to be reported here was undertaken in an attempt to supply direct evidence of the effect of raw soybean on intestinal proteolysis of the growing chick. Since very little is known about proteolysis in the intestine of the growing chick, this problem was investigated in a previous study (Nitsan and Alumot, '60).

MATERIALS AND METHODS

Diet. The percentage composition of the all-vegetable mash was as follows: soybean oil meal (45% protein), 30; sorghum, 30; corn, 28; wheat bran, 4.2; alfalfa meal, 3.2; ground shells, 2.0; dicalcium phosphate, 1.6; salt mixture, 0.3; commercial vitamin mixture, 0.3; vitamin B_{12} (3 mg/kg), 0.2; antioxidant 0.1; bifuron 0.1. Percentage composition of the mineral mixture was: NaCl, 94.70; MnO, 3.36; KI, 0.06; $Cu(OH)_2$, 0.14; $CoCl_2 \cdot 6H_2O$, 0.03; ZnO, 0.09; $FeCO_3 \cdot H_2O$, 0.33; $Na_2MoO_4 \cdot 2H_2O$, 0.01; Ca stearate to 100. Each gram of the vitamin mixture contained in milligrams: riboflavin, 2; Ca pantothenate, 4.8; niacin, 8; choline chloride, 60; menadione, 0.8; also vitamin B_{12} 3.2 μg; vitamin A, 3000 I.U., and vitamin D_3, 420 I.U.

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The soybean oil meal used for the control group was commercially processed and toasted. For the experimental treatment, raw extracted flakes from the same factory were used after evaporation of the solvent at room temperature. The crude protein content of the mash was 21.0%.

Experimental animals. In each of three trials, 100 three-day-old, White Leghorn male chicks were divided into two equal groups. One group received the experimental diet and the other served as control. Both groups were fed twice daily, from 7 to 9 A.M. and from 1 to 4 P.M. The purpose of the restricted feeding program was to obtain animals which had ingested approximately the same amount of feed at the same time. During the first two hours in the morning the trained chicks ate very intensively and consumed about 40% of the daily intake. In a preliminary experiment it was found that after two hours, feed consumption of trained chicks decreased markedly when the chicks had access to feed.

The daily food consumption of the control group was almost the same as that observed for chicks fed continuously. The experimental group consumed only 80% as much feed as the control.

The proteolytic activity in the intestines of the chicks of both groups was measured from two to 8 weeks of age at different intervals after feeding.

Chicks were killed by rapid bleeding, three at a time. The enzyme preparation and the slight modifications of Kunitz's procedure (Collowick and Kaplan, '55) for the determination of proteolytic activity are described in a previous paper (Nitsan and Alumot, '60). The activity is expressed in optical density at 280 mμ. One μEq of tyrosine corresponds to an optical density (O.D.) reading of 0.151.

Preparation of antitrypsin. The chyme suspension, remaining after determination of proteolytic activity (containing 100 mg of chyme per ml), was adjusted to pH 4.2 with hydrochloric acid and refrigerated overnight.

After centrifugation at 8000 rpm in a Servall refrigerated centrifuge, the supernatant was measured and 33 gm of ammonium sulfate for each 100 ml were added (about 52% saturation). The precipitate, containing the crude antitrypsin, was collected by centrifuging twice at 8000 rpm and then dissolved in a phosphate buffer, pH 7.6. The volume of buffer added was chosen arbitrarily as 1:15 of the volume of the initial supernatant. This procedure gave a sufficient amount of antitrypsin preparation for further determination and assured the same approximate concentration of it in each case. The antitrypsin was, in this way, 15 times as concentrated as in the original enzyme preparation.

Determination of antitryptic activity. Amounts from 0.1 to 0.5 ml of the above preparation were brought to 1 ml with the phosphate buffer, pH 7.6. One milliliter of 0.1% trypsin (1:250 Difco) was added and the reaction tubes were brought to 37°C in an incubator. One milliliter of 1% casein was added as described by Kunitz (Collowick and Kaplan, '55). After 20 minutes, the reaction was stopped by 5 ml of 10% trichloroacetic acid (TCA). After centrifugation the optical density of the filtered supernatant was read against a reagent blank at 280 mμ in a Beckman DU Spectrophotometer.

The readings were corrected for the values of blanks prepared in the same way but in a different order: the TCA was added before the addition of casein.

Along with each antitrypsin assay, the digestion of casein by trypsin was determined. The antitryptic activity is expressed as the depression of tryptic digestion of casein, caused by the antitrypsin preparation.

Antitryptic activity (in O.D. units) = O.D. (casein, trypsin) − O.D. (casein, trypsin, antitrypsin preparation).

Determination of peptic activity. The enzyme preparations were made by grinding gizzard contents with 5 volumes of distilled water in a Waring blender.

The peptic activity was tested by Anson's ('39) method with some modifications:

The enzyme preparation (0.3 to 1.0 ml) was brought to 2 ml with 0.06 N HCl and put in an incubator of 25°C. After reaching the temperature, 1 ml of 2% hemoglobin[2] in 0.06 N HCl was added and after

[2] May and Baker Ltd.

exactly 10 minutes the reaction was stopped by 5 ml of 10% TCA. After centrifugation and filtration the supernatant was read at 280 mμ and correction made for blanks (see trypsin determination).

RESULTS

Homogeneity of preparations and variability of results were tested in our previous study (Nitsan and Alumot, '60). It was found there, that chicks fed a commercial diet had similar intestinal proteolytic activity at all ages and intervals after feeding. Similar results were obtained in this study with chicks fed the control diet. The proteolytic curves (daily averages) are given in figure 1.

Strikingly different results, reproducible in all the trials, were obtained with chicks, fed the raw-soybean diet. The proteolysis was found to be dependent on age of chicks and intervals after feeding.

The enzyme curves obtained at two and 4 hours after feeding began are presented

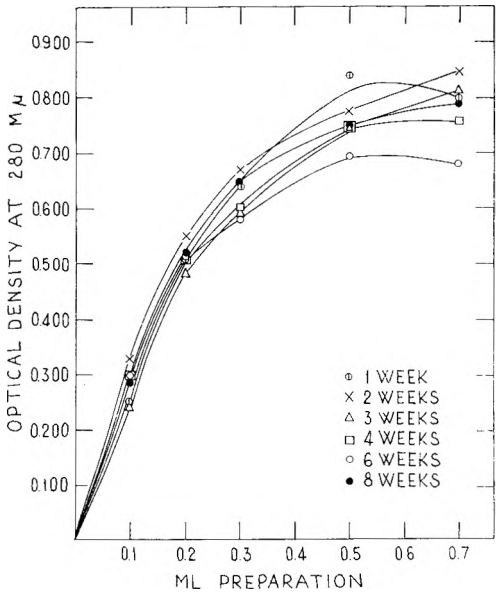

Fig. 1 Enzyme curves of control group (daily averages of three experiments).

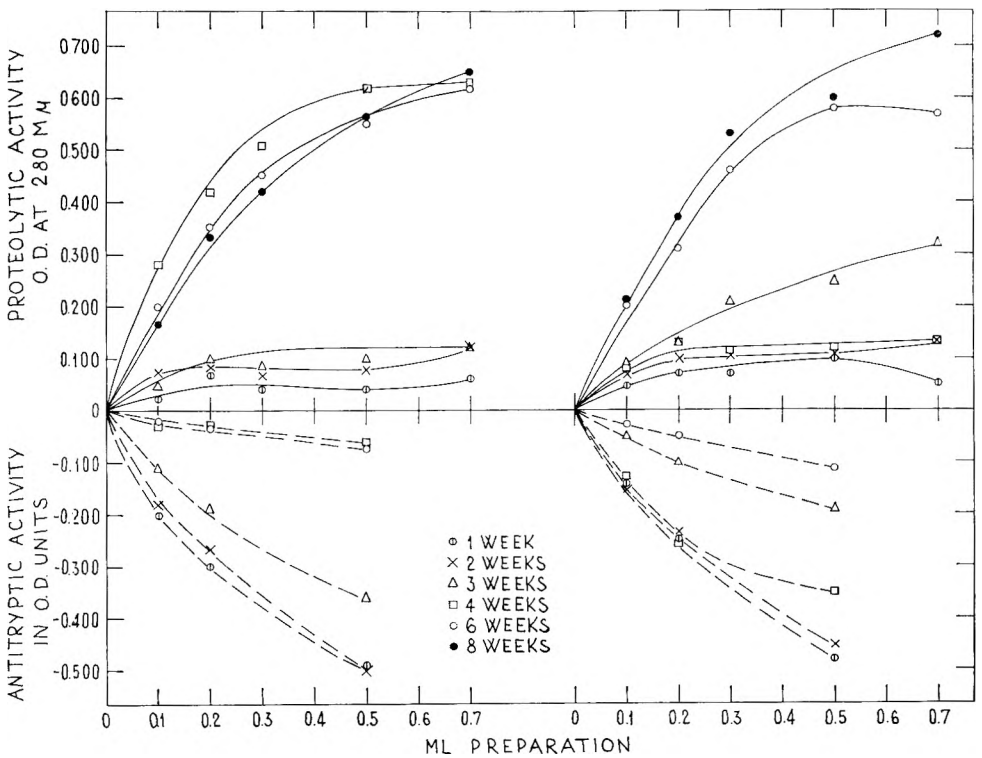

Fig. 2 Proteolytic and antitryptic activity of experimental group (average of 3 experiments); (left) two hours after feeding began; (right) 4 hours after feeding began. The antitryptic preparation is concentrated 15 times as compared with the proteolytic enzyme preparation.

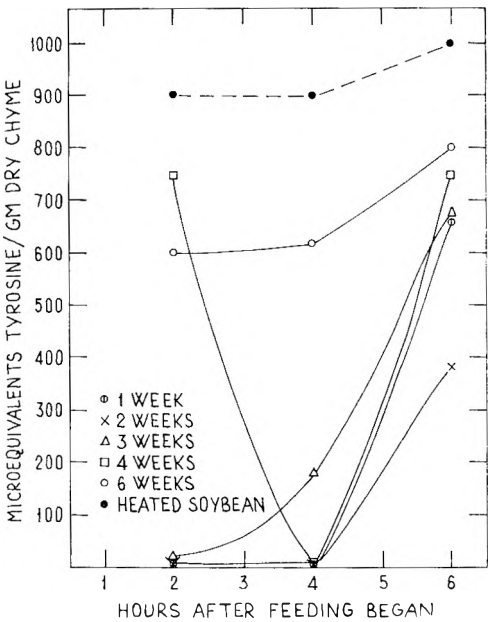

Fig. 3 Proteolytic activity of the experimental group in the different ages in comparison with that of the control (average of all ages).

in figure 2. In this figure, in addition to the proteolytic curves, the antitryptic activity (see methods section) is plotted on the negative side.

At 6 hours after the beginning of feeding, regular activity curves were obtained in all ages tested.

Figure 3 presents the age-dependent activity of the experimental group in comparison with the activity of the control (calculated in microequivalents of tyrosine per gram of dry chyme). Since the activity of the control group was similar at all ages, an average is presented. All the calculations are based on the activities of the initial concentrations (straight part of the enzyme curves). A complete lack of enzyme-curve was interpreted as zero-activity.

Chicks up to three weeks, fed the raw-soybean diet, showed practically no intestinal proteolysis during the first 4 hours after feeding began (figs. 2, 3). At 4 weeks of age some activity existed at the beginning, as well as at the end, of the determinations. However a drastic drop of proteolysis was found 4 hours after feeding began. Results obtained with 5-week old chicks were similar to those at 4 weeks. From 6 weeks of age normal enzyme curves were obtained in the experimental group throughout the day, but the values were lower than in the control group.

A close correlation was found between the proteolytic activity and the presence of antitrypsin in the intestinal content. Whenever a marked depression of proteolysis was found, considerable antitryptic activity occurred. On the other hand, when the proteolytic activity was normal, practically no antitryptic activity was found in the chyme.

The return to normal activity with age is probably connected with the hypertrophy of the pancreas, which was observed during all the experimental period (8 weeks) and was most conspicuous between the second and the third week. Data on chick and pancreas weights are given in table 1.

The mean weight of the pancreas of chicks from both groups was almost the same, whereas the mean body weight of the experimental chicks was much lower. The weight of the pancreas in percentage of total body weight was, therefore, higher in the experimental group (fig. 4). In agreement with the results of Chernick et al. ('48), other organs were not found to to be affected.

In order to examine whether the chicks, up to three or 4 weeks of age, suffer from protein deficiency, the following two points

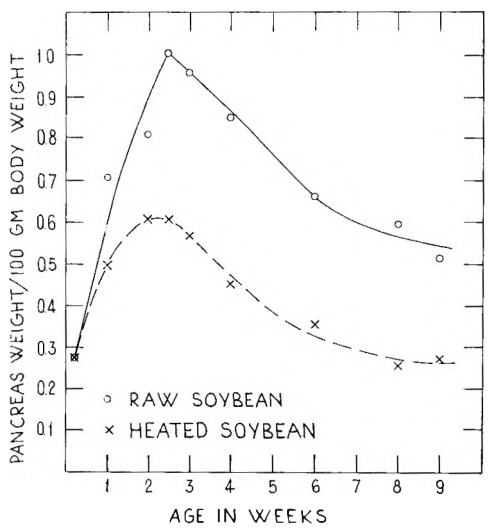

Fig. 4 Pancreas: body ratio in the two groups. Each point represents an average of 18 chicks.

TABLE 1

Mean body and pancreas weights

Age in weeks	Body weight		Pancreas weight		Dry matter in pancreas	
	Heated soybean	Raw soybean	Heated soybean	Raw soybean	Heated soybean	Raw soybean
	gm	*gm*	*gm*	*gm*	%	%
0	37± 1.6[1]	37±1.6	0.11±0.011	0.11±0.011	20.2	20.2
1	49± 1.3	42±1.7	0.28±0.007	0.30±0.018	22.7	23.4
2	82± 1.0	56±4.3	0.50±0.004	0.45±0.008	—	—
2.5	119± 5.9	73±4.3	0.72±0.006	0.77±0.025	26.7	25.5
3	144± 7.0	85±4.6	0.82±0.008	0.83±0.008	22.9	20.3
4	201±10.1	119±6.3	0.90±0.007	1.05±0.014	27.1	24.0
6	328±16.2	192±8.7	1.20±0.016	1.30±0.061	28.3	20.2
8	550±26.0	268±8.9	1.40±0.023	1.50±0.037	26.2	24.5

[1] Standard error of mean.

TABLE 2

Peptic activity in the gizzard and tryptic activity in the small intestine

Age in weeks	Peptic activity		Tryptic activity
	Heated soybean	Raw soybean	Heated soybean
	μEq tyrosine/gm dry content		
2	78	54	1116
3	99	92	936
4	121	113	930
6	86	67	1000

TABLE 3

Stability of inhibitor in intestinal preparation[1]

Age of preparation	Digestion time with casein	Optical density at 280 mμ	
		Raw soybean	Heated soybean
	minutes		
Fresh	20	0.057	0.735
1 hour	20	0.100	0.680
2 hours	20	0.063	0.640
3 hours	20	0.160	0.650
	hours		
Fresh	1	0.212	0.800
Fresh	2	0.417	0.820
Fresh	3	0.542	0.810
Fresh	4	0.587	0.830

[1] One-half milliliter of enzyme preparation, containing 50 mg of fresh chyme from three-week-old chicks, two hours after feeding began.

were tested: (1) peptic activity in the gizzard, and (2) the stability of the possible trypsin-antitrypsin complex, formed in the intestine of chicks, fed a raw soybean diet. Peptic activity of gizzard contents was tested in both groups. Slightly lower activities were observed in the experimental group. In general, peptic activity was found to be weaker than tryptic. The comparison, based on activity per gram of dry content, is given in table 2.

The stability of the trypsin-antitrypsin complex is shown in table 3. The results were obtained by leaving the enzyme preparations in an incubator at 37°C and testing the proteolytic activity, in the usual way (20 minutes' casein digestion), at different intervals. On the other hand, experiments were conducted with fresh preparation and casein digestion longer than 20 minutes.

The results indicate that the enzyme-inhibitor complex is stable under conditions similar to the natural ones for at least three hours. However, when an appreciable amount of soluble substrate (casein) is in contact with the preparation for a long time, some of the enzyme is released from the complex and the proteolytic activity reaches about 70% of that of the control group after three hours.

DISCUSSION

The growth inhibition of chicks by soybean antitrypsin can be explained after consideration of the following facts. (1) In the first three weeks of life intestinal proteolysis is almost completely inhibited. Although an increase in activity is observed 6 hours after feeding begins, the chick has little benefit from it, because at this time only residues of the food, initially ingested, are available. A confirmation of the initial inhibition of proteolysis can be found in a recent study of Lepkovsky et al. ('59). They found that feces of chicks fed raw-soybean diet contained in the first week much less proteolytic activity than of those in the heated soybean group. (2) The inhibition of proteolysis is stable for at least three hours, as shown in table 3. The average passage time of food through the small intestine is probably less than three hours (Halnan, '49). (3) The peptic activity of the gizzard is not increased in the experimental group. In addition, pepsin plays probably a minor role in chick digestion (Sturkie, '54); this fact was also confirmed in this study.

The above facts indicate that animals up to three weeks old are probably unable to fully digest the protein of the raw-soybean diet. This can explain the good growth of chicks up to two weeks of age, obtained by Fisher and Johnson ('58) with an amino acid-supplemented raw-soybean diet.

In some unknown way, the pancreas reacts to the presence of the trypsin inhibitor in the intestine and secretes more enzymes, compensating for the effect of the inhibitor. Hypertrophy of the pancreas develops in the experimental chicks as a result of this increased activity. This fact was already observed by Chernick et al. ('48), who also found increased amounts of proteolytic enzymes in the hypertrophic pancreas.

A distinct peak was observed in the experimental group between two and three weeks (fig. 4), when the pancreas probably made the greatest effort to overcome the inhibitor. It was actually shown that beginning with three weeks of age, the proteolytic activity increased continuously and approached the normal one at about 6 weeks of age.

In the 4th and 5th week a drop in activity was observed at about 4 hours after feeding had begun. It is possible, that at this point antitrypsin is released from the feed in the crop at a maximum rate. Considerable antitryptic activity at almost all ages, supports this observation (fig. 2, right).

Lyman and Lepkovsky ('57) observed an increase of pancreatic activity in rats fed a raw-soybean diet for a short time. They explained the growth depression, caused by the antitrypsin, by loss of endogenous nitrogen, rather than by insufficient proteolysis. In chicks, the two factors are probably involved, depending upon age: up to two-to-three weeks' unavailability of amino acids, caused by inhibited intestinal proteolysis, should be taken into account. This fact loses its importance at about 6 weeks. Increased requirement of protein, caused by the pancreatic hyperactivity, is the second possible cause of growth inhibition.

SUMMARY

The influence of a raw-soybean diet on the chicks' intestinal proteolysis was studied, in order to explain the growth-retarding effect.

In chicks up to three weeks old, intestinal proteolysis was almost completely inhibited. From the 4th week proteolysis increased, approaching the normal at 6 weeks. The presence or absence of antitryptic activity was closely correlated with depressed or normal proteolytic activity.

Hypertrophy of the pancreas developed in the raw soybean group, probably because of increased enzyme production to overcome the inhibitor.

The chick growth retardation by antitrypsin can be explained by the unavailability of protein especially in the first

three weeks of age and an increased protein requirement by stimulated pancreas.

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Relation of Vitamin E to Proteolytic and Autolytic Activity of Skeletal Muscle[1,2]

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Previous studies in our laboratory have shown the presence of at least two proteolytic enzymes in rat skeletal muscle. Whereas one of these enzymes catalyzes the hydrolysis of acid-denatured hemoglobin optimally at pH 3.8,[4] the other attacks endogenous muscle proteins as well as both hemoglobin and albumin optimally at pH 9.0 (Koszalka et al., '60b). The latter enzyme appears to be responsible for muscle autolysis which is also maximal at pH 9.0 (Koszalka et al., '60a).

It has also been reported that the proteolytic activity of skeletal muscle at pH 4.0 is increased in dystrophic rabbits deficient in vitamin E (Weinstock et al., '55), and also in mice with genetically determined dystrophia muscularis (Weinstock et al., '58). The studies presented in this report, based upon examination of skeletal muscles from vitamin E-deficient rats and hamsters, confirm and extend the observations of these investigators, and also indicate that vitamin E deficiency is associated with an increase in the autolytic activity of skeletal muscle.

MATERIALS AND METHODS

Syrian golden hamsters were fed from weaning a vitamin E-deficient diet composed (in per cent) of casein, 20; cornstarch, 25; rolled oats, 25; lard, 18; dried brewers' yeast, 7.5; salts, 2.5; and cod liver oil, 2. Albino rats were fed from weaning a vitamin E-deficient diet (as employed in the studies of Emmel, '57) composed of casein, 23.8; sucrose,[5] 48.9; dried brewers' yeast, 8.9; salts, 3; lard (containing 5000 I.U. vitamin A and 500 I.U. vitamin D per gm) 0.5; and linseed oil fatty acids, stabilized with propyl gallate and citric acid, 14.9%. In one series of studies with rats, summarized in table 2, vitamin E- free lard was used instead of linseed oil fatty acids Control animals were of two types; those fed the deficient diet but given oral supplements of α-tocopheryl acetate (12 mg daily) or α-tocopherylhydroquinone (10 mg daily) and those reared with a commercial stock diet,[6] with added meat.

At the end of the experimental period the animals were decapitated and bled before removal of tissues. Samples of skeletal muscles were removed and fixed for subsequent histological study, so that the degree of dystrophic change or the extent of repair after vitamin E therapy could be correlated with changes in enzymatic activity. The hind limbs were then skinned and samples of skeletal muscles weighing 5 to 10 grams, trimmed as far as possible of fat, fascia and tendon, were placed in ice-chilled beakers. To each gram of pooled muscle 5 ml of cold 2% KCl (4°C) were added and the mixture homogenized in a thoroughly chilled stainless steel blender.

The preparation was then filtered through two layers of surgical gauze to

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[1] These studies were supported by grants-in-aid from the Muscular Dystrophy Associations of America, Inc., and the United States Atomic Energy Commission.
[2] A preliminary report of these studies was made at the Annual Meeting of the American Institute of Nutrition, Chicago. (Koszalka, T. R., G. Krol and K. E. Mason 1960 Enzyme studies of skeletal muscle in vitamin E deficiency. Federation Proc., 19: 420 [abstract]).
[3] Postdoctoral Fellow of the Muscular Dystrophy Associations of America, Inc.
[4] Koszalka, T. R. 1959 The proteolytic activity of rat skeletal muscle. Thesis, University of Rochester, Rochester, New York.
[5] Cerelose, Corn Products Refining Company, New York.
[6] Purina Fox Chow, Ralston Purina Company, St. Louis.

remove strands of connective tissue which otherwise interfered with pipetting. The resulting homogenate was dialyzed with stirring against 2% KCl for 18 hours at 4°C. Aliquots of the dialyzed, filtered, muscle preparation were analyzed for protein, using the Folin reagent (Lowry et al., '51).

In one experiment, the major portion of the adductor magnus and brevis muscles of the thigh and the lower cervical and upper thoracic segments of the sacrospinalis muscles were selected for study. It was felt that more consistent data might be obtained if specific muscles or muscle groups were used rather than larger muscle masses which were not always comparable in terms of actual muscles represented. These particular muscles were selected because they are relatively fleshy and their connective tissue component is small and delicate. Each muscle sample, weighing from one to 5 gm, was placed on filter paper to remove moisture and homogenized with cold 2% KCl solution (10 ml per gm of muscle). After dialysis against 2% KCl, the final volume of the homogenate was recorded and the material filtered through surgical gauze. The preparations were then homogenized once more in a Dounce type[7] all-glass homogenizer immersed in an ice-bath. Aliquots of the latter preparation were used for the determination of enzymatic activity and protein content.

Assay of proteolytic activity. Three grams of hemoglobin[8] were mixed with 40 ml of 0.1 M acetate buffer, pH 3.8, with continuous stirring for 5 to 10 minutes. After the pH was readjusted to 3.8 with 4 M HCl, the solution was diluted to 50 ml with more acetate buffer. The preparation was centrifuged and the small amount of undissolved hemoglobin discarded. The resultant supernatant, a fresh 6% solution of acid-denatured hemoglobin, was prepared each day.

The assay for proteolytic activity was carried out as follows: one-milliliter aliquots of 0.2 M sodium acetate buffer, pH 3.8, were added to each of four 12-ml centrifuge tubes, two being used as controls and the other two containing the test systems. One milliliter of 6% hemoglobin solution was added to each of the two test systems, and 2.5 ml of the substrate solution were added to a 5th centrifuge tube. After incubating all tubes for 5 minutes at 37°C, one milliliter of muscle homogenate was added to each of the two control and two test systems, with mixing. Exactly 30 minutes after addition of the muscle homogenate, 5 ml of 5% trichloroacetic acid solution were added. One-milliliter aliquots of the 6% hemoglobin solution incubating in the 5th tube were added to each of the two control tubes. After mixing and standing at room temperature for one hour, the tubes were centrifuged and the optical density of the clear supernatant solutions was determined at 280 mμ in a Beckman DU Spectrophotometer. As shown earlier (Kunitz, '47), the enzymatic hydrolysis of a protein containing tyrosine and tryptophan can be followed spectrophotometrically by measuring the optical density at 280 mμ of trichloroacetic acid filtrates of the enzyme incubation mixture. Proteolytic activity was expressed as the optical density of the incubated complete test system minus that of the control. "Specific proteolytic activity" was defined as proteolytic activity per milligram of muscle protein multiplied by 10^4.

Preliminary experiments indicated that hamster skeletal muscle homogenates exhibited maximal proteolytic activity against hemoglobin at or near pH 3.5, whereas homogenates prepared from rat muscle were maximally active at pH 3.8. Consequently, the enzyme assays were carried out at these pH levels. Proteolytic activity was proportional to the protein concentration of the muscle homogenates in the ranges studied.

Assay of autolytic activity. Two milliliters of 0.1 M glycine buffer, pH 9.0, were incubated for 5 minutes at 37°C, after which one milliliter of dialyzed muscle homogenate was added to the system. After incubating the complete system for 30 minutes, 5 ml of 5% trichloroacetic acid solution were added to stop the reaction. The preparations were mixed and allowed to stand at room temperature for one hour; after centrifugation, the optical density of the resultant supernatant solu-

[7] Obtained from Blaessig Glass Specialties, Rochester, New York.

[8] Bovine Hemoglobin Enzyme Substrate, obtained from Nutritional Biochemicals Corporation, Cleveland.

tions was determined at 280 mμ in order to measure the release of acid-soluble peptides containing tyrosine and tryptophan from the endogenous muscle protein. Evidence for the view that the observed reaction is enzymatic as well as proteolytic in nature has been presented elsewhere (Koszalka et al., '60a). Autolytic activity was expressed as the optical density of the incubated complete system minus that of a nonincubated control. "Specific autolytic activity" was defined as autolytic activity per milligram of muscle protein multiplied by 10^4.

RESULTS

Table 1 shows the effect of dietary treatment on proteolytic and autolytic activity of the thigh muscles of hamsters and rats. The specific proteolytic activity of muscles from hamsters fed the vitamin E-deficient diet for 58 to 60 days, showing early onset of dystrophic lesions (rated + to ++, on a + to ++++ histological basis), was about the same as that of muscle from stock animals. The values obtained from littermate hamsters given the same deficient diet for 58 to 60 days but supplemented with α-tocopheryl acetate were lower than those obtained from animals fed a stock diet. The difference between the two values is statistically significant at the 5% level of significance as estimated by the "t" test (Dixon and Massey, '57). Whether this difference can be explained on the basis of different levels of dietary tocopherol intake of these two groups of animals, or on some other basis, will require further study.

On the other hand, the specific proteolytic activity of muscle from hamsters fed a vitamin E-deficient diet for 120 to 130 days (muscle lesions rated + to +++) and 327 to 354 days (muscle lesions rated ++ to ++++), respectively, was increased to approximately twofold and tenfold that observed in hamsters fed a deficient diet for 58 to 60 days. Although the proteolytic activity of muscle of hamsters receiving a deficient diet for 90 days and subsequently treated orally with α-tocopherylhydroquinone for 30 to 54 days was about the same as that of stock animals, oral administration of α-tocopheryl acetate for 10, 20, 30 and 40 days to hamsters previously fed a deficient diet for 330 days resulted in a pronounced diminution in proteolytic activity but not in a return to normal levels. All histologic evidence of degenerating fibers in the muscle of hamsters treated with either derivative of tocopherol was abolished, but there were present many fibers showing internal nuclear rowing; the latter presumably represented late phases of regeneration such as described by West and Mason ('55, '58).

The specific autolytic activity of hamster muscle changed in the same general direction as the specific proteolytic activity; however, the extent of the change was not as striking as that of the proteolytic activity (table 1).

Results similar to those described for hamsters were obtained when rats were used as experimental animals (table 1). The specific proteolytic activity of thigh muscle from rats maintained with a vitamin E-deficient diet for 293 to 355 days was nearly 10 times, and the specific autolytic activity was approximately twice, that of muscle from rats fed stock diets.

The data of table 2 record the proteolytic and autolytic activity of more specific muscle groups (adductors of the thigh; cervical and thoracic segments of the sacrospinalis) from rats maintained with a deficient diet for 5 to 6 months, with and without oral supplements of α-tocopheryl acetate. As might be expected from the results of the previous experiment, the specific proteolytic activity of muscles from deficient rats was 4 to 6 times that of vitamin E-treated controls. Proteolytic activity, when based on wet weight of muscle, was three to 5 times greater in deficient rats than in vitamin E-treated controls. Furthermore, the total proteolytic activity of the adductor and sacrospinalis muscles from vitamin E-deficient animals was two to three times greater than that of the control group. Yet, the wet weight and protein content of the muscles from the deficient animals were significantly less than that of the supplemented controls.

The specific autolytic activity of the adductor and sacrospinalis muscles of deficient rats was two and one-half to three times that of control rats. The autolytic activity expressed per milligram of wet muscle was increased in the vitamin E-deficient animals to about the same degree whereas the total autolytic activity of each muscle group was increased twofold.

TABLE 1

Effect of vitamin E deficiency on the proteolytic and autolytic activity of skeletal muscle

No. of animals	Dietary treatment (from weaning)	Specific proteolytic activity[1] pH 3.5 to 3.8	Specific autolytic activity[2] pH 9.0	Muscle histology (lesions rated + to +++)
Hamsters				
11	Stock diet	66.7 ± 9.0[3]	51.1 ± 11.6	normal
5	Vitamin E-deficient diet, with added α-tocopheryl acetate,[4] 58 to 60 days	38.4 ± 1.6	39.1 ± 4.4	normal
4	Vitamin E-deficient diet, 58 to 60 days	60.8 ± 1.6	39 ± 4.2	+ to + +
6	Vitamin E-deficient diet, 120 to 130 days	138 ± 18	95.4 ± 19.3	+ to + + +
8	Vitamin E-deficient diet, 90 days, then with added α-tocopherylhydroquinone,[5] 30 to 54 days	75.7 ± 7.3	46.6 ± 5.3	normal[6]
6	Vitamin E-deficient diet, 327 to 354 days	680 ± 76	205 ± 23	+ + to + + + +
8	Vitamin E-deficient diet, 330 days, then with added α-tocopheryl acetate, 10 to 40 days	191 ± 23	89 ± 12.9	normal[6]
Rats				
10	Stock diet	46 ± 1.6	39.5 ± 1.8	normal
12	Vitamin E-deficient diet, 293 to 355 days	446 ± 69	86 ± 11.4	+ to + + +

[1] Specific proteolytic activity is defined as optical density increment at 280 mμ per 30 minutes' incubation per mg protein × 10^4. Muscle homogenate was incubated with 2% denatured hemoglobin at pH 3.5 (hamster) or pH 3.8 (rat) in 0.1 M acetate buffer at 37°C.

[2] Specific autolytic activity is defined as the optical density increment at 280 mμ per 30 minutes' incubation per mg muscle protein × 10^4. Muscle homogenate was incubated in 0.067 glycine buffer pH 9.0, without the addition of exogenous substrate, at 37°C.

[3] Standard error of the mean.

[4] α-Tocopheryl acetate, kindly provided by Distillation Products Industries, Rochester, New York.

[5] α-Tocopherylhydroquinone, kindly provided by Distillation Products Industries, Rochester, New York.

[6] Normal, except for the presence of moderate numbers of fibers showing internal nuclear rowing, interpreted as regenerative response.

TABLE 2

Effect of vitamin E deficiency on the protein content, and on the proteolytic and autolytic activity, of rat skeletal muscle

No. rats	Dietary treatment	Wet weight	Total protein	Protein/gm wet weight	Proteolytic activity pH 3.8			Autolytic activity pH 9.0		
					Specific activity[1]	Activity/ mg wet tissue × 10^4	Total activity[2]	Specific activity[1]	Activity/ mg wet tissue × 10^4	Total activity[2]
		gm	*gm*	*gm*						
+ E 167 to 178 days										
9	Adductor[3]	4.04 ± 0.22	0.900 ± 0.026	0.221 ± 0.016	57.9 ± 7.6	11.9 ± 0.6	4.79 ± 0.35	15.2 ± 2.4	3.1 ± 0.3	1.22 ± 0.07
6	Sacrospinalis[4]	2.42 ± 0.11	0.481 ± 0.079	0.196 ± 0.028	47.5 ± 4.9	9.4 ± 1.1	2.41 ± 0.39	19.6 ± 1.2	3.7 ± 0.4	0.90 ± 0.11
— E 158 to 178 days										
9	Adductor	2.86 ± 0.22	0.545 ± 0.050	0.193 ± 0.012	212 ± 11	40.1 ± 2.7	11.09 ± 0.57	41.2 ± 2.8	7.7 ± 0.6	2.17 ± 0.08
6	Sacrospinalis	1.67 ± 0.15	0.309 ± 0.047	0.186 ± 0.027	263 ± 34	45.7 ± 3.4	7.43 ± 0.38	61.4 ± 1.7	11.4 ± 1.1	1.90 ± 0.24

[1] The specific proteolytic and autolytic activities have been defined in table 1.

[2] The total activity of a muscle or muscle group was calculated by multiplying the activity per milliliter of muscle homogenate by the total volume of homogenate.

[3] Representing fleshy adductor magnus and brevis muscle of the thigh.

[4] Representing fleshy portion of lower cervical and upper thoracic segments of sacrospinalis muscle (samples from 6 animals analyzed).

DISCUSSION

The studies described in this paper indicate that hamster skeletal muscle possesses proteolytic and autolytic activity similar to that of rat skeletal muscle, and also demonstrate that these enzyme activities are significantly increased in both species when the skeletal muscle is made dystrophic by vitamin E deficiency. This is in agreement with the observations of Weinstock et al. ('55) that the skeletal muscle of vitamin E-deficient rabbits shows increased proteolytic activity, at pH 4.0.

In our experimental hamsters it may be noted that as muscle lesions became progressively more severe with increased duration of vitamin E depletion a proportional increase was observed in proteolytic and autolytic activity of the muscle; furthermore, after 10 to 12 months of deficiency these activities were considerably greater in hamsters than in rats, which is in keeping with the fact that at comparable periods of deficiency the proportion of degenerating fibers or fiber segments, and of regenerating fibers, is considerably greater than in rats. Unfortunately, it has not been possible to determine whether this increase in proteolytic and autolytic activity precedes or follows the onset of muscle lesions, or whether it can related in any way to increased numbers of macrophages, known to be relatively rich in lytic enzymes, which appear in and around degenerating segments of muscle fibers but are not very much in evidence after repair of the lesions. The observations that all evidence of degenerating fibers is abolished after less than 10 days of vitamin E therapy, leaving only regenerating and presumably normal fibers (West and Mason, '55), and that periods of 10 to 40 days' therapy did not reduce proteolytic or autolytic activity to fully normal levels in the studies reported here, suggest that either degenerating fibers or macrophages, or both, represent the major source of increased enzyme activity; however, higher than normal activity after repair by vitamin E-therapy indicates that the possibility of increased enzymatic activity of regenerating fibers or of the connective tissue component of muscle cannot be ignored.

As pointed out by Weinstock et al. ('58), one of the manifestations of hereditary muscular dystrophy in mice is a reduction in noncollagen protein nitrogen content of the muscles; consequently, changes which are observed in muscle enzyme activities due to disease associated with muscle dystrophy and atrophy may be only a "reflection of overall functioning muscle mass." Thus, an apparent increase in proteolytic and autolytic activity when based on wet weight or protein content of muscle might be explained in animals deficient in vitamin E by loss of other specific protein from the affected muscles without loss of the enzymatic protein. That this is not the case in our experiments is shown in table 2. The proteolytic and autolytic activity of the adductor and sacrospinalis muscles of the rat were increased markedly per milligram of wet tissue or protein, as compared with vitamin E-treated controls. At the same time the total proteolytic and autolytic activity of each muscle group were increased two- to threefold despite a marked decrease in its wet weight and protein content. If the increase in proteolytic and autolytic activity of muscle were due simply to the loss of other protein, the total proteolytic and autolytic activity of each muscle group should not have increased in the deficient rats.

Although several explanations suggest themselves, it is premature to speculate further on the mechanism whereby muscle proteolytic and autolytic activity are increased in vitamin E deficiency. The observed increase in muscle autolytic activity is especially difficult to interpret since the endogenous protein of muscle which serves as substrate for the autolytic enzyme(s) is unknown. Presumably the increase in muscle autolysis could be attributed to a change in the amount or physicochemical property of the endogenous muscle substrate or to a change in the amount or nature of the autolytic enzyme(s) and their possible activators, namely cysteine, glutathione and Fe^{++} (Koszalka et al., '60b).

It will also be of interest to study the proteolytic and autolytic enzymes of muscle made dystrophic by procedures other than vitamin E depletion, namely, choline deficiency, selenium deficiency, trauma, local ischemia, and neurotomy—in order to investigate the possibility that the observed increase in enzyme activity is a

nonspecific response of muscle to metabolic or mechanical injury.

As evidence that the phenomenon of increased proteolytic activity may be a more general one in vitamin E deficiency than has heretofore been recognized, it has been demonstrated that the kidneys of vitamin E-deficient animals also have an increased rate of postmortem autolysis (Emmel, '57). A collaborative study currently in progress[9] indicates that increased proteolytic activity in the kidneys of deficient animals can be demonstrated in assay systems using specific protein substrates.

SUMMARY

Proteolytic and autolytic activity of dystrophic pooled samples of skeletal muscle of vitamin E-deficient hamsters and rats, in terms of protein content of muscle, were increased two- to tenfold in proportion to the duration of the deficiency state. Using more specific samples of skeletal muscle (adductors of thigh and sacrospinalis) from rats deficient in vitamin E for 5 to 6 months, it was observed that the total proteolytic and total autolytic activity, as well as the activity based upon protein content or wet weight of muscle, were appreciably increased. In addition, the total wet weight and protein content of the muscles of the deficient animals were significantly less than that of the unsupplemented controls.

Oral supplementation with antidystrophic derivatives of vitamin E for 10 to 54 days restored enzymatic activity to normal levels in hamsters showing early dystrophic lesions (three months); when dystrophy was more advanced (11 to 12 months) activity was significantly reduced but not restored to normal levels.

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Heated Fats

II. THE NUTRITIVE PROPERTIES OF HEATED COTTONSEED OIL AND OF HEATED COTTONSEED OIL FRACTIONS OBTAINED BY DISTILLATION AND UREA ADDUCT FORMATION[1]

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The effects of various heating procedures upon the nutritive value of food fats and oils have recently been under study in several laboratories. (Crampton et al., '51a,b,c, '53, '56; Kaunitz et al., '55, '56a,b, '59; Johnson et al., '56, '57; Perkins and Kummerow, '59; Alfin-Slater et al., '59; Keane et al., '59; Rice et al., '57). A loss in nutritive value accompanied by a variety of deleterious effects have been reported. The nature of the changes that take place is varied and depends upon specific processing conditions such as the duration of heating, the presence or absence of oxygen, the temperature, the presence of moisture, and the nature of the container, (Perkins and Kummerow, '59). As industrial cooking operations become more extensive, and as the consumption of foods prepared in industrial establishments rather than in home kitchens increases, questions concerning these effects assume greater importance.

The present study was initiated to develop additional information on the relationship of chemical changes to the changes in nutritive value that take place in fats subjected to prolonged heating at temperatures simulating those used in commercial deep fat frying operations.

EXPERIMENTAL

The general plan of the study was similar to that of Crampton et al. ('51c). Thirty pounds of U.S.P. cottonseed oil in an open aluminum vessel were heated by an immersion heater and stirred at 100 rpm continuously for approximately 190 hours at 225°C. The heated oil was stored under nitrogen in pint-size amber-colored bottles at − 15°C. Some of the chemical and physical constants obtained at different times during the heating process are shown in table 1. Of particular interest is the increase in molecular weight and viscosity, the decrease in the iodine value (from 109 to 73), the disappearance of peroxides, and the appearance of epoxides. The epoxides reached a peak value about half-way during heating and thereafter decreased. The carbonyl and hydroxyl oxygen content of the heated oil was relatively low. The chemical findings are being reported in greater detail elsewhere (Firestone et al., '60a).

Ethyl esters were prepared from the heated oil by interesterification according to the method of Crampton et al. ('53). In table 2 are shown the results of the analysis of these ethyl esters as compared with the ethyl esters obtained from the unheated oil. Particularly noteworthy is the decrease in the linoleic acid content from 49 to 9%, the very small change in the oleic acid content from 20 to 18, and the appearance of large amounts of dimer and higher polymers.

The fractionation procedure is summarized in figure 1. The esters were obtained from the heated oil by alcoholysis. These were distilled at 115°C and 7 μ. The distillate represents 63% of the starting material. The distilled esters were treated with urea and the insoluble fatty acid urea adduct separated. The adducting material represents 91% of the distillate or 57% of the starting material, and may be charac-

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TABLE 1

Some physical and chemical measurements of cottonseed oil heated in uncovered aluminum pot at 225°C

Heating time in hours	0	72	194
Molecular weight, cryoscopic	850	1080	1510
Viscosity in poises at 25°C	0.6	2.1	18.1
Refractive index at 30°C	1.4685	1.4724	1.4786
Iodine value, 30 min. (Wijs)	109.8	91.45	73.38
Peroxide value, mEq/1000 gm	2.5	1.5	0.0
Free fatty acids, as oleic, %	0.09	0.50	0.80
Epoxide oxygen, %	0.000	0.045	0.025
Unsaponifiables, %	0.54	0.51	0.43
Carbonyl oxygen, %	0.02	0.17	0.10
Hydroxyl oxygen, %	0.14	0.29	0.47
Urea filtrate values, %	2.3	17.5	31.0

TABLE 2

Effect of heating cottonseed oil at 225°C for 194 hours in aluminum pot, on polymer content and on fatty acid composition of monomer fraction

Component	Percentage of unheated oil	Percentage of heated oil
Linoleic acid	49.4	9.0
Linolenic acid	0.13	0.00
Oleic acid	20.4	18.1
Saturated acids (by difference)	30.1	31.1
Monomer	100.03	58.20
Dimer	—	29.8
Trimer and higher polymers	—	12.0
	100	100.0

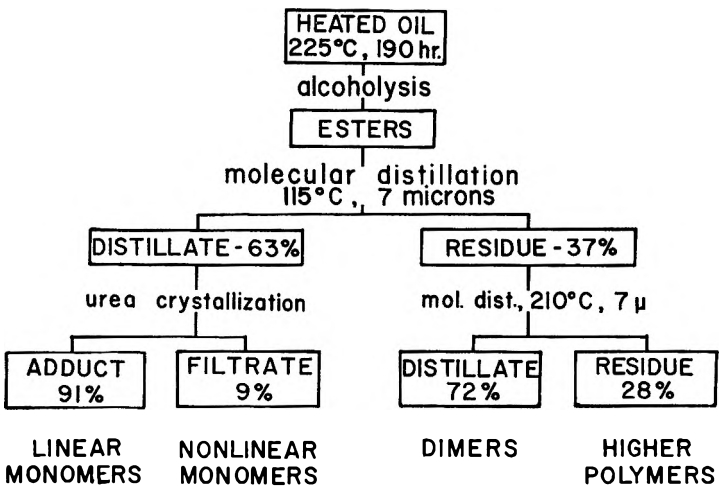

Fig. 1 Fractionation of ethyl esters from heated cottonseed oil by molecular distillation and urea adduction.

terized as linear monomers. The non-adducting fatty acid esters, or the urea filtrate, was 9% of the distillate, or approximately 6% of the original fat. The residue from the first distillation was further distilled at 210°C and 7 μ. The second distillate, equivalent to 26.7% of the original fat, may be characterized as dimer material, and the non-distillable residue, equivalent to 10.3% of the original fat, consists of trimers and higher polymers.

The heated cottonseed oil was fed to rats as part of a sucrose-casein diet.[2] The pyridoxine level was 1 mg per 1 kg of diet in this test. In our experience, this level is borderline for maximum growth and is about one-half to one-quarter of that usually used. This lower level was chosen because of the possibility (Witting et al., '57) that rats receiving low pyridoxine intakes are more susceptible to the deleterious effects of heated fats. Seven male rats and 5 female rats from the rat colony of the Division of Nutrition, were assigned to each group according to litter. The cottonseed oil control and the heated cottonseed oil were each fed at levels of 10 and 20% of the diet, the oil replacing an equivalent weight of carbohydrate. The animals were housed in individual cages with raised screen bottoms, and provided with food and water ad libitum.

RESULTS AND DISCUSSION

The average growth data and the food intake after a 30-day feeding period are shown in table 3 together with a covariance analysis (Snedecor, '46). Also given are the average weight gains of each group adjusted to the same food intake (the average food intake of all the groups). This adjustment makes possible a comparison of growth less complicated by differences in food intake. The effect of sex upon growth rate, food intake, and the adjusted weight gain is highly significant statistically, as is to be expected. The effect of the level of fat in the diet upon food intake and weight gain is also highly significant, but this effect disappears when the differences in food intake are removed by adjusting the weight gains to the same food intake. The effect of the heat treatment on food intake, on weight gain, as well as on the adjusted weight gain is very striking. The decrease in the nutritive value of the heated oil cannot be explained by a decreased food intake.

These animals were continued on experiment for 6 months at which time they were sacrificed, blood collected for lipid analyses and the weights of all organs obtained. A composite of the blood serum was prepared for each sex in each of the 4 diet groups, and this was analyzed for total cholesterol, ester cholesterol, phospholipid and total lipid. There were no differences between

[2] In grams: casein, 18; cystine, 0.2; sucrose-dextrin (1:1 mixture), 65.3 or 55.3; cottonseed oil, 10 or 20; USP salt mixture, 4; vitamin-Cellu Flour mixture, 2.5: Each 2.5 gm portion of vitamin mix contained in milligrams: niacin, 3.12; choline chloride, 125; inositol, 31.2; and in micrograms: riboflavin, 375; thiamine·HCl, 250; Ca pantothenate, 1250; biotin, 12.5; vitamin B_{12}, 3.12; folic acid, 250; pyridoxine·HCl, 100. In addition, 0.1 ml of a cottonseed oil solution containing 250 units vitamin A acetate, 25 units vitamin D, 0.7 mg *dl*-α-tocopheryl acetate and 20 μg of menadione was fed three times per week *per os*.

TABLE 3

Growth response and feed efficiency of rats fed cottonseed oil and heated cottonseed oil for 30 days

Diet	Av.[1] food intake	Av. weight gain	Av. adjusted[2] weight gains	Av.[3] F.E.
	gm	*gm*	*gm*	
10% CSO	424	156	134	0.362
10% HCSO	403	131	116	0.324
20% CSO	328	134	144	0.409
20% HCSO	277	86	112	0.312
	Covariance analysis[4]			
Effect of				
Fat level	***	***	—	
Heating, HCSO vs. CSO	**	***	***	
Interaction (fat level × heating)	—	*	—	

[1] Average of 6 male plus 4 female rats per group (littermate control).
[2] Average weight gain of each group adjusted to average food intake for all groups by covariance analysis.
[3] Food efficiency = weight gain/food intake.
[4] * P = 0.05, ** P = 0.01, *** P = 0.001.

the groups fed the 10% level of the heated and control cottonseed oil. With 20% of fresh cottonseed oil in the diet the serum levels of all 4 (fractions) measured dropped as compared with the 10% level; whereas on the 20% level of heated cottonseed oil, the values for the serum lipids did not change for the males and increased for the females. The differences noted were well outside the limits of experimental variation of the analytical procedures. However, since composite samples were used, it is not possible to determine the statistical significance of the results with respect to the populations from which the samples were obtained. Nevertheless, these data on the lipid fractions of blood, indicate that an alteration in lipid metabolism has occurred in animals fed heated cottonseed oil at 20% of the diet.

The average organ weights for liver, kidney, thymus, pituitary and adrenal are shown in table 4. In the case of the liver and thymus, there was a significant regression of organ weight on body weight and the average weights shown for these organs have been adjusted to the average body weight by covariance analysis. Even after 6 months, a difference in size is evident between the animals fed the heated oils and those fed the unheated oils. The increase in liver size of 25 to 30% due to the feeding of heated oil is highly significant. The effect of the heated oil upon the kidney size is difficult to understand. At the low level, kidney size decreased whereas at the high level it is somewhat greater than the control. The treatment effect is statistically significant at the 1% level of probability. The thymus is larger in the animals receiving the heated oils and this effect is statistically significant at the 1% level. In the case of the pituitary, no significant difference exists between the average of all the animals in each treatment.

Using techniques similar to those described by Rice et al. ('57), we have studied the heated cottonseed oil and the 4 fractions that were obtained by distillation and urea fractionation. In table 5 are shown the results of a calorie assay in which cottonseed oil and heated cottonseed oil are each fed at levels of one-half, one, and two grams per day as an addition to 5 gm of a basal ration.[3] Under the conditions of this test (Rice et al., '57), all nutrient requirements are completely satisfied, with the exception of energy. Therefore, the weight gain will be directly proportional to the calorie contribution of each of the foodstuffs fed. Since the food intake is strictly controlled and accurately known, determination of apparent digestibility is very conveniently carried out. The apparent digestibility of dry matter as well as of the heated fat supplement are also shown. It is quite clear that the caloric contribution of the heated cottonseed oil is approxi-

[3] Basal ration: sucrose, 42.7; casein 40, cystine 0.3, USP salt mix, 6; vitamin-Cellu Flour mixture, 6; vitamin A,D,E,K, solution, 2.4; cottonseed oil, 2.6. Each 6-gm portion of vitamin mix contained in milligrams: riboflavin, 0.9; niacin, 7.5; choline hydrochloride, 300; inositol 75; thiamine·HCl, 0.6; D-Ca pantothenate, 3; pyridoxine·HCl, 0.6; folic acid, 0.6; biotin, 0.03; cyanocobalamin, 0.0075. Each 2.4-gm portion of vitamin A,D,E,K, solution contained, vitamin A acetate 1400 I.U., vitamin D, 140 I.U.; *dl-α*-tocopheryl acetate, 4 mg; and menadione, 120 μg.

TABLE 4

Growth response and organ weights of rats fed cottonseed oil and heated cottonseed oil for 6 months

Diet	Av. body weight	Average organ weights				
		Liver[1]	Kidney	Thymus[1]	Pituitary	Adrenal
	gm	*gm*	*gm*	*mg*	*mg*	*mg*
10% CSO	433	10.1	2.7	154	12.2	39.3
10% HCSO	394	12.7	1.5	193	12.0	39.7
20% CSO	436	10.8	2.7	149	12.9	40.8
20% HCSO	398	13.9	3.1	187	12.4	37.1
Treatment[2] effect, HCSO vs. CSO	*	***	**	**	—	—

[1] Average organ weight adjusted to the average body weight by covariance analysis.
[2] Significant at * P = 0.05, ** P = 0.01, *** P = 0.001.

TABLE 5

Weight gain, apparent digestibility of dry matter and of added fats in rats fed cottonseed oil (CSO) or heated cottonseed oil (HCSO) in a 12-day "calorie" assay[1,2,3]

Sample	Level	Av. weight gain	Apparent digestibility	
			Dry matter	Added fat
	gm	*gm*	%	%
Basal	—	8.7	89.1	—
CSO	0.5	24.3	90.1	98.3
	1.0	32.4	90.8	98.5
	2.0	52.6	92.5	95.7
HCSO	0.5	17.7	86.1	86.6
	1.0	19.4	82.9	77.5
	2.0	27.3	77.8	63.4

[1] Each animal allowed 5 gm of basal diet per day and 0.5, 1.0 or 2.0 gm of "test" fat as indicated.

[2] Averages of 7 male rats per group.

[3] Dry matter digestibility % $= \frac{\text{11-day food intake} - \text{11-day feces (air-dried)}}{\text{11-day food intake}} \times 100$

Digestibility of added fat % $=$

$$\frac{\text{Added fat intake of test group} - \text{test group fecal fat} - \text{basal group fecal fat}}{\text{Added fat intake of test group}} \times 100$$

TABLE 6

Weight gain, apparent dry matter digestibility and liver weights of rats fed cottonseed oil (CSO) and heated cottonseed oil (HCSO) in a 10-day "calorie" assay[1,2,3]

Sample	Level	Av. weight gain	Apparent digestibility dry matter	Liver weight
	gm	*gm*	%	*gm/100 gm live weight*
Basal	—	12.7	89.7	3.64
CSO	0.5	28.1	90.8	3.84
	1.0	35.7	91.4	3.94
	2.0	46.5	92.1	4.14
HCSO	0.5	17.4	86.6	3.75
	1.0	22.8	83.3	4.10
	2.0	29.0	79.0	5.11

[1] Each animal allowed 5 gm of basal diet per day and 0.5, 1.0 or 2.0 gm of "test" fat as indicated.

[2] Averages of 6 male and 4 female rats per group.

[3] Dry matter digestibility % $= \frac{\text{9-day food intake} - \text{9-day feces (air dried)}}{\text{9-day food intake}} \times 100$

mately one-third that of the unheated oil.[4] This difference in nutritive value cannot be accounted for by the decreased digestibility of the heated cottonseed oil. Even at the high level, 63% of the daily 2-gm supplement was absorbed, but was not utilized for growth to the same extent as was an equivalent quantity of unheated cottonseed oil. A study of the digestibility figures and the food intakes reveals not only that the heated oil is poorly absorbed, but that it interferes to some degree with the absorption of other ingredients of the diet.

Repetition of this experiment with 10 rats per group for a 10-day test gave similar results (table 6).[4] In addition to measurements made in the first experiment,

[4] The data were subjected to an analysis of variance, which indicated that in addition to the obviously significant effects attributable to the heating of the oil and the different levels of oil fed that the interaction between the heating effect and the feed level effect was significant statistically at $P < 0.01$. This means that the caloric contribution per unit of heated oil fed decreases as the level of heated oil in the diet increases, and indicates the presence of a growth depressing component.

liver weights were also obtained. The feeding of the heated cottonseed oil at the 2-gm level resulted in an increase of the liver size of about 25%. It is of interest that this effect could be demonstrated in such a short time.

The fractions of the heated cottonseed oil obtained by distillation and urea fractionation were fed in a calorie assay similar to those just described. The distilled monomers were compared with the distilled ethyl esters from unheated cottonseed oil. The urea-adducting fatty acids were of equal caloric value and of the same degree of digestibility as the fatty acids of unheated cottonseed oil. However, in the female rats fed the urea-adducting fatty acids of the heated cottonseed oil, the size of the liver was increased. This effect was statistically significant at the 0.1% level of probability.

The fatty acids that do not form urea complexes, when added to the diet in the usual way, were not eaten by the rats. At the high level, fatalities occurred on the third or 4th day despite the very low food consumption. When these urea filtrate fatty acids were fed separately from the basal diet by tube, the rats did not eat, and died between the second and 4th days. The urea filtrate fatty acids were then fed to groups of rats at lower levels of the diet as indicated in table 7. The diet was fed ad libitum for 9 days. The average weight gains and the food intake for each group are shown, as well as the average weight gains of each group adjusted to the average food intake. The effect on food intake and weight gain is very striking. A very marked decrease of food intake and weight gain is noted as the dose is increased. However, the average adjusted weight gain for each group was not dependent upon the dose. The effect of the urea filtrate fatty acids on growth, therefore, seems to be mediated entirely through the effect on appetite. However, with larger doses the lethal effect is produced much more rapidly than by simple starvation.[5]

In figure 2 are presented the data for the liver weight:body weight ratios of the animals in this experiment. When the log of the liver weight:body weight ratio is plotted against the log of the dose, a linear regression exists which is significant at the 0.1% level. The effect upon the liver, therefore, is being produced at all doses.[6] The kidney weights were not significantly affected except at the 8% dietary level, and here the increase in kidney size, as compared with all the other groups, was

[5] Recent experiments by Douglass and Shue of this laboratory indicate that severe dehydration follows intake of toxic urea filtrate fatty acids. Forced feeding of water can prevent death.

[6] In a report at the 1960 Federation Meetings in Chicago (Mann, J. I., S. A. Koch and O. G. Fitzhugh 1960 Toxicity of a urea filtrate fraction from a fatty feed by-product. Federation Proc., *19:* 323 [abstract]), a urea filtrate material obtained from another source was fed for three months to rats. It is of interest in connection with the effect on liver size to note that a significant increase in serum alkaline phosphatase was observed at levels below those at which the increase in liver size was statistically significant. This finding supports the suggestion of early liver dysfunction resulting from the feeding of these materials.

TABLE 7

Growth response and food intake of rats fed nonlinear monomer fraction (urea filtrate) of heated cottonseed oil ad libitum for 9 days

Fraction 2	Av.[1] food intake	Av. weight gain	Av. adjusted[2] weight gain
% in diet	*gm*	*gm*	*gm*
0.0	115	63	44
0.5	107	60	47
1.0	98	53	47
2.0	95	51	48
4.0	76	34	46
8.0	55	12	42
Regression on dose[3]	***	***	—

[1] Four male and 4 female rats per group.

[2] Average weight gain of each group adjusted to the average food intake of all groups by covariance analysis.

[3] *** $P = 0.001$.

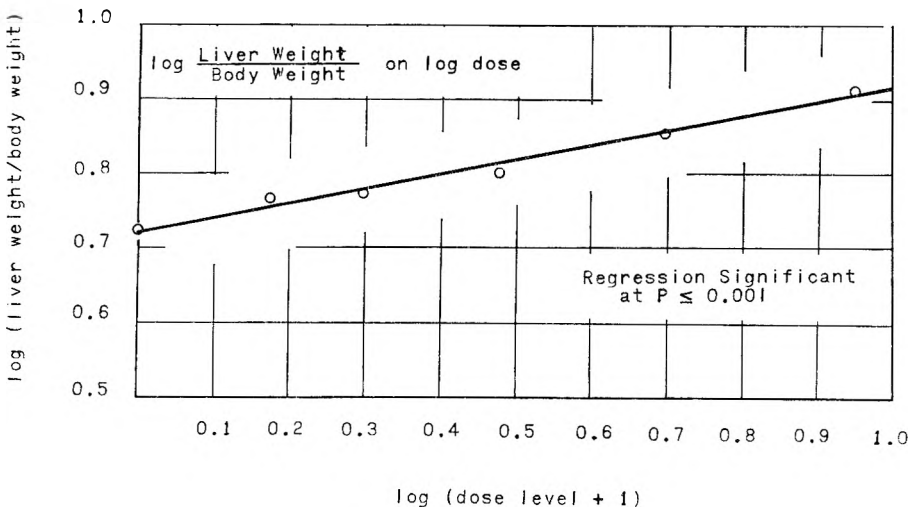

Fig. 2 Effect on rat liver size of feeding fraction 2 (urea filtrate) fatty acids (nonlinear monomers) from heated cottonseed oil, for 9 days; 4 males and 4 females per group. Regression of log liver weight:body weight ratio to log dose significant at $P \leq 0.001$.

statistically significant at the 1% level. The heart weights were also analyzed but no effect of the substance was observed at any dose.

The dimers and polymers were fed in a calorie assay and the apparent digestibility of dry matter and of fat were determined. The polymer, (as was to be expected) was probably not absorbed at all according to the growth data. The dimer had an apparent digestibility of about 74% at one level of feeding, and 60% at the higher level. The average weight gain at the higher feeding level was one-half that obtained on the basal diet alone, indicating a growth-depressing effect. The most drastic effects were observed with the urea filtrate material. These results confirm the earlier work of Crampton and associates ('51c, '53).

A rapid toxicity test for urea filtrates was devised, which depends upon the fact that weanling rats are very susceptible. A dose as low as 0.2 ml administered on two consecutive days will kill a large proportion of test animals; 0.3 ml fed on each of two consecutive days will kill test animals in from three to 4 days after the first dose.[7] A variety of urea filtrates prepared from different source materials has been tested in this way. The small amounts obtainable from beef and pork fat, cottonseed oil, and other sources of fresh fat or fatty acid have not been toxic in this test. However, urea filtrates from heated fat, a variety of fatty acids and commercial fatty acid esters, feed grade tallows, and others, have been toxic. Hydrogenation of toxic urea filtrates from an iodine number of 75 to one of 18 does not decrease toxicity. It appears that the urea filtrate fraction cannot be completely hydrogenated. It may be that this characteristic, namely, the inability to be completely hydrogenated (Perkins and Kummerow, '59), can be useful as an index of the presence of undesirable material in used fats as well as in food prepared by deep fat frying and the like.

It is true that the heat treatment studied here bears only a vague resemblance to the usual commercial processing operation, or to household usage. Several authors have pointed out that samples of fats from restaurants and commercial deep-fat frying operations have been tested and not found to give any evidence of deleterious effects (Deuel et al., '51; Melnick, '57; Alfin-Slater et al., '59; Keane et al., '59). Rice et al.[8] have stated that "Even the most severely used of these commercial fats cause only slight decreases in the calorie

[7] See footnote 5.

[8] Rice, E. E., P. E. Mone and C. E. Poling 1957 Effect of commercial frying operations on nutritive value of fats. Federation Proc., *16:* 398 (abstract).

availability of the fats and slight increases in liver size." The fact that it is difficult to demonstrate deleterious effects by feeding whole heated oils should not be cause for complacency. The evidence is clear that deleterious substances are formed and the problem is to devise techniques that will determine when the concentration of these substances has reached an undesirable level. Perkins and Kummerow ('59) have suggested that the proportion of non-urea-adduct (urea filtrate) forming material and the molecular weight are more reliable indicators of nutritive value than the iodine number used by Melnick ('57). Analytical procedures have been developed by Firestone ('60b) for the quantitative determination of urea filtrate fatty acids, and we suggest the desirability of developing authentic data on the production of these unnatural compounds during a variety of normal and unusual cooking operations. Such information will provide a basis for establishing objective criteria for evaluating industrial and commercial cooking practices.

SUMMARY

A sample of cottonsed oil heated for 190 hours at 225°C in the presence of air was fractionated into linear and non-linear monomers, dimers and higher polymers by molecular distillation and urea adduction. The heated oil, when fed to rats in a 6-month study or in 10- to 12-day "calorie assays," was shown to have decreased nutritive value for growth and food efficiency, and to produce enlarged livers in the test animals. The change most likely responsible for these effects is the appearance of fatty acids that have lost their capacity to complex with urea. It is suggested that the quantitative determination of urea-filtrate fatty acids may be useful in developing objective criteria for judging the acceptability of used fats and of the practices in specific industrial and commercial cooking operations.

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The Requirement and Tissue Distribution of Magnesium in the Rat as Influenced by Environmental Temperature and Dietary Calcium

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Faulty calcium metabolism appears to be one of the main characteristic symptoms found in animals fed a magnesium-deficient diet (Tufts and Greenberg, '36; Moore et al., '38; MacIntyre and Davidsson, '58). Frequently, however, this calcium disturbance does not occur in field cases of hypomagnesemia in ruminants (Allcroft, '47; Blaxter, Rook and McDonald, '54; Inglis et al., '59). In recent studies (McAleese and Forbes, '59) we found magnesium deficiency produced experimentally in lambs to be associated with concomitant hypocalcemia. This did not reflect a deficiency of calcium since bone calcium values were normal or slightly elevated and the hypocalcemia persisted in spite of supplementary calcium and vitamin D. Calcification of the soft tissues (e.g., kidney) is a common observation in cases of magnesium deficiency (O'Dell, '60; Thomas and Okamoto, '58; Tufts and Greenberg, '36; Watchorn and McCance, '37), but is not always found (Blaxter et al., '54). Furthermore this type of lesion is not specific to any particular deficiency or dietary adjustment since it has been found under a variety of conditions (Watchorn and McCance, '37; Lowenhaupt and Greenberg, '46; Follis et al., '42) and so cannot be used as a specific syndrome of magnesium deficiency except in carefully defined situations.

Many factors have been postulated to affect the magnesium requirement. There is some evidence that an increase in dietary calcium increases the magnesium requirement of the rat for survival and normal development (Tufts and Greenberg, '38b; Colby et al., '51; Lengemann, '59). Hegsted and associates ('56) found a high calcium intake to be detrimental only when magnesium was fed at a level below the minimum requirement. Blaxter and McGill ('56) reported that the maintenance of serum calcium at high levels in calves for long periods decreased the magnesium concentration in serum, despite an adequate dietary supply of magnesium.

Emotional distress or exaggerated response to cold wet weather are alleged to be associated with hypomagnesemia in livestock (Allcroft, '47; Inglis et al., '59). In addition blood serum magnesium has been observed to be very much elevated during hypothermia (Platner, '50; Platner and Hosko, '53), suggesting an increased requirement for magnesium at low temperatures. In support of this postulate Hegsted et al. ('56) have published data showing the magnesium required per gram of diet to produce equal weight gain in the rat to be about 4 times higher at an environmental temperature of 55°F than at 78°F (13° and 26°C), respectively.

In view of the conflicting evidence, the present investigation was undertaken to determine the magnesium requirement of the rat as affected by dietary calcium level and environmental temperature.

EXPERIMENTAL

Weanling male albino rats (522), of the Sprague-Dawley strain, were used in these studies. After being fed a dry stock ration for a one-week preliminary period, the animals weighed from 90 to 110 gm. They were confined to galvanized wire cages, three rats per cage. Food was supplied in amounts that would be con-

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sumed each day together with a constant supply of distilled water. The composition of the basal diet containing 20 ppm of magnesium by analysis, is given in table 1.

At the beginning of the experiment 18 rats were killed to serve as controls. The remaining 504 rats were divided into two groups, one kept at a constant temperature of 10°C and the other at 23°C. Each group was subdivided further into 21 subgroups of 12 rats each on the basis of dietary calcium (0.2, 0.5 and 0.8%) and magnesium (20, 80, 175, 285, 360, 420, and 510 ppm at each calcium level). To obtain these variations from the basal diet analytical reagent grade chemicals were used at the expense of glucose as described in the footnote to table 1. In view of the recent report by O'Dell ('60) it is important to note that the phosphorus content of all diets was 0.40%.

Each day at feeding time the animals were examined for signs of magnesium deficiency or related lesions. The rats were individually weighed initially and at 7-day intervals. The diets were compounded in stainless steel mixers, stored in plastic bags and refrigerated between feedings. The experiment extended over a 28-day feeding period and the criteria used for assessing adequacy of magnesium intake were weight gain, blood serum magnesium and bone ash magnesium. Calcium and magnesium analyses were also made on kidneys of all animals and hearts and skeletal muscle of several groups of animals. Since repeated observations on the same animal were impossible, three rats per sub-group were decapitated at 7-day intervals and the pooled blood used for calcium and magnesium determinations. A femur was removed from each rat and stripped of its adhering tissue. Three femurs were pooled for determination of calcium and magnesium in the bone ash, according to the same grouping of animals from which the blood had been pooled. The bones were defatted by Soxhlet extraction with alcohol for 48 hours and ether for 48 hours, dried to constant weight at 105°C, followed by ashing at 650°C until a white ash was obtained. The bone ash was taken up in the minimum of 1:4 HCl and made up to a suitable volume with deionized water.

TABLE 1

Composition of basal diet[1]

	%
Casein[2]	12
DL-Methionine	0.4
Corn oil	10
Vitamin A and D concentrate[3]	0.5
Powdered cellulose[4]	3.0
Vitamin mix 691[5]	5.0
Ca-Mg-free salts[6]	0.94
Glucose[7]	66.77
$CaHPO_4$	0.68
$NaH_2PO_4 \cdot H_2O$	0.71

[1] Variations in calcium and magnesium were made by additions of $CaCO_3$, $MgCO_3$ and alterations in amounts of $CaHPO_4$, $NaH_2PO_4 \cdot H_2O$ and cerelose so as to maintain constant amounts of all nutrients except Ca, Mg and Na. The small variation in the Na is considered to be without significance in this study.

[2] Labco, The Borden Company, New York.

[3] 2000 I.U. vitamin A and 250 I.U. vitamin D/gm.

[4] Brown Company, Chicago.

[5] Forbes and Vaughan ('54).

[6] The mineral mixture contained: NaCl, 39.6%; K_2CO_3, 53.5%; $FeSO_4 \cdot 7H_2O$, 4.23%; $MnSO_4 \cdot H_2O$, 1.28%; $CoCl_2 \cdot 6H_2O$, 0.42%; $CuSO_4 \cdot 5H_2O$, 0.64%; NaF, 0.08%; KI 0.04%; $ZnCO_3$, 0.21%.

[7] Cerelose, Corn Products Refining Company, New York.

Blood serum calcium and magnesium determinations were made using the method of Malmstadt and Hadjiioannou ('59a). The same authors' procedure ('59b) was used with slight modification for analysis of the diets, bone ash and other tissues. Modification of the method was required to ensure removal of all phosphorus with zirconium nitrate prior to titration.

The data relating to weight gain, blood serum magnesium and bone ash magnesium were analyzed by the method of least squares to determine the level of dietary magnesium required for maximum performance. The requirement was taken as the point of bending of a line rising linearly and then turning horizontally as dietary magnesium concentration increased. The values thus calculated were further treated by analysis of variance to detect significance of differences obtained among the variables employed. The remaining data were treated by analysis of variance where warranted.

RESULTS

The rats restricted to the basal diet soon developed the characteristic symptoms of

magnesium deprivation as first described by Kruse et al. ('32). Within three to 5 days reddening of the ears was evident. This was followed by edema of the ears, and later the nose and paws were reddened and edematous. The deficient animals were hyperexcitable but very few of them developed fatal convulsions. The hyperemia faded after 7 to 9 days, being replaced by a blanched appearance for a time, but the hyperemia then re-appeared and persisted until sacrifice of the animals. The early symptoms of magnesium deficiency were more difficult to identify in animals kept at 10° C since in some respects similar symptoms are produced by exposure to cold. However, by comparison with other groups receiving adequate dietary magnesium the symptoms became more strikingly evident with time at both temperatures. Many of the deficient rats developed trophic changes in the erythematous regions and 5 rats died before sacrifice. Obviously rats of this age can exist for quite some time when fed a diet containing 20 ppm of magnesium even though this is about 1/5th of the amount found to be required for maximum weight gain. During this period there was a major loss of magnesium from the skeleton, as will be discussed in more detail later.

The analyses of all tissues were made on pooled aliquots and therefore represent the arithmetic mean of three samples. To ascertain the extent of chemical change with time in blood and bone, data for the analysis of the control rats were obtained and are given in table 2. Results obtained from rats maintained for three weeks with a diet containing 0.4% of calcium and 2000 ppm of magnesium are also shown in this table for the purpose of indicating that the maximum values obtained for blood and bone magnesium in the main experiment are indeed maximum even though the magnesium intake be increased fourfold.

Weight gain. In figures 1 and 2 and table 3 are shown the average daily weight gains of the rats as influenced by the variables used in this experiment. The maximum weight gain attainable with magnesium-adequate diets was slightly, though statistically ($P = 0.02$) greater at 23°C than at 10°C, the average values being 5.11 and 4.80 gm, respectively. In order to make these gains, the average daily feed consumption was 17 and 23 gm daily at the warm and at the cool temperatures. The dietary calcium level did not affect maximum weight gain. Time on experiment had highly significant linear and quadratic effects on maximum weight gain. These effects were modified by temperature ($P = 0.02$), the gain being maximal during week 2 at 10°C and during week 3 at 23°C.

The magnesium requirement, estimated from weight gain, was not affected by temperature and averaged 112 and 118 ppm for rats at 23° and at 10°C, nor was it affected by time on experiment although there is a trend in the data toward a higher requirement as time progressed. The effect of increasing the concentration of dietary calcium was to increase linearly ($P = 0.02$) the magnesium requirement, which averaged 100, 116 and 130 ppm at 0.2, 0.4 and 0.8% of dietary calcium.

These data, showing no effect of temperature on magnesium requirement measured by weight gain, are in conflict with the interpretation of Hegsted ('56) who concluded that for maximum weight gain at 13°C (3.3 gm), 500 ppm of magnesium are required, whereas 250 ppm are sufficient for maximum gain (5.0 gm) at 26°C.

TABLE 2

Analytical data on control rats

	Starting rats	Rats kept 3 weeks at 2000 ppm Mg
Mg blood serum, mg/100 ml	2.57	2.31
Ca blood serum, mg/100 ml	11.1	10.7
Femur, weight, mg, dry fat-free	52	140
Femur, ash % of dry, fat-free	55.7	60.4
Femur, % Ca in ash	36.1	36.0
Femur, % Mg in ash	0.95	0.79

Figs. 1–6 Magnesium requirement estimated by the criteria and at the temperatures indicated. Each point on these charts represents the average of 12 rats.

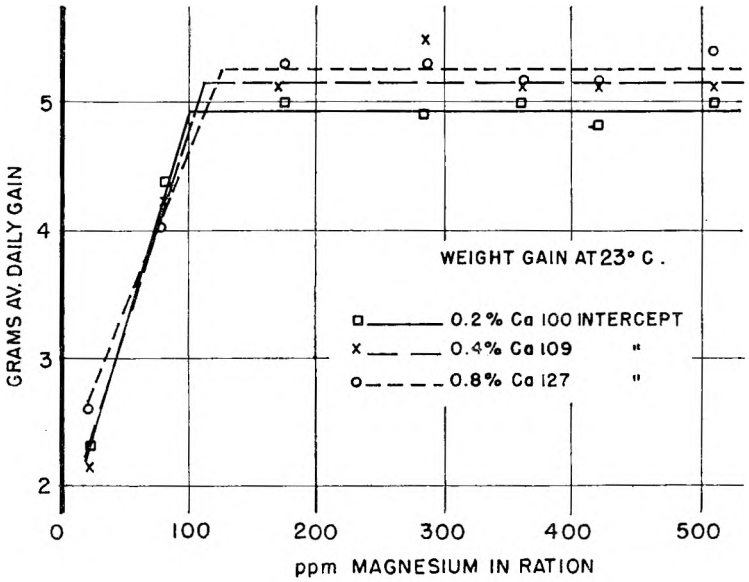

Figure 1

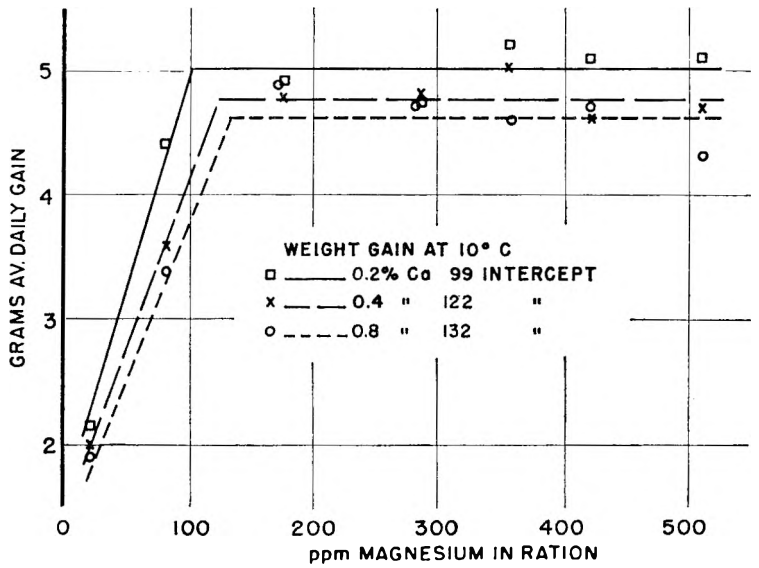

Figure 2

The effect, shown in our data, of increased magnesium requirement as a result of increased dietary calcium concentration is in agreement with our observation that the general appearance of rats fed low magnesium diets deteriorated most rapidly in groups receiving the highest calcium concentration.

Blood serum magnesium and calcium. The values obtained for blood serum magnesium and the magnesium requirement as affected by the imposed treatment are

TABLE 3

Magnesium requirement,[1] and maximum level of performance, as affected by criterion of requirement, calcium in diet, environmental temperature and time on diet

Criterion	% Ca in diet	Requirement				Maximum level of performance			
		Weeks on exp.				Weeks on exp.			
		1	2	3	4	1	2	3	4
		ppm/mg				*Av. daily gains*			
						gm	*gm*	*gm*	*gm*
Av. daily gain									
23°C	0.2	116	84	92	108	4.3	5.3	5.3	4.8
	0.4	116	92	120	108	4.0	5.6	5.7	5.3
	0.8	88	120	124	176	4.0	5.7	6.1	5.2
10°C	0.2	100	88	96	112	4.6	5.3	4.9	5.2
	0.4	84	124	160	120	4.4	5.2	4.7	4.8
	0.8	80	176	100	172	4.4	5.2	4.5	4.4
						Blood serum Mg mg/100 ml			
Blood Mg level									
23°C	0.2	304	304	376	440	2.9	2.3	2.4	2.5
	0.4	360	304	360	456	2.3	2.2	2.1	2.2
	0.8	280	440	416	464	2.7	2.1	2.1	2.1
10°C	0.2	120	248	256	232	2.8	2.2	2.5	2.4
	0.4	504	360	352	496	2.7	2.1	2.4	2.3
	0.8	504	408	408	384	2.8	2.1	2.3	2.2
						Bone ash Mg			
						%	%	%	%
Bone ash Mg									
23°C	0.2	320	288	272	504	0.93	0.91	0.76	0.79
	0.4	304	232	376	384	0.87	0.85	0.77	0.74
	0.8	264	416	392	496	0.92	0.81	0.80	0.82
10°C	0.2	96	112	232	288	0.81	0.79	0.87	0.75
	0.4	176	192	360	224	0.84	0.79	0.83	0.82
	0.8	80	312	288	276	0.77	0.88	0.78	0.85

[1] Each requirement figure at a given week and calcium level is derived from 21 animals.

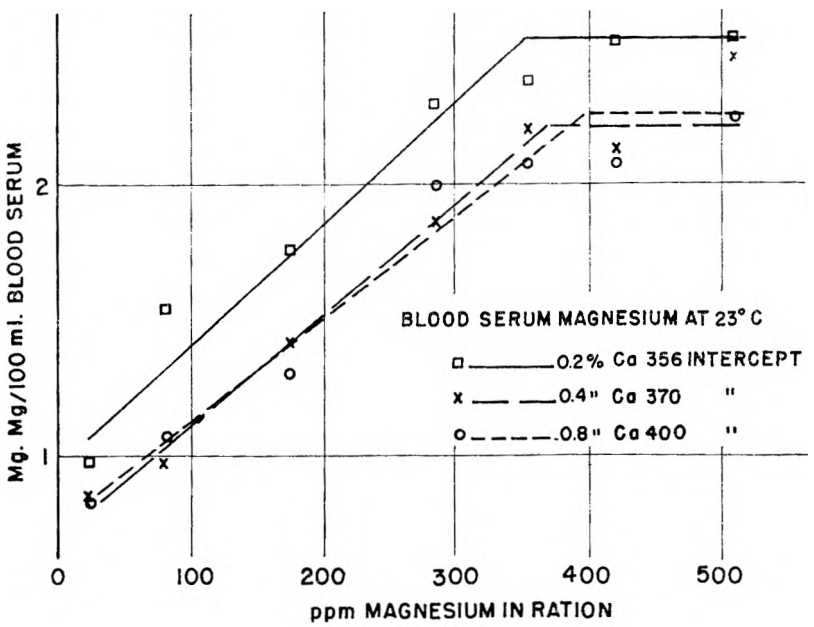

Figure 3

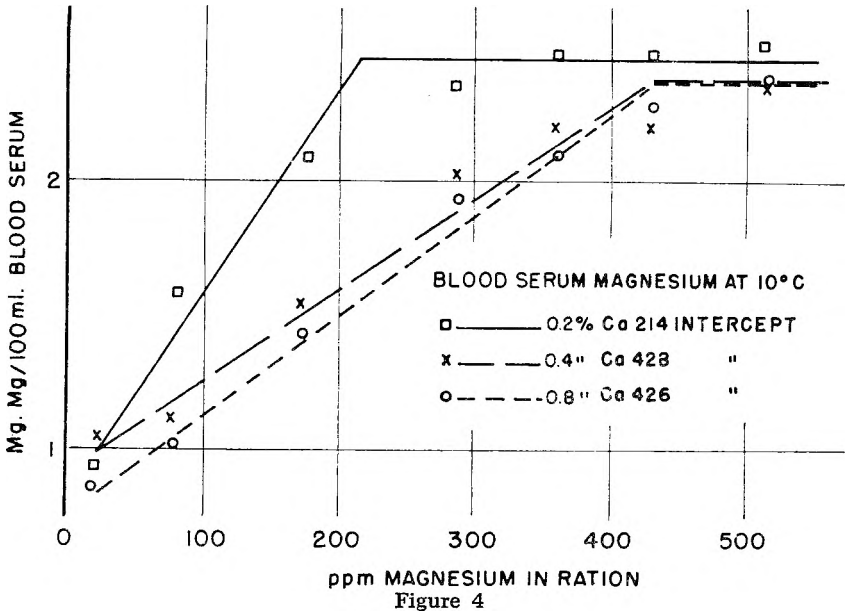

Figure 4

shown in figures 3 and 4 and table 3. Data are not shown for blood calcium since these values were remarkably constant and were affected in this experiment only by dietary calcium level. At 0.2% of dietary calcium, blood serum calcium averaged 11.1 mg/100 ml, while at both 0.4 and 0.8% of dietary calcium the value averaged 11.4 mg/100 ml. This difference possessed a P value less than 0.01, and indicates that the lowest calcium level was below the requirement for this element.

The maximum value obtainable for blood magnesium was slightly higher ($P = 0.02$) at 10° (2.40 mg/100 ml) than at 23°C (2.33 mg/100 ml). The maximum level of blood magnesium was inversely related to the dietary calcium level ($P < 0.01$), being 2.49, 2.29 and 2.30 mg/100 ml at 0.2, 0.4 and 0.8% of dietary calcium. A qualitatively similar finding has been reported for rats by Colby and Frye ('51). There was also a significant trend with time, the maximum blood magnesium level being 2.70, 2.17, 2.30 and 2.28 mg/100 ml at weeks 1, 2, 3 and 4 of the experiment. Reference to table 2 also bears this out, showing that starting rats had higher levels of blood magnesium than rats three weeks older, even though the latter had been kept on a ration containing 2000 ppm of magnesium. The calcium effect on maximum blood serum magnesium was less marked ($P = 0.02$) at 10° than at 23°C.

The requirement for magnesium, measured by blood magnesium level, was not affected by time on experiment, nor by temperature, being 375 and 356 ppm, respectively at 23° and at 10°C. It was, however, affected by dietary calcium ($P = 0.01$) in a manner similar to the requirement judged by weight gain. As calcium level increased, the magnesium requirement increased from 285 to 399 and then to 413 ppm for maintenance of maximum blood serum magnesium. The effect of calcium appeared to be mediated by temperature ($P = 0.05$), this being particularly noticeable in the low requirement found by the least squares method for rats kept at 10°C on the low calcium level. Inspection of these data, however, reveals that they would best be expressed by a curve, rather than by a bent line. In this case, the asymptote of the curve would approximate 350 ppm rather than the 215 shown in fig. 4.

Bone calcium and magnesium. The data obtained on bone ash magnesium percentage as affected by the treatments of this experiment are presented in figures 5 and 6 and table 3. The maximum level of magnesium in the bone ash was not influenced by temperature, dietary calcium

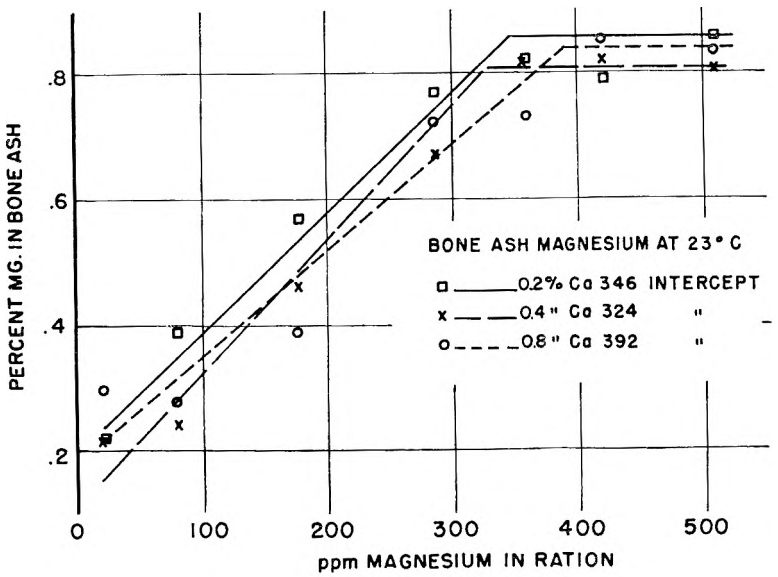

Figure 5

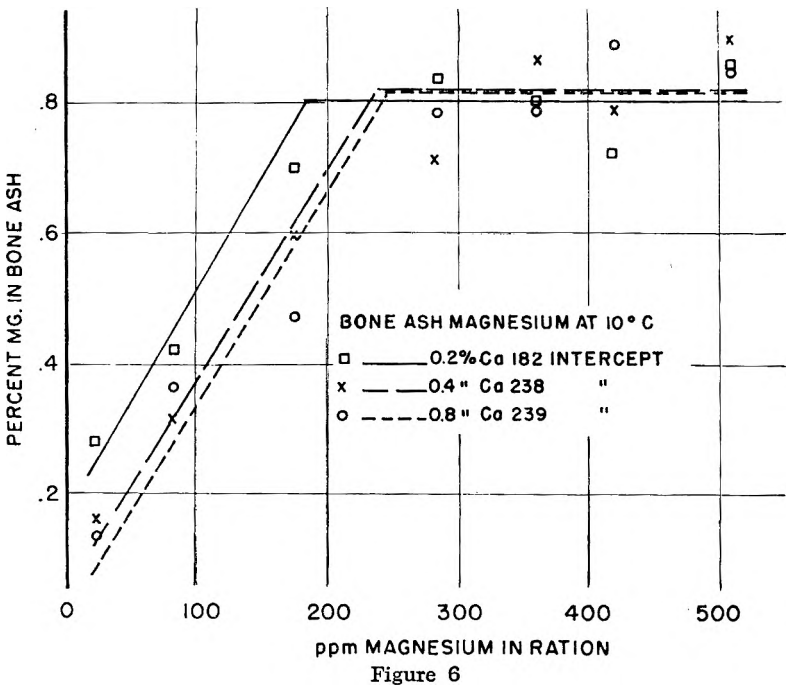

Figure 6

level, time on experiment or any of their interactions.

The requirement for magnesium, measured by bone ash magnesium concentration, was the only one of the three criteria used to be affected by temperature. In this comparison the requirement was lower ($P < 0.01$) at 10°C (221 ppm) than at 23°C (354 ppm). The requirement measured in this manner was affected by the dietary calcium concentration ($P < 0.01$), as was that measured by weight gain and

by blood serum magnesium concentration. At dietary calcium concentrations of 0.2, 0.4 and 0.8% the magnesium requirement was 264, 281 and 318 ppm, respectively. Time on experiment also affected the requirement measured by bone ash magnesium ($P < 0.01$), being 207, 258, 320 and 325 ppm at successive weekly intervals.

The percentage of ash in the dry, fat-free femurs was affected by time on experiment, dietary levels of calcium ($P < 0.01$) and magnesium, and by environmental temperature ($P = 0.05$). The average ash values were 55.9, 56.4, 58.5 and 58.8% of the dry, fat-free femur at successive weeks of the experiment. Rats fed 0.2% of calcium had an average of 55.1% ash, whereas those fed higher calcium concentrations had ash values of 58.7%. These data parallel those relating blood calcium level to dietary calcium and again show the insufficiency of the lowest level of dietary calcium. In rats fed the low-calcium diets the bone ash concentration was less ($P = 0.05$) at 23° than at 10°C, the average values being 54.4 and 55.8%, but temperature did not affect bone ash at the higher dietary calcium levels.

The percentage of calcium in the bone ash increased from 36.6 to 37.5% from weeks 1 to 4 on experiment. Temperature had no effect on concentration of calcium in the bone ash. As the experiment progressed, and bone ash magnesium decreased in the magnesium-deficient animals, the bone ash calcium increased ($P = 0.01$). This is illustrated by a comparison of bone ash calcium from rats fed the two lowest magnesium levels with the corresponding value for those fed the 5 highest levels, 37.9 and 37.1%, respectively. It is significant that, in agreement with Duckworth and Godden ('41) and Blaxter ('56), this increase in calcium concentration is equivalent to the decrease in magnesium concentration in the ash.

The effect of treatment on net accumulation of calcium and magnesium in rat femurs is seen in table 4. Again it is evident that 0.2% of calcium is insufficient for maximum calcium deposition. It is of particular note that the two lowest magnesium levels failed to maintain the amount of magnesium present in the bones of the starting rats. An effect of low temperature on increasing bone mineral deposition is seen to be greatest at sub-optimal levels of dietary minerals.

The relationship between magnesium levels in bone and blood is shown in table 5 where Y represents the percentage of magnesium in bone ash and X represents milligrams of magnesium per 100 ml of blood serum. The correlation is obviously linear showing no evidence of curvilinearity, and the standard error of estimate is of similar magnitude in all 4 equations. However, the regression coefficient for week 1 differs highly significantly from that for each of the other weeks, showing that during the early stages of magnesium

TABLE 4

Total femur calcium and magnesium (mg per femur, average of all animals)

Dietary calcium	Dietary magnesium	Dry, fat-free femur (52-mg original)		Calcium (11-mg original)		Magnesium (0.28-mg original)	
		23°C	10°C	23°C	10°C	23°C	10°C
%	*ppm*						
0.2	20	82	97	16	20	0.08	0.12
	80	96	106	19	22	0.18	0.20
	175	96	112	19	24	0.29	0.42
	285–510	98	108	20	23	0.42	0.49
0.4	20	110	110	24	24	0.12	0.09
	80	124	123	28	27	0.15	0.20
	175	129	127	28	28	0.29	0.42
	285–510	125	127	28	28	0.56	0.62
0.8	20	118	118	26	25	0.17	0.08
	80	126	136	28	30	0.19	0.24
	175	137	134	31	30	0.24	0.36
	285–510	136	132	30	29	0.61	0.65

TABLE 5

Regression of magnesium concentration in bone ash (Y=%) on that in blood serum (X=mg/100 ml)

Week of experiment		S.E. of estimate of Y	S.E. of regression coefficient
1	Y = 0.27X + 0.13	0.13	0.035
2	Y = 0.41X − 0.02	0.10	0.028
3	Y = 0.46X − 0.26	0.12	0.028
4	Y = 0.44X − 0.25	0.14	0.037

TABLE 6

Concentrations of ash and of calcium in rat kidneys (% of dry matter)

Dietary calcium	Dietary magnesium	23°C		10°C	
		Ash	Calcium	Ash	Calcium
%	*ppm*				
0.2	20–175	7.16	0.43	6.18	0.33
	285–510	5.95	0.04	5.61	0.05
0.4	20–175	8.50	0.78	6.51	0.37
	285–510	5.93	0.08	5.31	0.06
0.8	20–175	7.88	0.68	5.75	0.24
	285–510	6.01	0.07	5.45	0.07

deficiency there is a time lag between the reduction of blood magnesium and mobilization of bone magnesium.

Soft tissue calcium and magnesium. The analysis of the kidneys for ash, calcium and magnesium revealed a remarkably early reaction of this organ to magnesium deprivation (table 6). As early as 7 days after initiation of the experimental diets the percentage of ash in the dry matter was increased in the kidneys of rats fed the three lowest levels of magnesium, above that in kidneys of those more adequately fed. This effect was more noticeable in rats kept at 23°C than at 10°C and in the former group became greater as the experiment progressed. The same trends were found to be highly significant in kidney calcium concentration and the increase accompanying low dietary magnesium concentrations was, in percentage, much greater than for kidney ash. The magnesium concentration in the kidneys was not influenced by calcium or magnesium intake or by temperature or time on experiment, and averaged 0.076% ± 0.016 (S.D.) of the dry matter.

The hearts of 24 groups of deficient and of normal animals were analyzed for calcium and magnesium. Hearts from animals fed the basal level of magnesium had lower magnesium concentration (0.059% of dry matter) than those from animals fed higher levels (0.069%). The opposite trend was noted for heart calcium content (0.053 vs. 0.041%). Analysis of muscle from 16 groups of rats revealed that, on the dry matter basis, calcium was significantly higher (0.094%) and magnesium significantly lower (0.072%) in rats receiving the basal magnesium level than in those receiving the highest magnesium level (0.058% and 0.078%, respectively).

Watchorn and McCance ('37), on the other hand, found no change in magnesium or calcium concentration in rat heart as a consequence of magnesium deficiency but found a doubling of the calcium content of muscle.

DISCUSSION

Weight gain is a common criterion for assessing the adequacy of diets. The results observed in this study point to the wisdom of using more than one criterion for this type of research. The magnesium required to promote maxium weight gain averaged 115 ppm, whereas maximum levels of blcod serum magnesium and bone ash magnesium were attained only by

feeding much higher dietary levels of magnesium. One might well question the necessity of maintaining blood serum levels of magnesium at the maximum in view of the apparent good health and lack of observable signs of magnesium deficiency in rats fed magnesium concentrations half as great as required for maximum blood serum or bone ash values. It is well to remember, however, that in this relatively short experiment there was a trend toward higher requirement with time as measured by weight gain and a significant increase in requirement measured by bone ash magnesium as the experiment was prolonged. These trends indicate that if the experiment had been carried out for a longer period, the requirement measured by weight gain might have increased and approached that measured by the other criteria.

Loss of magnesium from the femurs of rats fed the basal level of magnesium amounted to nearly 80% of that originally present (table 4). Half of the bone samples obtained during weeks 3 and 4 from rats fed the two lowest levels of magnesium actually contained barely detectable amounts of magnesium and thus contained no more than 0.10% of magnesium in the ash. The practical limit, in ratio of calcium to magnesium, for magnesium determination is about 400:1 by the method of analysis used in this experiment. This loss of magnesium from bone is in contrast to the report of Orent and co-workers ('34) who concluded that the lower concentration of magnesium in bone of rats deficient in magnesium, was a result of bone growth without magnesium accretion rather than a net loss of magnesium from the bone. Duckworth and Godden ('43) and Blaxter et al. ('54) observed that the skeleton can function as a reservoir for magnesium, releasing from 30 to 60% of this magnesium during dietary magnesium inadequacy. In both of these investigations it was found that loss of magnesium from various parts of the skeleton was uniform, thus the measurements obtained from a single bone may be safely assumed to represent the general situation.

In agreement with Smith ('59) and Blaxter ('56) we have found a good general correlation between magnesium levels in blood and in bone (table 5). In view of our observation that the magnesium requirement to maintain maximum bone magnesium (207 ppm) was initially much lower than that for maintenance of maximum blood magnesium (345 ppm), and that the former value increased with time to approach the latter, it would appear that the magnesium in bone serves as a reserve supply being mobilized upon stimulus to a lowered concentration of blood magnesium.

The evidence presented in this study is consistent with the view that magnesium is retained on the surface of the bone crystallites in a loose chemical binding from which it may be released in times of shortage to meet the needs of this essential ion for enzyme systems and for maintenance of proper mineral ratios in nerve and muscle. The possibility of very small amounts being occluded in the bone crystal voids during crystal formation and growth cannot be precluded (Hendricks and Hill, '50). If a large part of the magnesium were within the apatite structure (Dallemagne and Fabry, '56), then removal of 87% of the magnesium from bone, as our evidence indicates is possible, would require dissolution and subsequent loss of calcium from the bone. As our data show, in severe magnesium deficiency, not only are essentially all detectable amounts of magnesium removed from the bone but, also there are actual accumulations of calcium in the bone, expressed both in absolute terms and as a percentage of the ash. The latter increase is in amounts proportional to loss of magnesium.

Duckworth and Godden ('41) found the repletion of bone magnesium to be a slow process. It may be inferred that the slow rate of repletion is due to a mechanical blocking of the exchange sites by the excess calcium laid down during magnesium depletion. At pH 7 the magnesium ion exists as $Mg(OH)^+$ and not Mg^{++} indicating why the magnesium ion has such difficulty regaining its former position on the surface of the bone crystal lattice. Using Mg^{28},[2] our evidence confirms this slow magnesium uptake by bone of magnesium-deficient lambs relative to the rapid uptake by soft tissues, teeth and

[2] Unpublished data.

blood serum and the rapid uptake (exchange) of Mg^{28} in bone of non-deficient lambs. Under these conditions bone magnesium cannot be used as an accurate index of the current magnesium status (nutritional) of the animal.

The finding that the rat can satisfy its requirement for magnesium, judged by bone magnesium concentration, at a lower concentration of dietary magnesium at 10° than at 23°C is probably explainable by the fact that at the lower temperature total feed and hence magnesium intake was 35% greater than at the higher; yet the skeletal growth as measured by weight of dry, fat-free femur was not significantly greater at the cooler temperature (table 4) and total body weight gain was slightly less (table 3). Since there is thus a relative excess of magnesium being supplied per unit of body weight it would be reasonable to expect it to be stored in bone and to saturate the bone sites available for magnesium absorption at a lower dietary concentration. The concept that magnesium absorption in bone is limited by the number of exchange sites available for its binding is strengthened by the observation that femur ash of rats receiving for three weeks 2000 ppm of magnesium contained no more magnesium, as seen in table 2, than was found in the femur ash of rats receiving 300 to 400 ppm.

Other criteria indicating that moderate deficiencies of bone-forming nutrients may be overcome by temperature-induced increased caloric intake may be derived from table 4. There was a greater deposition of dry, fat-free material and of calcium at 10° than at 23°C only in femurs of rats fed the insufficient level of 0.2% calcium. While there seemed to be a similar temperature effect on magnesium levels, the greatest increase occurred in rats fed "border line" concentrations (i.e., 175 ppm) of magnesium. In these cases there was approximately a 50% increase in absolute amounts of magnesium per femur.

With one exception (MacIntyre and Davidsson, '58) all reports in the literature refer to the unchanged magnesium concentration in muscle, even in the most deficient state. Our data agree qualitatively with those of MacIntyre and Davidsson although the reduction in muscle magnesium is so small as to escape notice except by use of a sensitive and reproducible method of analysis. In magnesium deficiency, there is a reduction in the red blood cell magnesium, but this may be due to dilution of the blood cells by new or recently formed cells with low magnesium content. The deposition of calcium in the soft tissues and particularly in the kidney in magnesium deficiency has been referred to previously. Greenberg et al. ('38) report kidney calcification in magnesium deficiency to occur first in the corticomedullary zone and pyramids, and later in the cortex. Gershoff et al. ('58) state that calcium deposition in renal tubules and the outer portion of the renal medulla is a borderline indication of magnesium deficiency. Histological studies were not made in our investigation but gross observation of the kidneys revealed only one case of obvious abnormality, this occurring in both kidneys from an animal fed for three weeks a diet with 0.4% of calcium and 80 ppm of magnesium. These organs were light yellow in color and had three times the dry weight and 15 times the ash weight of those of the other rats given the same treatment. We cannot at this time offer a sound theory to account for kidney calcification in magnesium deficiency, but submit the hypothesis that it is a consequence of the tubular degeneration and hence an interference with the normal reabsorptive capabilities of the tubules.

SUMMARY

Five hundred twenty-two rats were used in a single investigation of the effect of environmental temperature (10 and 23°C), duration of experiment, (one to 4 weeks) and dietary calcium level (0.2, 0.4, and 0.8%) on the requirement of the rat for magnesium. For maximum weight gain the average requirement was 115 ppm, a value not affected by environmental temperature or time on experiment, but averaging 100, 116 and 130 ppm at increasing calcium levels. For maintenance of "normal" blood magnesium levels an average of 365 ppm was required. This value was unaffected by time or temperature, but was influenced by dietary calcium, being 285, 399 and 413 ppm at increasing calcium levels. For maintenance of "normal" bone

magnesium levels an average of 288 ppm was required. This value varied with temperature, being 221 ppm at 10°C and 354 ppm at 23°C, with calcium level, being 264, 281 and 318 ppm at increasing calcium concentrations, and with time, being 207, 258, 320 and 325 ppm at successive weekly intervals.

It was noted that when supplied the basal diet (20 ppm of magnesium), rats could lose 80% of the magnesium originally present in their femurs while still gaining in femur calcium. Calcification of kidneys of rats on magnesium-deficient diets was marked as early as 7 days on experiment and amounted to as much as an 11-fold increase in calcium concentration, while kidney magnesium was unchanged.

ACKNOWLEDGMENT

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