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WILBUR O. ATWATER

(1844–1907)

Wilbur O. Atwater

Wilbur O. Atwater

— *A Biographical Sketch*

(May 3, 1844 — October 6, 1907)

A minister's son born a century and a quarter ago, a graduate of a small New England liberal arts college and later a school teacher, who was stimulated by some farmers interested in scientific agriculture and by professors under whom he pursued graduate study to undertake a career in agricultural and physiological chemistry — such was the early life of the man who was to become the founder of the science of nutrition in the United States — Wilbur O. Atwater. He was born May 3, 1844 in a small village, Johnsburg, New York, later moving with his family to Vermont where most of his boyhood was spent. After attending public schools he entered the University of Vermont at Burlington. Later he transferred to Wesleyan University, Middletown, Connecticut where he was graduated in 1869.

After a short period of service as a high school teacher, Atwater entered the Sheffield Scientific School of Yale University for graduate study with Samuel Johnson, Professor of Agricultural Chemistry. He also came under the influence of W. H. Brewer, Profressor of Agriculture. His thesis for the Ph.D. degree which he received in 1869 was entitled, "The proximate composition of several varieties of American maize." In this study he employed the Weende method, not previously used in the United States, the special feature of which was the separation of the carbohydrate fraction into crude fiber and nitrogen-free extract.

Following the completion of his work at Yale, Atwater spent the next two years in study of agricultural and physiological chemistry in Berlin and Leipsig. He also became acquainted with the organization and activities of agricultural experiment stations. This period in Germany had a large influence in determining his future career. He was to revisit frequently centers in that and other European countries to learn about experimental methods and results of special interest to his own program, and to gather other information to bring back and publish for the use of investigators and to stimulate public interest in nutrition. In 1871 he was appointed to a professorship in chemistry at East Tennessee University (now University of Tennessee) and later to a similar post at Maine State College (now University of Maine). While there he met Marcia Woodward whom he married in 1874. Two children, Helen and Charles, resulted from this marriage. In the fall of 1873 he returned to Wesleyan as Professor of Chemistry, a post which he held until his death in 1907. Upon his return he became acquainted with Orange Judd, founder and for many years editor of the American Agriculturist, who was a trustee of Wesleyan and had given that university the Orange Judd Hall of Natural Sciences in which Atwater had his laboratories. Mr. Judd encouraged him to undertake chemical research on farmers' problems. Thus, he began laboratory studies of fertilizers, and these studies were accompanied by field tests with the cooperation of farmers.

Ever since his return from Germany Atwater had urged the need for agricultural experiment stations in the United States. In 1873 he joined with his former teacher, Professor Johnson, who had long been working to this end, to persuade the Connecticut Legislature to appropriate money for the establishment of a state station. To encourage such action Wesleyan offered laboratory facilities and Atwater's part-time services, and Mr. Judd agreed to make an initial contribution of $1,000 toward the support of the work. Thus the first agricultural experimental station in the United States was set up at Wesleyan in 1875, for a two-year period,

with Atwater as Director. In 1877 the Station was permanently established at the Sheffield Scientific School of Yale with Johnson as Director. While the station was located at Wesleyan, Atwater expanded his fertilizer program and began experiments on the growth and composition of field crops, with the assistance of E. H. Jenkins, W. H. Jordan and A. T. Neale. These investigations were continued, with increased private support which included the free use of a nearby farm, after the state appropriation ceased. Atwater became particularly interested in the nitogen metabolism of plants and was at least one of the first investigators to produce definite proof, from experiments begun in 1881, that legumes assimilate nitrogen from the air. His first series of studies, with peas grown in nutrient solution, was published in the American Chemical Journal in 1885. Several later studies were made during the next five years. Charles D. Woods, who was graduated from Wesleyan in 1880, was closely associated with Atwater in these studies as well as in many later ones.

As Atwater's broad interests and accomplishments became known he was asked to assist in a variety of projects. During 1879–82 he and his assistants carried out a series of analyses under the auspices of the United States Fish Commission, resulting in a report entitled, "Contributions to the knowledge of the chemical composition and nutritive values of fish and invertebrates." Similar studies were made of the flesh of animals for the Smithsonian Institution. In the winter of 1882–83 he was given a leave of absence for work in Voit's laboratory where he studied the digestibility of "lean fish" by men and dogs and found it comparable to that of lean beef. During this period he became acquainted with methods the Germans were using to study nutrition and thus was stimulated to undertake such studies himself upon his return. In 1885–86, at the request of the Massachusetts Bureau of Statistics of Labor, Atwater made a study of extensive data which the Bureau had collected on family food purchases and their costs. He calculated the daily per capita supplies of protein, fat and carbohydrates thus provided and, taking account of the cost data, made recommendations as to how more economical diets adequate in nutritive value could be selected. A report of some 90 pages, which he prepared, was published in the Bureau's Annual Report for 1886.

Following the lead of Connecticut, certain other states established experiment stations during the next 15 years. Several of the country's agricultural leaders, including Atwater, campaigned for federal support for these stations and for the encouragement of their establishment in other states. As a result, such support was provided in the Hatch Act of 1887. One-half of the sum available to Connecticut was allocated to the station at New Haven, and one-half for the support of a new station at the Agricultural School (now University of Connecticut) at Storrs, with Atwater as Director. In assuming this post he maintained his headquarters at Wesleyan, some 35 miles away.

As a consequence of the enactment of the Hatch Act, a U.S.D.A. Office of Experiment Stations was created with Atwater as Director, with the understanding that he would not have to give up his professorship at Wesleyan or his directorship at Storrs. During three years of part-time service he established policies for the Office and assisted states in formulating programs. At the outset he stated, "The future usefulness of the stations will depend upon what they discover of permanent value and this must come largely from the most abstract and profound research; to forget this would be fatal." He further stated, "I insist that this enterprise ought to be kept out of politics." At the outset he also established a journal, the Experiment Station Record, to keep station workers informed of what their colleagues were doing, and the Farmers' Bulletin to furnish farmers with a readable and understandable presentation of the findings of the stations and other scientific institutions. The Record was later expanded to provide abstracts of research in the field of agriculture wherever conducted and Atwater made a special effort to obtain abstracts, and also full reports, of European work. In 1897 he made a special trip to Russia to learn what was going on in the scientific field and to arrange to have the results

abstracted in English for publication in the Record.

With the establishment of the Storrs station most of the field research on fertilizers and crops previously carried out at Wesleyan were transferred to the new institutions and expanded under the supervision of C. D. Woods who was appointed Vice-Director. Most of the chemical work continued to be done at Wesleyan. It is evident that Atwater maintained a guiding role in these agricultural research programs through his 14 years as Director of the station, but from 1890 on, more and more of his personal time and activities were devoted to the field of nutrition. He drew up an extensive plan for nutrition investigations which included: food analyses, dietary studies, experiments on energy requirements of human subjects doing various kinds of work, experiments on the digestibility of foods and investigations on the economics of food production and use. He inaugurated programs at Wesleyan and Storrs accordingly. Major support for expanding programs in these areas, with wider participation, was provided by a special fund initially appropriated by Congress in 1893 for studies of problems of human nutrition, to be carried out with the cooperation of the experiment stations. Awater was placed in direct charge of these studies with headquarters at Wesleyan. Continuing appropriations supported a very extensive cooperative program at some 20 stations.

Recognizing the need for data on the composition and nutritive value of American foods comparable to the information available in European tables, Atwater and his associates early began some studies in this field, such as those on fish and the flesh of animals previously mentioned. When the Storrs station was established these studies were expanded and other stations were encouraged to participate in the program. With the support of the federal fund for human nutrition investigations a cooperative project was organized in which several stations participated. As a result of these various activities Atwater and Woods published in 1896 a compilation of approximately 2,600 analyses of American foodstuffs. This bulletin was reissued in 1899 by Atwater and Bryant, based on over 4,000 analyses, including approximately 1,000 made at Wesleyan and Storrs. It was reissued with corrections in 1906. The data included: maximum, minimum and average values for refuse, water, protein, fat, total carbohydrates, ash, and "fuel value" calculated by Rubner's factors. In addition to the studies of the proximate composition of foods, extensive bomb calorimetric determinations on various fats, carbohydrates and proteins and on specific foods were carried out in the Wesleyan laboratory beginning in 1891.

The Massachusetts food purchase study previously mentioned interested Atwater to undertake dietary studies which would measure foods and nutrients actually consumed. Thus he and his associates began such studies in 1890, with the support of the U. S. Department of Labor. In these studies, kitchen and food wastes were accounted for and other features were included to make the data more meaningful. Most of the foods were sampled and analyzed to provide a more exact basis for computing nutrient intakes. The studies dealt with individuals, families and groups in public institutions. Special attention was given to working people with low incomes. As an example, studies were made in Chicago in 1895–96 with the cooperation of Jane Addams and Caroline Hunt of Hull House. The objective was to obtain information regarding the conditions of living and the "pecuniary economy" of the food of the "poor" of different nationalities residing in the worst congested districts of the city. Similar studies were made in New York City during 1896–98, from which suggestions were made for improving the food habits of the city's poor, in the interest of getting a palatable diet which would be both more nutritious and less expensive. Mention was made in the reports of the need for public education along these lines. Atwater's principal associates in these studies were C. D. Woods, A. P. Bryant and H. B. Gibson.

In some of the publications of the many studies made, Atwater discussed the European dietary standards and made a recommendation of his own which closely followed those of Voit. His standard called for large increments of protein as muscu-

lar work increased. Apparently he continued to hold the view that such increments were desirable in practice despite the results of his calorimetric studies which showed that muscular activity caused minimal increases in nitrogen elimination. In discussing the question he pointed out that such short-time observations might not hold in practice and that extended studies were needed, combining controlled feeding experiments, metabolic studies and observations regarding physical and mental efficiency, and general health, strength and welfare. He stated that dietary standards were estimates only and that while "tables of composition of food materials and dietary standards are rational and useful, the housewife who attempts to bring the daily meals of herself and her family into exact mathematical accord with any given standard will not be making the best use of what the science of nutrition has to offer." From a review of his dietary studies Atwater was led to make comments, similar to those made today, on the possible dangers of overeating. After referring to the "modern" commercial developments as regards the food supply and to the tendency to less manual labor and exercise, he stated, "it is a fair question whether the results of these things have induced among us in a large class of well-to-do people, with little muscular activity, a habit of excessive eating and may be responsible for great damage to health, to say nothing of the purse." He was particularly concerned about the high consumption of fats and sweets.

In addition to the dietary studies Atwater and his associates carried out, he was instrumental in organizing a cooperative project for such studies by various states, through the Office of Experiment Stations. As a result some 350 studies were conducted more or less under his general direction, during the 15-year period when he was actively interested in the field. While the scientific value of these studies may not have been sufficient to justify the cost and effort involved, they had an important influence in arousing widespread interest, both among investigators and the public, in nutrition problems and in encouraging governments to increase their support for investigations in food and nutrition.

Atwater's most basic contributions to the science of nutrition resulted from his metabolic studies. In 1887 he visited Voit's laboratory where he became acquainted with Rubner's experiments with a respiration calorimeter. He returned with the determination to build such an apparatus which would be large enough for a variety of human experiments. The task was begun in 1892 and completed in 1897 largely through skill of E. B. Rosa, Professor of Physics at Wesleyan, who later became chief physicist of the Bureau of Standards. The apparatus provided for the direct determination of the amount of heat given off by the body, as well as for the conduct of digestion trials and nitrogen-carbon balances to measure the gain or loss of protein and fat. It enabled Atwater to achieve his goal of finding out what happened to food in metabolism, as distinguished from the end results indicated by dietary experiments. The accuracy of the apparatus was amazing, as shown by the finding that when a given weight of alcohol was burned in the chamber the carbon dioxide recovered was 99.8 per cent of the theoretical value, and the heat produced, 99.9 per cent. The apparatus is described in detail by Atwater and Rosa in bulletin 63 of the Office of Experiment Stations (1899).

The first experiments were reported in 1896, representing nitrogen-carbon balance studies carried out before the calorimetric feature of the apparatus was completed. In 1897 direct-calorimetric studies were begun. A total of some 500 experiments were carried out with the apparatus up to the time of Atwater's death. Most of them were made for a period of four days and five nights, some for considerably longer periods. The subjects ate and slept in the apparatus and performed various levels of physical work using various diets, as prescribed for the experiment in question. The chamber contained a bed, table and folding chair. Among the objectives were determinations of the quantities of nutrients and energy metabolized under different conditions of rest and muscular activity, the relations between external work and the energy and nutrients

metabolized in its performance, the capacities of different classes of nutrients to supply the body with material and energy and the proportions in which they may replace each other for these purposes, and the digestion and assimilation of food materials. Students taking examinations in the calorimeter were studied in an attempt to measure the energy expenditure in mental effort. The results of the various studies were published in the annual reports of the Storrs Station, in bulletins of the Office of Experiment Stations and in various journals. Among those associated with Atwater in the experiments special mention should be made of F. G. Benedict who became his research assistant in 1895 and later Professor of Chemistry. Most of the publications were made under their joint authorship. Another joint leader in some of the projects was C. F. Langworthy who succeeded Atwater in 1906 in charge of the federally supported nutrition investigations. Others associated in the experiments at various times were C. D. Woods, A. P. Bryant, R. D. Milner, P. B. Hawk, H. C. Sherman, A. W. Smith, O. F. Tower, H. M. Burr, J. F. Snell, H. E. Wells, and E. H. Hodgson. Among the findings in fundamental metabolism the one of greatest theoretical importance resulted from the series of studies on energy intake and output which showed that the law of conservation of energy held in the transformation of matter in the human body, as welll as in the inanimate world. The results of these studies were published in the Storrs Annual Report for 1902–03. A series of 13 studies of popular as well as scientific interest dealt with the nutritive value of alcohol. This work was undertaken at the request of the "Committee of Fifty for the Investigation of the Drink Problem," to obtain accurate and scientific information concerning the physiological action of alcohol. The experiments proved conclusively that alcohol was oxidized in the body to the extent of 98 per cent, providing energy for warmth and probably for work and protected body tissues from catabolism. Its value relative to other energy sources was expressed by the statement that 4 grams were isodynamic with 7 grams of sugar or starch, and with 3 grams of fat. The results were published in 1902 by Atwater and Benedict in a Memoir of the National Academy of Sciences. They brought a storm of criticisms from temperance organizations. The breadth of Atwater's interest in investigating the relations between diet and muscular activity is indicated by the studies made on three subjects during a 6-day bicycle race held at Madison Square Garden in 1898. The results were published by him and H. C. Sherman under the title, "Food consumption, digestion, metabolism and mechanical work on bicycles" (Office of Experiment Stations bulletin 98).

The many nitrogen balances carried out in the calorimeter confirmed and extended previous European results showing that muscular work didn't significantly increase the protein catabolism provided the energy intake was adequate. It was also shown that the maintenance of nitrogen equilibrium was possible at levels little above one-third of the Voit Standard. As reported by Atwater in one of his publications "Men with more or less activity have maintained nitrogen equilibrium on 7 g of nitrogen or 44 g protein per day." Nevertheless, as previously mentioned, he did not feel that these short-time observations justified lowering the standard.

As early as 1887, Atwater had compiled the results of several European digestion experiments. When he was placed in charge of the special fund for nutrition investigations which Congress appropriated in 1893, he encouraged the experiment stations to undertake such studies. He and his associates began digestion experiments at Wesleyan in 1896, some of which were made in connection with the metabolism investigations with the calorimeter. During the following eight years more than 100 experiments with various pure nutrients, individual foods and mixed diets were published in the annual reports of the Storrs' Station. Atwater's associates in these studies were F. G. Benedict, A. P. Bryant, C. F. Langworthy, R. D. Milner and H. C. Sherman. Many other publications were issued by workers in other stations, who had been stimulated to undertake similar studies.

Atwater used the results of the digestion studies and of bomb calorimetric determi-

nations to arrive at factors for calculating the "fuel values" of individual foods, food groups and mixed diets. Having worked out the factors for various individual foods he summarized 185 dietary studies made throughout the United States to arrive at the food and nutrient composition of the "average diet." He then applied his factors for individual foods to the make-up of this diet to arrive at the general factors: protein, 4; fat, 8.9; carbohydrates, 4. These values were lower than the Rubner factors which did not take account of digestion losses. In his publication (Storrs Annual Report for 1899, pp. 69–110) Atwater detailed his procedures for arriving at these factors and discussed their applicability. In later use the factor for fat was rounded off to 9. Following the general report mentioned above, a table of fuel values for specific foods was published, calculated by the use of the individual factors. For many years Atwater's data were not properly applied in many cases, in that the general factors were used for certain foods and combinations for which individual factors should have been employed.

Atwater's nutrition interests were not limited to man. Based on his visits to European experiment stations in 1770–71, he published, in 1774, an article of some 60 pages entitled, "Results of late European experiments on the feeding of cattle." In this article he included data on feed composition from Wolff's table and described Wolff's feeding standard. This was the first publication in English, at least for American readers, of this classic standard. During the next 15 years he wrote several other articles dealing with the feeding of animals and some feed analyses were made in his laboratory at Wesleyan. With the establishment of the Storrs Station, feed analyses, feeding trials and digestibility studies with dairy cattle and sheep became an important part of the program. C. S. Phelps was in direct charge of most of these animal experiments.

Throughout his life Atwater wrote many popular articles to encourage the application of scientific knowledge in practice. From 1875 to 1881 he contributed a series of 69 articles to the American Agriculturist, entitled, "Science Applied to Farming." During 1887 and 1888 he published a series in the Century Magazine under the title "The Chemistry of Foods and Nutrition." In 1894 his Farmers' Bulletin 23 was issued under the title, "Foods, nutritive value and cost." This bulletin served a very important purpose as a guide to housewives. It was revised and reissued as Bulletin 142 in 1902 and reprinted in 1906 and 1918. Following the furor created among opponents of liquor by his studies on the nutritive value of alcohol, Atwater wrote articles in Harpers Magazine and in various journals for teachers, explaining his findings, emphasizing that the quantity effectively used was small, that it could have deleterious effects as well and advocating rational methods of temperance reform. In his popular writing he tried to reach all groups who might aid the cause of better nutrition, as illustrated by his article in a religious magazine, entitled, "What the Church can do toward improving the nutrition of the masses."

In 1903 Atwater visited certain European universities to discuss the possibility of setting up experiments on an international scale to study the relation of diet to labor power and the success and health of nations. This proposal evoked considerable interest and he returned to work out details accordingly. In late 1904 however, he became ill and largely incapacitated for further work. During the last three years of his life the program at Wesleyan was continued by his associates. Atwater had wanted to develop an apparatus for the direct determination of oxygen as a feature of the calorimeter and in 1902 and 1903 the Carnegie Corporation of Washington gave him grants for this purpose. The task became the primary responsibility of Benedict, the completion of which represented one of his very important contributions. The Carnegie Corporation had also agreed to build a new laboratory for Atwater and endow his program, but when it became evident that he was permanently incapacitated this proposed support was transferred to the establishment of the Nutrition Laboratory in Boston of which Benedict became Director in 1907.

The many accomplishments here reviewed make it clear that Atwater must have had outstanding ability to organize and direct research. Quite evidently also

he had rare judgment in selecting associates and assistants and a genius for inspiring them to help carry out the programs which he envisioned. Many of them had been his students as undergraduates. Several, desirous of working with him, came from foreign countries. Many of his associates later became leading scientists in agricultural experiment stations and in other institutions. Thus, though the program at Wesleyan ceased with Atwater's death, it went forward elsewhere throughout the country. In referring to his scientific associates and assistants mention should also be made of his daughter, Helen, who helped in the preparation of many of his manuscripts, particularly those designed to popularize nutrition findings. She served as editorial assistant in the Office of Experiment Stations from 1898 to 1903. Later she had a notable career in her own right, which included the editorship of the Journal of Home Economics.

An insight into the character of Atwater as a man is revealed in the following excerpt from the minutes of the Wesleyan Faculty, recorded after his death: "Professor Atwater was one of the most genial and companionable of men. With a buoyant, elastic temper, exhaustless energy, always ready to talk from a full mind, eager to give and receive, always hopeful and in good humor, he seemed to bring life and vigor wherever he went. His enthusiastic devotion to his chosen scientific work had not narrowed his culture or his sympathies. He had an intelligent interest in all humanist studies, all matters of art and literature; but his keenest concern was enlisted in whatever pertained directly to the welfare of society. All measures for the amelioration of social conditions, for the reform of evils or the relief of hardship among all classes of men, always had his hearty support."

ACKNOWLEDGMENTS

The writer is greatly indebted to Mr. Wyman W. Parker, Librarian of Wesleyan University, and Emeritus Professor Karl Van Dyke of that institution, for the loan of a voluminous collection of papers, correspondence and other material preserved from Atwater's files. Mrs. Edith M. Fox, Archivist, Cornell University, was instrumental in obtaining this loan, as well as encouraging the writer in his task.

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Nitrogen Balances of Young Women Fed the FAO Reference Pattern of Amino Acids and the Oat Pattern[1]

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A provisional pattern of essential amino acid requirements of adults has been formulated by the Food and Agriculture Organization Committee on Protein Requirements ('57) as an aid to estimating the biological values of different proteins. With such a pattern as a reference for comparison it was envisioned that the amino acid content of a single food, a combination of foods, or a country's entire food supply can be used to appraise the quality of the protein, to identify inadequacies, and where needed, to plan effective supplementation.

Swenseid et al. ('61, '62) have reported on studies made with young men and women to evaluate the FAO pattern of essential amino acids for maintaining nitrogen equilibrium and to compare it with the amino acid pattern in egg. The present report is a comparison of the FAO pattern and a good quality plant protein, oats, using young women as subjects in the measurement of nitrogen balance. The study was undertaken at Oklahoma State University in 1957. Other laboratories have studied milk and peanut butter (Kirk et al., '62) and wheat.

EXPERIMENTAL PROCEDURES

The 6 women college students who served as subject were in good health as determined by a medical examination. Their ages ranged from 19 to 22 years, their heights from 159 to 175 cm, and their weights from 57.4 to 74.1 kg.

The study was 48 days in length. The first 8 days were used for the subjects to adjust to the daily nitrogen intake of approximately 9 gm supplied first by ordinary foods and then by the semi-purified diet plus purified amino acids and diammonium citrate. To determine the lowest intake of essential amino acids that would maintain nitrogen equilibrium in each subject, all of the subjects were given, during successive 8-day periods, the FAO pattern at a level that supplied 240 mg of tryptophan, then 160 mg, and then 200 mg. For another 8 days following these three periods, the purified essential amino acids were fed proportioned as in the oat pattern to supply 240 mg of tryptophan for subjects 1, 4, and 6 and to supply 200 mg for subjects 2, 3, and 5. During the last 8 days of the study the oat pattern was supplied by cooked rolled oats in amounts that supplied 240 mg of tryptophan (120 gm dry rolled oats) for subjects 1, 4, and 6, and 200 mg of tryptophan (100 gm dry rolled oats) for subjects 2, 3, and 5.

The amounts of the important amino acids supplied in each pattern and at each level of tryptophan are shown in table 1 together with the source of the total nitrogen intakes.

The semi-purified diet which was planned to be adequate in all known dietary essentials included: (1) small amounts of a few foods of low nitrogen content: canned applesauce, orange and tomato juice, peaches, pineapple, pears, jelly, and fresh bananas and lettuce; (2) wafers and pudding made with cornstarch, butter oil, sugar, and nondigestible flakes; (3) mineral and vitamin supplements; and (4) purified amino acids and diammonium

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[2] Present address: Agricultural Research Service, U. S. Department of Agriculture, Washington, D. C.

TABLE 1

Amino acid and nitrogen intakes

Constituent	FAO pattern[1]				Oat pattern		
	Ratio	Amounts fed			Ratio	Amounts fed	
			mg/day			*mg/day*	
Essential amino acids							
Tryptophan, estimated	1.0	160	200	240	1.0	200	240
Tryptophan, analyzed		158	198	245		195	233
Isoleucine	3.0	476	596	716	3.8	793	942
Leucine	3.4	544	679	814	6.7	1,388	1,647
Lycine	3.0	477	579	717	3.4	767	901
Methionine and cystine	3.4	648	809	975	4.2	900	1,129
Phenylalanine and tyrosine	4.5	737	919	1,102	8.5	1,730	2,061
Threonine	2.0	317	397	477	3.2	689	814
Valine	4.0	642	802	962	5.4	1,121	1,332
Nitrogen from			*gm/day*			*gm/day*	
Essential amino acids[2]		0.455	0.566	0.684		0.851	1.010
Nonessential amino acids[3]		1.406	1.757	2.108		1.761	2.109
Glycine		3.429	3.194	2.961		2.881	2.595
Diammonium citrate		3.429	3.194	2.961		2.881	2.595
Total		8.719	8.711	8.714		8.374	8.309

[1] Preliminary pattern secured by personal communication. In the pattern published by FAO ('57) the ratio of the sulfur-containing amino acids is 3.0, aromatic 4.0, and valine 3.0.
[2] Includes 0.078 gm from basal diet.
[3] Includes 0.477 gm from basal diet.

citrate, or cooked rolled cats. To maintain each subject at constant body weight, the total energy intake was adjusted to meet individual needs with additional amounts of butter oil, jelly and sucrose, and a carbonated drink.

The quantities of the amino acids to be fed in each period were mixed in a ball mill. Portions were weighed for each subject for each meal (one-third of the day's total ration), mixed with 20 gm of sucrose, and made into solution. Diammonium citrate and glycine were added so that the total daily nitrogen intake would approximate 9.0 gm as closely as possible. Because of the insolubility of cystine and tyrosine, these were weighed separately and mixed with 100 gm of the applesauce. Approximately one-third of the applesauce was eaten at each meal.

The amounts of the nonessential amino acids included with the FAO reference pattern corresponded to those present in the quantity of rolled oats that supplied the amount of tryptophan in the level being tested — 240, 160, or 200 mg.

The rolled oats were of the type commonly used as a breakfast cereal but not the precooked or quick-cooking type. The same lot of oats was used throughout the study. One-third of the day's portion of oats for each subject was cooked in an individual-size casserole and eaten directly from the casserole at each meal.

Urine and feces were collected throughout the 48 days of the study. For each subject urine collections were combined into 24-hour composites and feces collections, marked with carmen, were combined into 4-day composites and analyzed for nitrogen. Food composites and amino acid mixtures were also analyzed for nitrogen as fed. Nitrogen was determined by the AOAC method ('55).

The amino acids were checked for purity and the amino acid contents of the basal diet and of oats were determined by the microbiological methods of Henderson et al. ('48) and Henderson and Snell ('48).[3]

RESULTS

The mean nitrogen balances of the subjects on the different patterns, amounts, and sources of amino acids are shown in table 2. The actual analyzed nitrogen intakes differed slightly from the calculated planned intakes given in table 1. The mean daily balance for each subject

[3] We are indebted to Dr. M. J. Horn, Human Nutrition Research Division, U. S. Department of Agriculture for the amino acid analysis of the proteins in the basal diet and for the nonessential amino acid analysis of the oats.

TABLE 2

Mean nitrogen intakes and balances

Period	No. days	Description of intake			Nitrogen[2]			
					Subjects 1, 4, 6		Subjects 2, 3, 5	
		Pattern	Tryptophan[1]	Source of amino acids	Intake	Balance	Intake	Balance
			gm/day		*gm/day*	*gm/day*	*gm/day*	*gm/day*
A	5	Ordinary foods			9.59	−0.66	9.48	−0.12
B	3	Transition to semi-purified			8.66	−2.08	8.74	−0.74
C	4	Reference	240	purified	8.97	−0.85	9.00	−0.94
D	4	Reference	240	purified	8.94	0.54	8.97	0.47
E	8	Reference	160	purified	8.94	−0.33	8.95	−0.43
F	8	Reference	200	purified	8.98	−0.44	9.00	−0.11
G	8	Oat	200	purified	—	—	8.97	−0.01
H	8	Oat	200	rolled oats	—	—	9.06	0.28
G	8	Oat	240	purified	8.98	0.36	—	—
H	8	Oat	240	rolled oats	9.11	0.10	—	—

[1] Amount of tryptophan supplied with other essential amino acids proportioned as in the pattern being fed.
[2] Figures are for last 6 days of each 8-day period E through J.

for each period after adjusting to the semi-purified diet (periods D through H) is charted in figure 1.

After a 4-day foreperiod (period C), the FAO pattern fed at a level to supply 240 mg of tryptophan maintained nitrogen balances ranging from 0.19 to 0.84 gm daily (period D, 4 days) in the 6 subjects. When the level was reduced to supply only 160 mg of tryptophan the 6 subjects went into negative balance which ranged from − 0.20 to − 0.59 gm of nitrogen daily (period E). Increasing the level of the FAO pattern to supply 200 mg of tryptophan (period F) improved the nitrogen balances for subjects 2, 3, and 5 to − 0.07, − 0.17, and − 0.10 gm of nitrogen daily, respectively, with a mean of − 0.11 gm. On the basis of the balances in period F, the level of 200 mg of tryptophan was used for subjects 2, 3, and 5 for studying the effect of the oat pattern supplied by purified amino acids and by the intact protein of cooked oatmeal.

At the 200 mg tryptophan level, subjects 1, 4, and 6, however, had negative nitrogen balances of − 0.88, − 0.22, and − 0.22 gm, respectively, with a mean of − 0.44 gm. For this reason these subjects were returned to a level of 240 mg of tryptophan for subsequent periods when the oat pattern was fed.

Pattern of amino acids. When the FAO pattern and the oat pattern were supplied by purified amino acids and fed at the same tryptophan level there were no significant differences among the nitrogen balances of the subjects. The mean daily

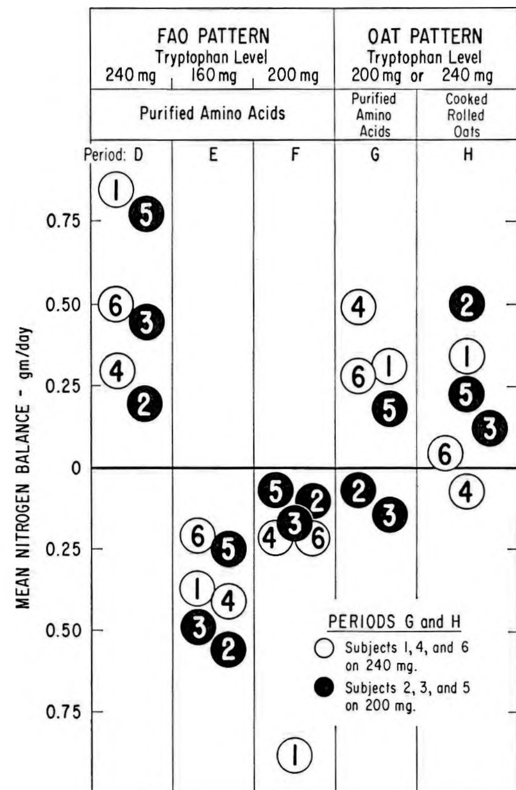

Fig. 1 Nitrogen balance of each subject when fed the FAO pattern and the oat pattern at different levels of tryptophan.

balances with the 240 mg tryptophan level for subpects 1, 4, and 6 were 0.54 gm of nitrogen with the FAO pattern and 0.36 gm with the oat pattern. Corresponding balances at the 200 mg tryptophan level for subjects 2, 3, and 5 were − 0.11 gm fed the FAO pattern and − 0.01 gm with the oat pattern. These results suggest that one pattern was as effective as the other when supplied by purified amino acids at the lowest level of tryptophan that would maintain approximate nitrogen equilibrium.

Source of amino acids. When subjects 2, 3, and 5 were receiving the oat pattern supplied by purified amino acids (period G), they had a mean nitrogen balance of − 0.01 gm daily (− 0.07, − 0.14, and 0.18 gm, respectively). Their mean balance increased to 0.28 gm of nitrogen daily (0.50, 0.12, and 0.23 for subjects 2, 3, and 5, respectively) when the oat pattern was furnished by cooked rolled oats (period H). Although the difference was significant at the 5% level, data on three subjects are insufficient to permit concluding that the intact protein supports better nitrogen retention than the purified amino acids.

There was no significant difference, however, between the mean balances of subjects 1, 4, and 6 when the oat pattern, to supply 240 mg of tryptophan, was furnished by purified amino acids (period G) and by cooked rolled oats (period H). With the purified source the mean nitrogen balance was 0.36 gm daily (0.31, 0.49, and 0.28 for subjects 1, 4, and 6, respectively) as compared with 0.10 gm when cooked rolled oats supplied the amino acids (0.34, − 0.07, and 0.04 gm, respectively).

Essential amino acid nitrogen. The amounts of nitrogen supplied by the essential amino acids (plus cystine and tyrosine) in the two patterns and at the different tryptophan levels are shown in table 1. At the same level of tryptophan the oat pattern supplied 50% more nitrogen from essential amino acid than the FAO pattern. When the FAO pattern supplied 160 mg of tryptophan daily, the essential amino acid nitrogen totaled only 0.46 gm and the 6 subjects were in negative nitrogen balance.

In studies of the amino acid requirements of young women Leverton et al. ('56) found that 150 mg of tryptophan maintained approximate nitrogen equilibrium when other dietary essentials were supplied in adequate amounts. The semi-purified diet used in that study, however, contained approximately 0.9 gm of nitrogen from essential amino acids as compared with 0.46 gm supplied in this study by the FAO pattern at the level of 160 mg of tryptophan. Further study needs to be made of the FAO pattern to determine whether at this level of tryptophan the limiting factor is a particular amino acid or the total essential amino acid nitrogen.

At the level of 200 mg of tryptophan daily the essential amino acid nitrogen in the FAO pattern and the basal diet totaled 0.57 gm. This amount maintained only three of the 6 subjects (2, 3, and 5) in an approximate nitrogen equilibrium of − 0.11 gm nitrogen daily. Subjects 1, 4, and 6 had a mean balance of − 0.44 gm nitrogen daily on 0.57 gm of essential nitrogen but had had a mean daily retention of 0.54 gm when the FAO pattern was fed at the level of 240 mg of tryptophan and the daily intake of essential nitrogen was 0.68 gm.

Under the conditions of this study and with these subjects, 0.6 to 0.7 gm of essential amino acid nitrogen daily was necessary to maintain approximate nitrogen equilibrium or slight retention. This is more than the 0.5 gm which is the sum of the minimal requirements of young women for the individual amino acids as summarized by Leverton ('59). Swenseid et al. ('62) reported that young women fed the FAO pattern and the egg pattern required from 0.65 to 0.85 gm of essential nitrogen to maintain equilibrium.

No relationship was found between the weights of the young women and their nitrogen balances, using the different amounts and sources of the amino acids.

SUMMARY

The nitrogen balances of 6 young women were determined when they were fed a semi-purified diet with the essential amino acids proportioned as in the FAO reference pattern based on tryptophan levels of 240, 160, and 200 mg daily.

With this pattern the 240-mg tryptophan level most consistently supported nitrogen equilibrium or slight retention in all of the subjects. The 160-mg level caused all subjects to be in negative balance. At the 200-mg level three subjects approached nitrogen equilibrium.

Following these levels the subjects were fed the amino acids in the proportions in which they occur in oats supplied first by purified amino acids and then by cooked rolled oats and based on 240 mg of tryptophan for three subjects and 200 mg for the other three subjects.

When the FAO pattern and the oat pattern were supplied by purified amino acids and fed at the same tryptophan level, there were no significant differences among the nitrogen balances of the subjects.

With the semi-purified diet that supplied about 9 gm of nitrogen daily by a combination of essential and nonessential amino acids, glycine, and the diammonium citrate, 0.6 to 0.7 gm of nitrogen from essential amino acids was necessary to maintain these subjects in approximate nitrogen equilibrium or slight retention.

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Effects of Ubiquinones and Phytyl-Ubichromenol upon Encephalomalacia and Muscular Dystrophy in the Chick[1]

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Encephalomalacia in chicks, since its characterization by Pappenheimer and Goettsch in 1931, has been recognized as a classical symptom of vitamin E deficiency. However, encephalomalacia can be accelerated or suppressed by dietary changes which are unrelated to the vitamin E content of the diet (Dam, '44). Thus, when vitamin E-low diets were used no symptoms occurred unless highly unsaturated fats were added to the diets. Whether the vitamin E-deficient chicks showed encephalomalacia or exudative diathesis depended upon the presence or absence of certain other substances in the diet. Scott et al. ('55) showed that when chicks were fed a vitamin E-deficient diet containing a high level of Torula yeast, exudative diathesis occurred in 100% incidence but encephalomalacia was observed only rarely. It appeared, therefore, that although Torula yeast was severely deficient in vitamin E and other factors needed for prevention of exudative diathesis, it nevertheless contained a principle capable of preventing encephalomalacia. More recently, Schwarz et al. ('57) and Patterson et al. ('57) showed that complete prevention of exudative diathesis is achieved upon supplementation of the vitamin E-deficient, Torula yeast diet with as little as 0.1 mg of selenium (as sodium selenite) per kg of diet. However, the factor in yeast responsible for prevention of encephalomalacia is still unknown.

Recently Edwin et al. ('61) and Diplock et al. ('61b) suggested that both α-tocopherol and selenium may function in controlling the ubiquinone levels in rats, and have further suggested that this may represent the main function of vitamin E and selenium in animal metabolism.

Recently, Diplock et al. ('61a) determined the ubiquinone and ubichromenol content of various yeasts. This study showed Torula yeast to be an excellent source of both ubiquinones and ubichromenols. They claimed that ubichromenol is the substance in yeast observed by Cowlishaw and Prange ('56) to have some properties similar to those of tocopherol and to protect erythrocytes of vitamin E-depleted rats from hemolysis *in vitro*. Johnson et al. ('61) have shown that DL-ubichromenol, but not ubiquinone-50, had a definite effect in permitting the birth of live young when administered to proven vitamin E-deficient female rats at the time of mating. It therefore seemed worthwhile to determine the effects of ubiquinone and ubichromenol in comparison with the effect of yeast upon encephalomalacia in vitamin E-deficient chicks. Studies were conducted also on the effects of the ubiquinones and ubichromenol upon muscular dystrophy in chicks. These studies are the subject of this report.

EXPERIMENTAL

Day-old cross-bred New Hampshire × White Leghorn chicks of mixed sex were given a vitamin E-low starter ration for 7 days. They were thereafter grouped

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[2] On leave (1961) from Cornell University.

TABLE 1

Composition of basal diets[1]

Diet no.	2502	2503	2504	2505	2535	2574
	%	%	%	%	%	%
Casein, crude[2]	—	—	—	—	—	15
Casein, vitamin-test[3]	30	—	—	—	—	—
Torula yeast 3N[4]	—	60	—	—	60	—
Fleischmann's yeast 50B[5]	—	—	60	—	—	—
Soybean protein[6]	—	—	—	30	—	—
Gelatin	3	3	3	3	3	10
Salt mixture[7]	5.17	5.17	5.17	5.17	5.17	5.17
Vitamin mixture[7]	0.1	0.1	0.1	0.1	0.1	0.1
Choline chloride	0.2	0.2	0.2	0.2	0.2	0.2
Cornstarch	31.43	1.43	1.43	31.43	31.43	—
Sucrose	—	—	—	—	—	69.52
Lard	30	30	30	30	—	—

[1] To diets 2502, 2503, 2504, 2505 and 2535 were added 1.4 mg SeO_2/kg. Diet 2574 was supplemented with 0.14 mg SeO_2/kg. All the diets were supplemented with 10 mg Synkavit Roche (dicalcium salt of 2-methyl-1,4-naphthohydroquinone-diphosphoric acid ester) per kg. Vitamins A and D_3 were given as a solution prepared from crystalline vitamin A acetate (Roche), 1 gm; crystalline vitamin D_3 (Roche), 0.058 gm; Tween 80 (polyoxyethylene sorbitan monooleate), 64 gm; ethyl alcohol, 100 ml; and distilled water to a total volume of 330 ml. Twice a week 0.1 ml per animal furnished 250 IU vitamin A and 20 IU vitamin D_3 per day.

[2] Dairinex, from A/S Dansk Mejeri Industri and Export Kompagni, Stege, Denmark.

[3] From Genatosan Ltd., Loughborough, England.

[4] From Lake States Yeast Corporation, Rhinelander, Wisconsin.

[5] From Standard Brands, Inc., New York.

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

[7] Dam and Søndergaard ('53).

(10 chicks per lot) and given the experimental diets.[3]

The basal experimental diets are shown in table 1. The encephalomalacia-producing diets contained a high level of lard, but sufficient selenium dioxide to prevent exudative diathesis. Feed and water were supplied ad libitum. Vitamins A and D_3 were administered orally to each chick twice weekly. Daily observations were made of symptoms of encephalomalacia. Encephalomalacia was diagnosed not only by occurrence of gross symptoms of ataxia but also by macroscopic examination of the brain. A thorough scoring of incidence and severity of muscular dystrophy was made by postmortem examination of all chicks at the end of the experimental period, which was usually 5 weeks.

RESULTS AND DISCUSSION

Studies on encephalomalacia. The results are shown in figures 1–4. Abscissae indicate number of days from the beginning of the experimental feeding, ordinates the number of chicks showing clinical signs of encephalomalacia and the total number of chicks. Furthermore, the ordinates also register chicks that died without having encephalomalacia. Thus the staircase-like ascending front line shows the

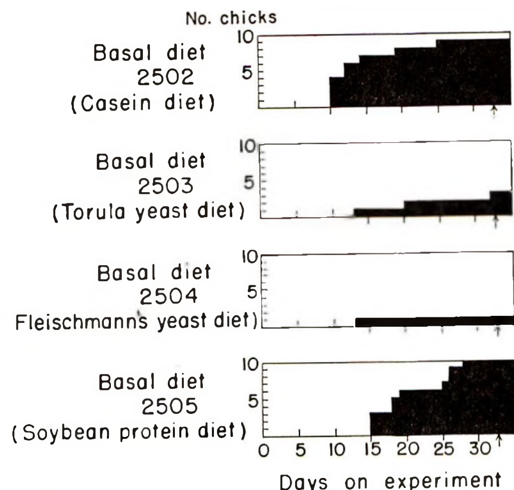

Fig. 1 Development of encephalomalacia in chicks receiving various vitamin E-deficient basal diets. Black areas, starting at bottom and working up, show when each individual chick developed encephalomalacia. The arrow marks the end of the experimental period.

[3] The authors wish to thank Dr. O. Wiss, F. Hoffmann-La Roche and Company, Basle, Switzerland, for samples of ubiquinone-30, ubiquinone-50 and phytylubichromenol used in the studies on encephalomalacia and one of the studies on muscular dystrophy. They also wish to thank Dr. Karl Folkers, Merck and Company, Rahway, New Jersey, for kindly supplying the ubiquinone-50 (coenzyme Q_{10}), and Dr. P. L. Harris, Distillation Products Industries, Rochester, New York, for the injectable vitamin E used in the second experiment on muscular dystrophy.

development of the disease, whereas the descending front line indicates chicks that died without having encephalomalacia. The arrow marks the end of the experiment.

The graphs presented in figure 1 demonstrate the occurrence of an encephalomalacia-protecting principle in both Torula yeast and Fleischmann's yeast which was absent in both the casein and soybean protein diets. These results showed, therefore, that either the casein diet or the soybean protein diet could be used as basal diet for the study of the effects of ubiquinones and ubichromenol upon encephalomalacia.

It should be pointed out that the Torula yeast contains 2.4% of dienoic and 0.14% of trienoic fatty acids (Dam et al., '57), the soybean protein contains 0.65% of dienoic and 0.07% of trienoic (Bieri et al., '61), whereas casein and Fleischmann's yeast are practically devoid of polyenoic fatty acids.

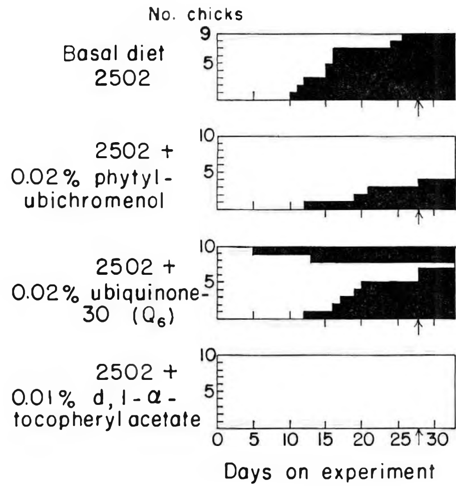

Fig. 3 Effects of phytyl-ubichromenol, ubiquinone-30 and vitamin E upon encephalomalacia in chicks receiving the casein basal diet. Black areas at the bottom denote occurrence of encephalomalacia in individual chicks. Black area at top indicates mortality without encephalomalacia.

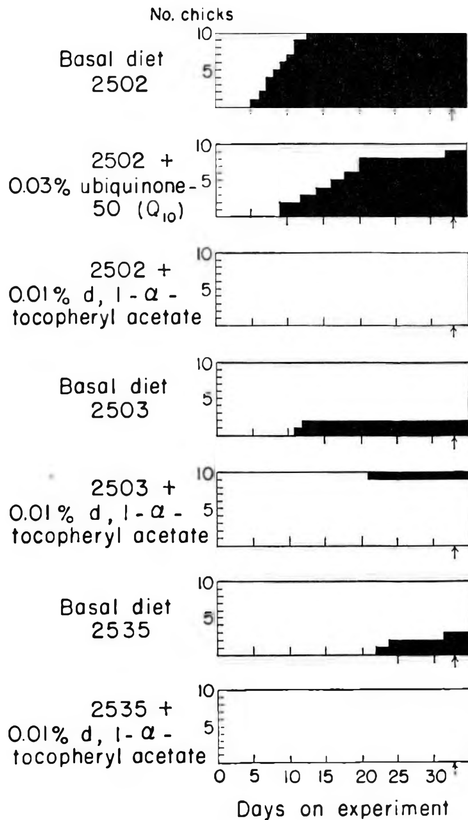

Fig. 2 Effects of ubiquinone-50 (coenzyme Q_{10}) and vitamin E upon encephalomalacia in chicks receiving various experimental diets. Areas in black at the bottom show individual chicks with encephalomalacia. Black area at top shows mortality without symptoms of encephalomalacia. Arrow marks end of experiment.

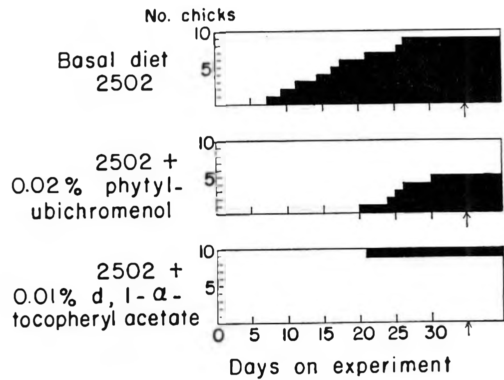

Fig. 4 Effect of phytyl-ubichromenol upon encephalomalacia (second experiment). Black areas at bottom show occurrence of encephalomalacia in individual chicks. Black area at top shows mortality without symptoms of encephalomalacia.

The graphs presented in figure 2 show that the addition of 0.03% of ubiquinone-50 (coenzyme Q_{10}) produced a slight delaying effect upon the development of enceph-

alomalacia in chicks receiving the casein diet (2502), that the addition of 0.01% of *dl*-α-tocopheryl acetate completely prevented encephalomalacia in chicks receiving this diet and that only a low incidence of encephalomalacia occurred in chicks receiving the Torula yeast basal diets (2503,2535).

The results presented in figure 3 show that addition to the casein-lard diet (2502) of 0.02% of phytyl-ubichromenol was more effective in preventing encephalomalacia than addition of 0.02% of ubiquinone-30.

Phytyl-ubichromenol prevented encephalomalacia not quite to the same extent as it was prevented by feeding the Torula yeast diet which, according to the analyses of Diplcck et al. ('61) supplied approximately 0.02% of ubichromenol when added to the diet at the level of 60%. The ubichromenol used in these studies was prepared[4] from phytyl-ubiquinone and has the same side-chain as vitamin E.

The results of the second experiment comparing the effect of 0.02% of phytyl-ubichromenol with that of 0.01% of *dl*-α-tocopheryl acetate in basal diet 2502 are presented in figure 4. These results confirm those presented in figure 3 and show that phytyl-ubichromenol is only slightly less effective than yeast for prevention of encephalomalacia, thereby supporting the suggestion that ubichromenol is the main substance in yeast responsible for the encephalomalacia-protecting properties of yeast.

It remains to be determined whether ubichromenol has a primary metabolic effect or merely a sparing effect upon vitamin E. It should be emphasized that some non-tocopherol antioxidants are capable of counteracting encephalomalacia in chicks. Thus, methylene blue (Dam et al., '51), N,N'-diphenyl-*p*-phenylenediamine (DPPD) (Singsen et al., '55; Bunnell et al., '55) and 1,2-dihydro-6-ethoxy 2,2,4-trimethylquinoline[5] (Machlin et al., '59, '60) prevent this disease.

Studies on muscular dystrophy. Phytyl-ubichromenol was considerably more effective than the ubiquinones in the prevention of encephalomalacia in vitamin E-deficient chicks. In view of the suggestion of Edwin et al. ('61) and Diplock et al. ('61b) that the principal function of vitamin E is simply in the synthesis or control of the metabolic levels of ubiquinone in the animal body, it was considered necessary to investigate the effects of these substances on another vitamin E-deficiency symptom, namely, muscular dystrophy. If the main function of selenium and vitamin E is in the synthesis or control of ubiquinones and their derivatives, then ubiquinone and ubichromenol should be equally effective in prevention of muscular dystrophy and encephalomalacia in chicks receiving vitamin E-deficient diets. An experiment was conducted, therefore, using basal diet 2574 (table 1) which had previously been shown by Dam et al. ('52) to produce a high incidence of muscular dystrophy. This basal diet was fed alone and supplemented with 0.02% of ubiquinone-30, ubiquinone-50 or phytyl-ubichromenol, or with 0.01% of *dl*-α-tocopheryl acetate.

The results of the experiment, presented in table 2, show that whereas *dl*-α-tocopheryl acetate completely prevented muscular dystrophy in the chicks receiving this diet, no prevention occurred from supplementing the diet with 0.02% of ubiquinone-30 or with 0.02% of ubiquinone-50. Since addition to the diet of 0.02% of phytyl-ubichromenol reduced the incidence of muscular dystrophy from 100 to 70% and also reduced the severity of dystrophy, it appears that this substance had some preventive effect upon muscular dystrophy in the vitamin E-deficient chicks.

It is quite possible that the beneficial effect of ubichromenol upon dystrophy is due to some indirect sparing effect upon vitamin E.

Assuming that ubiquinone-50, the particular ubiquinone found in most animal tissues, may not be efficiently absorbed from the intestinal tract, an experiment was conducted in which a solution of ubiquinone-50 was administered by daily intraperitoneal injection into chicks receiving the basal dystrophy-producing diet. As a control, one lot of chicks was given similar injections of *d*-α-tocopheryl polyethylene glycol 1000 succinate (a water-soluble, injectable vitamin E).

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[4] Prepared in the laboratories of the F. Hoffmann La Roche Company, Basle, Switzerland.

[5] Santoquin, Monsanto Chemical Company.

TABLE 2

Influence of ubiquinone and phytyl-ubichromenol on white striation of breast muscles

Addition to basal diet 2574	Incidence of white striation of breast muscles	Avg score[1] of severity of white striation of breast muscles
None	10/10[2]	2.3
Ubiquinone-30, 0.02%	9/10	2.0
Ubiquinone-50, 0.02%	10/10	2.2
Phytyl-ubichromenol, 0.02%	7/10	1.3
dl-α-Tocopheryl acetate, 0.01%	0/10	0

[1] Zero indicates no striation; 1, slight; 2, moderate; and 3, marked striation of the breast muscles.
[2] Numerator shows number of chicks with muscular dystrophy; denominator shows total number of chicks per treatment.

TABLE 3

Failure of injected ubiquinone-50 to prevent muscular dystrophy

Material injected daily (as % of feed consumed)	Avg weights, 4-week	Feed consumed	Incidence of muscular dystrophy
	gm	*gm/chick*	
None	355	618	26/28[1]
Ubiquinone-50, 0.00007%	358	640	27/27
Ubiquinone-50, 0.00025%	366	672	25/28
Ubiquinone-50, 0.00094%	396	627	29/30
Ubiquinone-50, 0.0027%	356	602	30/30
d-α-Tocopheryl polyethylene glycol, 1,000 succinate, 0.0027%	362	641	0/30

[1] Numerator shows number of chicks with muscular dystrophy; denominator shows total chicks per treatment.

The results of this experiment, presented in table 3, show that the injected ubiquinone-50 was ineffective in reducing the incidence of muscular dystrophy, whereas the injected vitamin E compound produced complete prevention of dystrophy.

The fact that injections of fairly high levels of ubiquinone-50 produced absolutely no effect upon muscular dystrophy in chicks strongly indicates that ubiquinone-50 is not the primary metabolic substance required for prevention of muscular dystrophy.

SUMMARY

Ubiquinone-50 (coenzyme Q_{10}) or ubiquinone-30 (Q_6) supplementation of vitamin E-deficient chick diets has been shown to produce a slight delaying effect upon symptoms of encephalomalacia but no effect upon muscular dystrophy.

Supplementation of encephalomalacia-producing chick diets with 0.02% of phytyl-ubichromenol prevented encephalomalacia to approximately the same extent as that observed when the diet was supplemented with 60% of Torula yeast or Fleischmann's yeast, which represents the amounts of these yeasts needed to contribute approximately (calculated) 0.02% of ubichromenol to the diet. These results support, therefore, the suggestion that ubichromenol is the chief substance in yeast responsible for its encephalomalacia-preventing activity.

Although dietary phytyl-ubichromenol had a slight effect in the prevention of nutritional muscular dystrophy in chicks receiving a diet low in vitamin E and sulfur amino acids, the effect of this compound in the prevention of muscular dystrophy was not as great as its effect in prevention of encephalomalacia.

The results presented here show, therefore, that phytyl-ubichromenol is capable of reducing the incidence and severity of two vitamin E-deficiency diseases in the chick. Whether this is a primary metabolic effect or merely a sparing effect upon vitamin E remains to be determined.

ACKNOWLEDGMENTS

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Effect of Carbohydrates on Utilization of Protein and Lysine by Rats [1,2]

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Many investigators have demonstrated that the nature and amount of carbohydrate in the diet influence the rate of protein utilization. Monson et al. ('50) reported that chicks fed a dextrin diet grew markedly faster than those supplied with a sucrose diet. Yoshida and Morimoto ('55) found that feeding of raw-potato starch to rats decreased the digestibility of dietary protein. Harper et al. ('53) studied the influence of various carbohydrates on utilization of protein by rats and found that dextrin supported better growth of rats than sucrose. Womack and Marshall ('55) reported that rats fed extra threonine, in diets containing corn, rice or wheat starch, or corn dextrin, had a lower negative nitrogen balance than did rats fed diets containing sucrose and a low level of amino acids. Harper and Katayama ('53) found that rats fed diets containing 9% of casein grew faster when cornstarch replaced sucrose as the dietary carbohydrate.

Lysine is commonly low in protein of vegetable origin. Supplementation with lysine to improve the quality of plant protein has been intensively investigated. Guthneck et al. ('53) found greater utilization of lysine from dried eggs and skim milk (91%) and from fresh meats (84%) than from cereal and legume food (71%). Gupta et al. ('58) reported that the availability of the lysine in beef was 76% , from gelatin 74% , and in the other purified protein listed was 90 to 104%. Deshpande et al. ('57) reported that the presence of amino acid in cereal protein, although in sufficient quantity, does not adequately determine its nutritional quality. Heller et al. ('61) studied the effect of heat treatment on lysine in meat and found that the percentage of available lysine in pork, beef, and lamb muscle ranged from 74 to 92% in either raw samples or after standard cooking procedures. Little is known concerning the effect of various carbohydrates on utilization of lysine. The comparative effects of several carbohydrates on utilization of lysine in wheat gluten by rats have been investigated. The results are reported in this paper.

EXPERIMENTAL

Diets. Twelve diets were prepared and divided into two series of 6 each. The composition of the diets is shown in table 1. The 6 diets in series 1 included the following carbohydrates: potato starch (raw), cornstarch, sucrose, dextrin, glucose, and potato starch (cooked). The 6 diets in series 2 contained the same sources of carbohydrates as in series 1 except that 0.7% of L-lysine·HCl was added to each diet. Wheat gluten was used as the source of protein at an 18% protein level in all diets. The cooked-potato starch was prepared according to the method of Womack and Marshall ('55). Vitamins A and D were provided by cod liver oil (USP). The other vitamins known to be essential for rats were added (mg/kg) to the ration: thiamine, 5; pyridoxine, 5; menadione, 5; cobalamin concentrate (3 mg/gm), 5; riboflavin, 10; *p*-aminobenzoic acid, 10; nicotinic acid, 20; Ca pantothenate, 20; D-α-tocopheryl acid succinate, 75; folic acid, 5.5; biotin, 0.3; inositol, 400; and choline chloride, 1,000. The protein-free ration had the same general composition as the basal ration except that the different carbohydrates tested were used respectively to replace the pro-

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TABLE 1

Composition of diets

	Series 1 (without lysine)	Series 2 (with 0.7% L-lysine)
	% diet	*% diet*
Wheat gluten[1]	24	24
Carbohydrate[2]	61	60.3
Cottonseed oil, hydrogenated[3]	8	8
Salt mixture USP, 14	4	4
Cod liver oil, USP	2	2
Vitamin mix[4]	1	1
L-Lysine	—	0.7

[1] Wheat gluten contained 75% of protein (24 × 0.75 = 18% of protein in the diet).

[2] Carbohydrates and dietary groups:

A-Potato starch (raw)	G-Potato starch (raw) with lysine
B-Cornstarch	H-Cornstarch with lysine
C-Sucrose	I-Sucrose with lysine
D-Dextrin	J-Dextrin with lysine
E-Glucose	K-Glucose with lysine
F-Potato starch (cooked)	L-Cooked potato starch with lysine

[3] Crisco, Procter and Gamble Company, Cincinnati.

[4] See Experimental section in text.

tein. The minerals were furnished by USP 14 salt mixture.

Feeding experiments. Ninety-six male Sprague-Dawley weanling rats were divided into 12 groups of 8 each according to body weight and litter origin. Each of the 12 experimental diets were fed simultaneously when the rats weighed 70–80 gm at 4 weeks of age. Water and food were supplied ad libitum. Body weight and food consumption were recorded weekly during the 9-week period. The influence of various carbohydrates on the digestibility of wheat gluten and on the availability of lysine was investigated according to the method of Mitchell ('24, '54). At the end of 9 weeks, the animals were then fed a protein-free ration.

Feces excreted during the first three days of the experiment of the protein-depletion period were discarded. Thereafter, the feces from each rat were collected daily for 5 days for the determination of metabolic nitrogen and lysine. After the protein-depletion period, each group was fed its previous test ration containing wheat gluten or wheat gluten with lysine and the respective carbohydrates. The feces were again collected for a 5-day period for determination of total nitrogen and lysine. In changing from one diet to another, at least three days were allowed to elapse before the feces were again collected.

Method for collection and treatment of feces. The animals were placed in metabolic cages; the feces were collected daily, preserved with 2 N HCl, composited for the 5-day collection period, and stored in the refrigerator. The 5-day collection of feces from each rat in each period was homogenized with 2 N HCl in a glass homogenizer. The homogenates were diluted to 100 ml with 2 N HCl except for the homogenates of feces from the rats fed raw-potato starch, which were diluted to 250 ml. For the analysis of lysine, 20 ml of the homogenates were transferred to a bottle, 20 ml of concentrated HCl added, and the bottle sealed and autoclaved for 10 hours at 120°C. The HCl was evaporated under reduced pressure, the residue thus obtained dissolved in distilled water, deodorized with 0.5 gm of activated charcoal, filtered by suction, and diluted to volume. The lysine content of the feces was determined microbiologically by the method of Dunn et al. ('49). For determination of total nitrogen, 2 ml of the homogenate were transferred to a micro-Kjeldahl flask. The total nitrogen was determined by the micro-Kjeldahl method ('60). All data were analyzed statistically by analysis of variance and Duncan's multiple range test. (Steel, '60).

RESULTS

The average increase in weights of the rats fed the different carbohydrate diets is shown in table 2. Without lysine supplementation the growth responses of rats fed sucrose were lower ($P = 0.05$) than the responses of the rats fed glucose and dextrin. There were no significant differences in growth responses of rats fed diets containing raw-potato starch, cooked-potato starch, cornstarch, or dextrin. Addition of 0.7% of L-lysine to the diets greatly increased the rate of growth of rats regardless of the source of the carbohydrates. With lysine supplementation, however, rats fed cooked-potato starch grew better than those fed raw-potato starch ($P = 0.05$); the growth rates of rats fed the other 4 sources of carbohydrates were similar.

The ingestion of raw-potato starch caused a distention of the cecum and intestine. Diarrhea was a problem in the

TABLE 2

Total weight gain and protein-efficiency ratios for rats fed various carbohydrate diets[1,2]

Carbohydrate sources	Total weight gain	Total protein eaten	Protein-efficiency ratio[3]	Food eaten daily/rat
	gm	*gm*		*gm*
Series 1 (without lysine)				
Potato starch, raw	$123.0 \pm 10.4^{4}_{bc}$*	196.8	$0.72 \pm 0.05^{4}_{d}$*	14.9
Cornstarch	$105.0 \pm 5.6_{ab}$	128.3	$0.83 \pm 0.05_{cdf}$	11.3
Sucrose	$94.4 \pm 4.9_{a}$	117.8	$0.80 \pm 0.05_{cd}$	10.3
Dextrin	$124.7 \pm 7.2_{bc}$	138.4	$0.90 \pm 0.03_{bc}$	12.2
Glucose	$131.9 \pm 6.8_{c}$	141.1	$0.93 \pm 0.03_{bf}$	12.4
Potato starch, cooked	$110.7 \pm 6.0_{abc}$	116.0	$0.96 \pm 0.04_{bf}$	10.2
Series 2 (with 0.7% L-lysine)				
Potato starch, raw	$303.6 \pm 5.5_{d}$	282.4	$1.08 \pm 0.03_{e}$	24.9
Cornstarch	$320.0 \pm 4.9_{de}$	198.7	$1.61 \pm 0.07_{a}$	17.5
Sucrose	$322.0 \pm 11.3_{de}$	192.8	$1.67 \pm 0.06_{a}$	17.0
Dextrin	$322.8 \pm 6.0_{de}$	204.8	$1.58 \pm 0.04_{a}$	18.0
Glucose	$310.0 \pm 5.1_{de}$	201.7	$1.54 \pm 0.03_{a}$	17.7
Potato starch, cooked	$327.0 \pm 6.8_{e}$	197.1	$1.66 \pm 0.07_{a}$	17.3

[1] Eight rats/group.
[2] Nine weeks on experiment.
[3] Weight gain in grams/gram of protein eaten.
[4] Mean ± standard error.
* Numbers that do not contain the same subscript are statistically different at the 0.05 level.

rats fed the potato starch. Also, the rats fed this ration excreted white, bulky feces in large amounts.

Table 2 also shows the protein efficiency ratio for rats fed each of the experimental diets. These were determined by dividing the grams of protein eaten by the average gain in weight during a period of 9 weeks. Lysine supplementation in the diets containing cornstarch, sucrose, dextrin, glucose, and cooked-potato starch greatly increased the protein efficiency ratio for rats. With lysine supplementation, the protein efficiency ratio for rats fed raw-potato starch was found to be the lowest ($P = 0.05$) of all the groups, whereas the other carbohydrates tested were similar in protein-efficiency ratio. Without lysine supplementation, the protein-efficiency ratio for rats fed raw-potato starch was lower than that for the dextrin, glucose, and cooked-potato starch groups ($P = 0.05$), and the protein-efficiency ratio for rats fed sucrose was lower than that for the rats fed glucose and cooked-potato starch.

The digestibility of protein as affected by the various carbohydrates is shown in table 3. Rats fed raw-potato starch excreted larger amounts of fecal-food nitrogen and of metabolic nitrogen. Such nitrogen excreted by the rats fed cooked-potato starch and dextrin was about one-half as much as the excreted nitrogen of those rats fed raw-potato starch and twice as much as the excreted nitrogen of the rats fed cornstarch, sucrose, and glucose. As a result of the large amount of fecal-nitrogen excretion, the digestibility of protein was lowest when the rats were fed the diet containing raw-potato starch. The protein was slightly more digestible when the rats were fed diets containing cooked-potato starch and dextrin. The highest protein digestibility ($P = 0.05$) occurred when the rats were fed sucrose, glucose, or cornstarch. Lysine supplementation greatly increased the digestibility of protein in rats fed raw-potato starch and slightly increased the digestibility of protein in rats fed cooked-potato starch and dextrin, but did not noticeably affect the digestibility of protein when the other three carbohydrates were used.

The lysine availability for rats fed various carbohydrate diets is shown in table 4. In both series, when raw-potato starch was used as the source of carbohydrate in the diets, the lysine availability was low, particularly when the diets were not supplemented with L-lysine. The lysine availability for rats fed cooked-potato starch

TABLE 3

Digestibility of protein by rats fed various carbohydrates

Carbohydrate sources	Total N intake	Total N in feces	Metabolic fecal N	Fecal food N[2]	Total N absorbed[3]	True digestibility of protein[4]
	gm	*gm*	*gm*	*gm*	*gm*	%
Series 1 (without lysine)						
Potato starch, raw	2.494	0.518	0.197	0.321	2.173	$87.13 \pm 0.58^{5}{}_{c}$*
Cornstarch	2.136	0.118	0.047	0.071	2.065	$96.68 \pm 0.10_{b}$
Sucrose	2.348	0.101	0.038	0.063	2.285	$97.31 \pm 0.18_{b}$
Dextrin	3.012	0.225	0.059	0.166	2.846	$94.50 \pm 0.05_{a}$
Glucose	2.902	0.124	0.037	0.087	2.815	$97.00 \pm 0.13_{b}$
Potato starch, cooked	2.254	0.258	0.105	0.153	2.101	$93.21 \pm 0.25_{a}$
Series 2 (with 0.7% L-lysine)						
Potato starch, raw	4.218	0.639	0.374	0.265	3.953	$93.72 \pm 0.70_{a}$
Cornstarch	3.138	0.161	0.069	0.092	3.046	$97.06 \pm 0.08_{b}$
Sucrose	3.476	0.173	0.081	0.092	3.384	$97.35 \pm 0.17_{b}$
Dextrin	3.381	0.259	0.131	0.128	3.253	$96.21 \pm 0.05_{b}$
Glucose	3.466	0.159	0.062	0.097	3.369	$97.20 \pm 0.08_{b}$
Potato starch, cooked	3.487	0.331	0.144	0.187	3.300	$94.64 \pm 0.37_{a}$

[1] Five-day period.
[2] Fecal food N = total N in feces – metabolic N.
[3] Total N absorbed = total N intake – fecal food N.
[4] True digestibility of protein = $\frac{\text{total N absorbed}}{\text{total N intake}}$.
[5] Mean ± standard error.
* Numbers that do not contain the same subscript are statistically different at the 0.05 level.

TABLE 4

Lysine availability for rats fed various carbohydrate diets[1]

Carbohydrate sources	Total lysine intake	Total lysine in feces	Metabolic fecal lysine	Fecal food lysine[2]	Total lysine absorbed[3]	Total lysine availability[4]
	mg	*mg*	*mg*	*mg*	*mg*	%
Series 1 (without lysine)						
Potato starch, raw	238	119	49	70	168	$70.5 \pm 6.1^{5}{}_{d}$*
Cornstarch	193	31	10	20	173	$89.5 \pm 1.0_{b}$
Sucrose	200	23	7	16	184	$91.9 \pm 1.6_{b}$
Dextrin	273	72	28	45	229	$83.6 \pm 3.0_{a}$
Glucose	224	24	9	15	209	$93.3 \pm 0.8_{bc}$
Potato starch, cooked	208	73	37	36	172	$82.5 \pm 1.6_{a}$
Series 2 (with 0.7% L-lysine)						
Potato starch, raw	1185	156	81	76	1110	$93.6 \pm 1.0_{bc}$
Cornstarch	848	50	21	29	814	$96.6 \pm 0.6_{c}$
Sucrose	980	58	19	39	941	$96.0 \pm 0.9_{c}$
Dextrin	944	103	40	62	882	$93.4 \pm 1.0_{bc}$
Glucose	1010	47	14	33	977	$96.7 \pm 0.3_{c}$
Potato starch, cooked	1023	116	40	77	946	$92.5 \pm 0.3_{bc}$

[1] Five-day period.
[2] Fecal food lysine = total lysine in feces – metabolic fecal lysine.
[3] Total lysine absorbed = total lysine intake – fecal food lysine.
[4] True lysine availability = $\frac{\text{total lysine absorbed}}{\text{total lysine intake}}$.
[5] Mean ± standard error.
* Numbers that do not contain the same subscript are statistically different at the 0.05 level.

and dextrin diets was higher than that of the raw-potato starch groups, whereas the lysine availability for rats fed cornstarch, sucrose, and glucose diets was the highest ($P = 0.05$) of all groups. Lysine supplementation increased the lysine availability in rats fed diets containing raw-potato starch, cornstarch, sucrose, dextrin, and glucose.

DISCUSSION

Since lysine supplementation did not produce much improvement in the digestibility of protein in wheat gluten for rats fed most of the carbohydrate diets but markedly increased their growth responses, it appears that the added lysine had an effect on the utilization of the other amino acids in wheat gluten. The increase in body weight of rats may have been caused by the added lysine combining with the other amino acids from wheat gluten to increase the formation of tissue protein. Previous studies in our laboratory[3] indicated that, although wheat gluten was low in methionine content, supplementation of methionine to a diet containing wheat gluten did not support better growth of the rats. This observation shows that lysine is the first limiting amino acid in wheat gluten.

The inferiority of sucrose to glucose and dextrin as shown by the slower growth rate of rats is in accord with many of the observations reported previously (Monson et al., '50; Harper et al., '53). Harper and Katayama ('53) postulated that the slower growth of rats fed sucrose in comparison with the growth rate of rats fed cornstarch was caused by the lower availability of certain amino acids. In the present study the digestibility of protein and the availability of lysine in rats fed sucrose and glucose were practically equal. Possibly the lower food intake of the sucrose group might be the principal factor that caused the slower growth of the rats.

The protein-efficiency ratio, the digestibility of protein, and the availability of lysine varied to a greater extent among the rats fed the 6 different carbohydrate diets without lysine supplementation than among the rats fed the same diets with lysine supplement. Evidently the nature of the carbohydrate affects the utilization of protein more markedly when the protein in the diets is of poor quality or when the protein is inadequate. Harper et al. ('53) reported that when rats were fed various carbohydrates with 9% of casein as the source of protein, sucrose was inferior to cornstarch by the criterion of growth rate. When the casein level in the diets was increased to 18%, there was no marked difference in growth responses of rats fed these two carbohydrates. In planning diets for underfed groups it may be important to consider not only the amount and quality of protein but the nature of the carbohydrate as well.

Experimenters have reported (Langworthy and Deuel, '20, '22) that certain starches, mainly derived from tubers, were more poorly utilized than the starch derived from cereals. Harper ('52) explained that the lower growth rate of rats fed a raw-potato starch diet was caused by the poor utilization of raw-potato starch and the decrease in the digestion of the dietary protein. Booher et al. ('51) and Jelinek et al. ('52) hypothesized that a heat-labile substance present in raw-potato starch, which was digestive-resistant, was responsible for stimulating peristalsis. As a result, the swift passage of food through the digestive canal caused diarrhea and low protein utilization. The data reported in this paper confirm these interpretations. The digestibility of protein was lowest in rats fed a diet containing raw-potato starch. When the potato starch was cooked, the digestibility of the protein was improved, the amount of feces excreted by the rats was reduced, and the diarrhea disappeared.

Rats fed the diet containing raw-potato starch would be expected to grow more slowly than the rats fed cooked-potato starch. Such, however, was not the case in the unsupplemented groups. The discrepancy in the growth of rats fed raw-potato starch may be explained by the compensating effect of their high food intake. The daily food intake of rats fed the diet containing raw-potato starch was 30% greater than that of rats fed the diet containing cooked-potato starch. The inclusion of indigestible raw-potato starch lowered the energy-to-protein ratio of

[3] Unpublished data.

the diet and made it essentially a higher protein diet. Thus more protein and lysine were available for the synthesis of tissue protein, making more rapid growth possible. When 0.7% of L-lysine was added to the diets, the total lysine ingested by the rats was augmented. Under such conditions, the rats fed raw-potato starch, despite their high food intake, showed a lower weight gain than the rats fed the cooked-potato starch diet.

The higher levels of nitrogen and lysine excreted in the feces of rats fed diets containing raw-potato starch, dextrin, and cooked-potato starch were caused partly by the low digestibility of the protein and partly by the great amount of metabolic nitrogen and lysine excreted by the rats fed these carbohydrates. If the metabolic fecal nitrogen and lysine are derived mainly from the intestinal secretions and intestinal microorganisms, it appears that the nature of the carbohydrate also has an effect on the excretion of nitrogen and amino acids in the feces of the rats. Similar results have been reported by Harper et al. ('52).

In table 4, it is apparent that the metabolic fecal lysine excreted by rats fed diets with lysine supplementation was higher than that excreted by rats fed diets without lysine supplementation. This may be explained by the fact that rats in the lysine-supplemented groups are larger animals than those in the unsupplemented groups. Since larger animals contributed more intestinal secretions and intestinal microorganisms, the feces of the experimental rats contained a larger amount of lysine. When L-lysine was included in the diets, the lysine intake of rats in various groups was greatly augmented; the fecal-food lysine excreted by rats in such groups was only slightly increased. This indicated that the added lysine was almost completely absorbed by rats regardless of the source of the carbohydrate. The increase in lysine availability for rats with lysine supplementation may have been caused by the larger doses of lysine ingested by the rats rather than by any change in metabolic effect.

SUMMARY

The effect of various carbohydrates (raw-potato starch, cornstarch, sucrose, dextrin, glucose, and cooked-potato starch) on utilization of dietary protein as well as the availability of lysine from wheat gluten were determined in rats.

Without lysine supplementation, the digestibility of protein by rats fed raw-potato starch was 87% and by those fed cooked-potato starch, 93%. With the other three sources of carbohydrates tested, the digestibility of wheat gluten-protein ranged from 94 to 97%. With the addition of 0.7% of L-lysine to the diets, the digestibility of the protein for the rats fed raw-potato starch was 94%, for the rats fed cooked-potato starch, 95%, and for those fed dextrin, 96%. There was no significant change in the digestibility of the protein with L-lysine supplementation when the rats were fed the other three carbohydrates tested.

Raw-potato starch, cooked-potato starch, dextrin, sucrose, cornstarch, and glucose, without the addition of lysine, had different effects on the availability of lysine. The availability of lysine increased in the order indicated. With the addition of 0.7% of L-lysine, the lysine availability in the rats fed cornstarch, sucrose, and glucose was similar and was considerably higher than the lysine availability in the rats fed raw-potato starch, cooked-potato starch, and dextrin.

Lysine supplementation increased by lysine availability, weight gain, food intake, and protein efficiency ratio of rats fed all sources of carbohydrates.

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Investigation of Calcium, Phosphorus and Vitamin D_3 Relationships in Rats by Multiple Regression Techniques[1]

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Many experiments have been conducted to determine the relationships of calcium, phosphorus, and vitamin D in animal nutrition, and physiology. Most of the work has been concerned more with qualitative than quantitative information. The complex relationships of calcium, phosphorus, and vitamin D have been reviewed by Wasserman ('60). The Ca/P ratio is important but the absolute levels of these elements in the diet must also be studied. It was concluded that there is no simple relationship between the metabolism of calcium and phosphorus although the interaction of these elements in certain instances can be explained primarily on the basis of physico-chemical concepts. This relationship becomes exceedingly complex as biological factors are introduced into a given system.

Mraz ('61) studied dietary calcium, phosphorus, and vitamin D_3 effects upon the metabolism of Ca^{45}, P^{32}, and Sr^{85} in chicks. A central composite experimental design was used where these three dietary ingredients were varied over a wide range. However, the statistical and mathematical possibilities inherent in the design (Box and Wilson, '51; Box, '54) were not fully explored.

The primary objectives of this investigation were to quantitatively relate dietary calcium, phosphorus, and vitamin D_3 to certain physiological responses and to determine the combination of these factors for optimal absorption, retention, and growth in young laboratory rats. A secondary objective was to check the validity of the use of mathematical techniques for describing physiological responses and for finding optimal conditions in animal experiments.

EXPERIMENTAL

Ninety weanling male rats, 21 to 25 days of age, were randomly assigned in two groups of three each to each of 15 treatments. The rats were fed ad libitum for 5 days a basal ration containing 0.5% of calcium, 0.4% of phosphorus, and 3,000 IU of vitamin D_3/kg of diet (table 1).

TABLE 1

Basal purified ration

	%
Casein	20
Cornstarch	60
Cellulose	3
Corn oil	4
Mineral mixture (Ca and P-free)[1]	2
Vitamin mixture (vitamin D-free)[2]	1
Additive (Ca, P, and vitamin D_3)[3]	10

[1] Mineral mixture described and used by Hansard et al. ('51).

[2] Vitamin mixture is a modification of the one used by Mraz and Patrick ('57) in that vitamin D was deleted.

[3] The additive contained the desired levels of calcium, phosphorus, and vitamin D_3 plus enough cornstarch to bring the weight to 10% of the total mixture. Calcium and phosphorus were added in the form of $CaCO_3$, KH_2PO_4, $CaHPO_4$, and $CaH_4(PO_4)_2 H_2O$. KCl was added to some rations to keep the potassium content of all diets constant. Vitamin D was added in the form of a vitamin D_3 oil.

At the end of this period, the animals were placed on 15 different treatments (table 2) for a three-week experimental period. In this experiment, calcium was varied from 0.125 to 2.0%, phosphorus from 0.1 to 1.6%, and vitamin D_3 from 30 to 300,000 IU/kg of ration (table 2).

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TABLE 2

Dietary variables and code values

Ration no.	X_1 Calcium		X_2 Phosphorus		X_3 Vitamin D	
	Dietary level	Code	Dietary level	Code	Dietary level	Code
	%		%		*IU/kg*	
1	0.25	−1	0.2	−1	3×10^2	−1
2	1.00	1	0.2	−1	3×10^2	−1
3	0.25	−1	0.8	1	3×10^2	−1
4	1.00	1	0.8	1	3×10^2	−1
5	0.25	−1	0.2	−1	3×10^4	1
6	1.00	1	0.2	−1	3×10^4	1
7	0.25	−1	0.8	1	3×10^4	1
8	1.00	1	0.8	1	3×10^4	1
9	0.50	0	0.4	0	3×10^3	0
10	0.125	−2	0.4	0	3×10^3	0
11	2.00	2	0.4	0	3×10^3	0
12	0.50	0	0.1	−2	3×10^3	0
13	0.50	0	1.6	2	3×10^3	0
14	0.50	0	0.4	0	3×10^1	−2
15	0.50	0	0.4	0	3×10^5	2

The levels of dietary variables (coded as —2, −1, 0, 1, 2) of calcium and phosphorus were doubled from one level to the next. Each successive level of vitamin D_3 was 10 times as much as the previous level. The equations for interpolation are given in footnote 2.[2] One dietary level to the next was assumed to result in an equal physiological response although not necessarily a linear one. Using the coded values, a multiple regression model was constructed to estimate linear, quadratic, and interaction effects.[3]

These 15 treatments form a central composite design as described by Box and Wilson ('51). Treatments 1 through 8 form a 2^3 factorial experiment and the additional treatments 9 through 15, form a fractional 3^3 factorial experiment. With this design, good estimates can be made of the main effects, the two factor interactions, and the quadratic effects. It is assumed that the quadratic × quadratic (4th degree) effects found in a complete 3^3 factorial experiment are negligible, as also may be the linear × quadratic and quadratic × linear (3rd degree) effects (Cragle et al., '55).

Sufficient numbers of animals were not available to conduct the entire experiment at one time. Therefore, groups of animals were used in parts of the total experiment at 4 different times over a three-month period. No additional variation was apparent due to this procedure. Weekly weights were taken of all animals and at the end of the three-week experimental period, each group of three animals was placed in a metabolism cage as described by Hansard and Comar ('53). They were allowed 24 hours to adjust to the cage and were then dosed orally with 40 μc of Ca^{45} and 20 μc of P^{32} given simultaneously in 1 ml of solution. A 24-hour metabolism period was then conducted during which the feces and urine were collected separately. At the end of the metabolism period, the animals were anesthetized with ether, bled by a heart puncture, killed, and sampled for liver, kidney, muscle, and femur.

The percentages of uptake of Ca^{45} and P^{32} were measured in liver, kidney, muscle, femur, and blood. The percentages of Ca^{45} and P^{32} excreted in urine and eliminated in feces were measured. Femur ash per cent was determined on both a wet and dry basis. Total calcium and phosphorus were determined in femur and blood.

[2] The following equations may be used to convert dietary values to code values:

$$\log \text{dietary Ca } \% = 0.301X - 0.301$$
$$\log \text{dietary P } \% = 0.301X - 0.389$$
$$\log \text{IU vitamin } D_3/\text{kg of feed} = X + 3.477$$

where X is the coded value.

[3] The model was:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3$$

where Y is the estimated response, the b's are the partial regression coefficients and x_1, x_2 and x_3 are the dietary code values.

The Ca^{45} and P^{32}, both beta emitters, were counted with an end-window Geiger-Müller tube. Ca^{45} was prepared for counting by the oxalate procedure as described by Comar et al. ('51). P^{32} was determined by a wet counting procedure using an aluminum absorber to shield out the beta particles of Ca^{45}. Stable calcium was determined by the complexometric titration as described by Kamal ('60). Phosphorus was determined by the colorimetric analysis of Fiske and SubbaRow ('25).

All data were subjected to analysis of variance procedures.[4] Response equations, which were estimates of all first and second degree effects, were calculated for 18 different responses. These equations were solved for a maximum or minimum if one existed within the system.[5]

RESULTS

The results are presented in the following sequence. (1) The observed data are presented (table 3). (2) Equations describing the various phenomena were derived from the original data. The constants and standard errors of constants for these equations are presented in table 4. (3) The responses are predicted by use of the response equations and the values are compared with the observed values (table 3). In those instances where it is evident that the equations fit the actual data, curves are drawn which describe responses under a number of arbitrary conditions and in greater detail than would be possible from the unprocessed actual data (figs. 1 and 2).

Response equations are of considerable importance in predicting responses for any untried combination of experimental variables within the limits of the experimental design. The net result is an economy in the number of experiments that must be conducted, and flexibility in making predicted responses. If a statistically significant deviation from regression of the model from the experimental data exists,[6] the validity of these predictions is impaired but in most cases reasonable, reliable predictions can still be made. In this experiment, only Ca^{45} and P^{32} in both fecal elimination and urinary excretion can be described by their respective response equations with nonsignificant deviation from regression.

The results presented in figure 1 are predicted responses derived through the use of the response equations. Vitamin D_3 was held at the zero code value, i.e., 3×10^3 IU of vitamin D_3/kg of feed, whereas either dietary calcium or phosphorus was varied over the entire experimental range. The mineral that was not being varied was held at the zero code value also, i.e., 0.5% of Ca or 0.4% of P. A deviation from this procedure was followed in figure 2. Both dietary Ca and P were varied simultaneously so that three Ca/P ratios as well as total intake of Ca and P could be observed.

The dietary effects of calcium and phosphorus upon the uptake of Ca^{45} and P^{32} in blood are illustrated in figure 1a. As dietary calcium increases, the Ca^{45} in blood decreases.[7] This decrease is more rapid at lower dietary levels and is practically zero

[4] The sums of squares for treatments were subdivided into linear, quadratic, interaction and deviation from regression components. The analysis of variance headings were as follows:

Source	Degrees of freedom			Sum of squares	Mean square	F value
Total						
Mean	1					
Treatments	14					
Linear		3				
x_1			1			
x_2			1			
x_3			1			
Quadratic		3				
Interaction		3				
x_1x_2			1			
x_1x_3			1			
x_2x_3			1			
Deviation from regression		5				
Error						

The degrees of freedom for total and error, respectively, were 90 and 75 for all measurements on individual rats. For urine and feces data these values were 30 and 15, respectively, since calculations were made using cage averages.

[5] The maximum or minimum response point (stationary point) for each dependent variable (Y) is calculated by taking the derivatives of the equation of the response with respect to x_1, x_2, and x_3 and equating them to zero. Then, the equations with x_1, x_2, and x_3 are solved simultaneously to produce the estimated co-ordinates of the point. A stationary point may be represented by all maxima, maxima and minima, or all minima, responses of Y in relation to x_1, x_2, and x_3 (Box, '54).

[6] See footnote 4.

[7] Probability levels for a response being significant are purposely omitted in the written portion of results. All response relationships which are pointed out are significant ($P < 0.05$). However, in some cases significant linear, quadratic, and interaction effects are incorporated into a single response. These individual significances are indicated in table 4.

TABLE 3

Observed and predicted responses by rats to various calcium, phosphorus and vitamin D_3 diets

Responses	1[1]	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Blood Ca^{45}, $10^3 \times$ % dose/ml	21[2]	15	18	16	20	14	16	13	16	19	15	17	27	19	15
	19[3]	15	20	19	18	14	17	15	15	19	13	19	23	19	15
Blood P^{32}, $10^2 \times$ % dose/ml	10.4	8.0	6.2	6.8	7.7	8.9	6.6	6.7	9.4	8.3	14.2	9.8	9.2	7.7	8.8
	9.3	9.4	7.1	8.2	8.0	9.8	6.9	9.6	7.7	9.0	11.8	9.8	7.4	7.3	7.5
Blood P, mg/100 ml	44	47	48	51	45	47	44	43	48	44	37	42	48	46	48
	43	44	49	49	47	45	46	43	49	41	40	44	47	48	46
Feces Ca^{45}, % dose	1.0	5.6	0.5	7.3	0.4	5.5	0.6	15	14	1.2	9.1	8.7	3.6	9.7	6.5
	6.7	7.7	1.8	8.4	2.6	7.6	2.0	13	10	−2.3	9.3	4.4	4.5	6.4	6.5
Feces P^{32}, % dose	1.9	4.9	0.6	5.3	1.0	4.4	0.9	11.0	5.3	0.7	1.0	3.1	2.1	4.4	3.7
	4.4	3.6	0.9	4.3	1.6	3.6	1.7	7.9	5.8	−1.6[4]	3.8	2.4	3.3	4.0	4.7
Urine Ca^{45}, % dose	0.20	6.5	0.06	0.58	0.33	5.6	0.14	0.7	0.33	0.10	7.4	3.5	0.67	0.41	2.2
	−0.34	5.9	−0.06	0.97	0.02	5.8	0.79	1.4	0.25	0.30	7.1	4.1	−0.05	0.87	1.6
Urine P^{32}, % dose	5.8	0.6	14	9.1	5.7	0.42	25	12	10	15	0.23	0.17	14	8.7	12
	6.2	3.1	14	7.1	7.6	−0.01	22	11	10	15	0.40	−2.10	17	8.0	13
Liver Ca^{45}, $10^2 \times$ % dose/gm	1.3	1.2	1.5	0.85	1.20	0.85	1.0	0.80	0.99	1.1	1.1	0.94	1.7	1.1	0.69
	1.2	1.0	1.4	1.10	0.91	0.86	1.1	0.96	—[5]	—	—	—	—	—	−0.08
Liver P^{32}, % dose/gm	0.79	0.74	0.56	0.60	0.75	0.82	0.69	0.69	0.83	1.00	1.3	1.00	0.79	0.89	0.77
	0.85	0.92	0.68	0.76	0.80	0.90	0.71	0.83	0.63	0.98	1.2	0.91	0.67	0.73	0.73
Kidney Ca^{45}, $10^3 \times$ % dose/gm	21	14	32	24	20.00	11.0	25	48	17.0	23	28	20	250	19.0	19.0
	11	−2	63	65	0.17	1.5	63	79	−5.0	13	16	58	190	6.6	9.6
Kidney P^{32}, % dose/gm	0.66	0.58	0.46	0.50	0.58	0.63	0.57	0.56	0.68	0.79	1.0	0.81	0.66	0.70	0.67
	0.70	0.72	0.55	0.61	0.64	0.70	0.59	0.68	0.51	0.77	0.9	0.73	0.57	0.59	0.60
Muscle Ca^{45}, $10^2 \times$ % dose/gm	2.1	1.3	1.9	1.1	2.8	1.4	1.9	1.1	1.6	2.0	1.6	1.4	2.4	1.6	1.3
	1.8	1.3	2.0	1.6	2.2	1.4	1.9	1.3	1.6	2.4	1.2	1.9	2.0	1.4	1.5
Muscle P^{32}, % dose/gm	0.24	0.26	0.18	0.18	0.23	0.26	0.22	0.19	0.25	0.27	0.43	0.33	0.24	0.29	0.27
	0.25	0.32	0.20	0.23	0.24	0.29	0.23	0.24	0.19	0.28	0.36	0.30	0.20	0.25	0.25
Femur Ca^{45}, % dose/femur	4.1	2.3	3.9	2.3	4.7	2.6	3.3	2.0	2.9	3.6	2.5	3.2	3.2	2.9	3.0
	3.6	2.3	3.6	2.7	4.2	2.8	3.2	2.3	3.0	4.2	2.0	3.6	3.0	3.0	3.1
Femur P^{32}, % dose/femur	1.9	1.5	1.4	1.4	1.9	2.0	1.7	1.7	2.2	2.2	2.7	2.6	1.7	2.3	2.0
	2.1	2.1	1.7	1.7	2.1	2.2	1.7	1.9	1.7	2.1	2.3	2.3	1.6	1.9	2.0
Bone ash, %, dry-matter basis	45	49	45	54	48	50	45	51	47	39	45	46	45	49	48
	45	48	44	52	47	48	44	49	49	39	47	47	46	50	49
Bone ash, %, wet basis	21	24	22	28	22	25	22	26	23	17	22	23	22	25	25
	21	24	22	26	23	24	21	25	24	17	23	22	23	25	25
Avg weekly gain, gm	31	28	30	31	32	31	30	33	31	31	19	34	20	32	34
	34	28	29	27	35	31	29	29	33	29	23	31	24	33	35

[1] Ration number.
[2] Observed experimental value.
[3] Predicted value.
[4] Negative values should be considered as zero.
[5] No value was obtained because of print out failure.

TABLE 4

Response equations, coefficients of variation and standard errors of constants for dietary responses in rats[1]

Responses	b_0	Standard error b_0	Linear b_1	b_2	b_3	Standard error b_1, b_2, b_3	Quadratic[2] b_{11}	b_{22}	b_{33}	Standard error b_{11}, b_{22}, b_{33}	Interaction b_{12}	b_{13}	b_{23}	Standard error b_{12}, b_{13}, b_{23}	Coefficients of variation
Blood Ca^{45}, 10^3 × % dose/ml	14.75	2.961	−1.48[4]	−0.80	−1.04[3]	0.839	0.38	1.56[3]	0.48	1.009	0.68	−0.13	−0.54	1.188	34.02
Blood P^{32}, 10^2 × % dose/ml	7.69	2.139	0.71[4]	−0.61[3]	0.04	0.607	0.68	0.23[3]	−0.07	0.729	0.23	0.41	0.26	0.858	20.13
Blood phosphorus, mg/100 ml	49.23	2.864	−0.42[3]	0.86[4]	−0.41[3]	0.813	−2.19	−0.88[4]	−0.52	0.976	−0.23	−0.61[3]	−0.20[4]	1.148	13.13
Feces Ca^{45}, % dose	10.32	4.135	2.91[4]	0.03	0.03	1.172	−1.71	−1.47	−0.97	1.408	1.42	1.01	1.06	1.658	77.43
Feces P^{32}, % dose	5.76	2.368	1.36[4]	0.21	0.18	0.672	−1.16	−0.73	−0.36	0.807	1.04	0.71	0.91	0.949	63.55
Urine Ca^{45}, % dose	0.25	0.717	1.71[4]	−1.05[4]	0.19	0.203	0.87	0.45[4]	0.25	0.243	−1.31[4]	−0.12	0.12	0.287	46.11
Urine P^{32}, % dose	10.11	2.265	−3.60[4]	4.67[4]	1.30	0.643	−0.63	−0.71	0.13	0.773	−0.93	−1.11	1.75	0.908	65.73
Liver Ca^{45}, 10^2 × % dose/gm	0.98	1.195	−0.09[3]	0.08[3]	−0.11[4]	0.339	0.03	0.08[3]	−0.02	0.409	−0.04	0.03	0.00	0.480	19.74
Liver P^{32}, % dose/gm	0.63	0.185	0.05	−0.06[3]	0.00	0.055	0.11	0.04[4]	0.03	0.064	0.00	0.01	0.02	0.071	21.65
Kidney Ca^{45}, 10^3 × % dose/gm	−4.97	43.079	0.76	32.45[4]	0.75	12.217	4.96	31.95[4]	3.26	14.683	3.77	3.68	2.65	17.278	118.82
Kidney P^{32}, % dose/gm	0.51	0.151	0.03	−0.04[3]	0.00	0.045	0.08	0.03[4]	0.02	0.055	0.01	0.01	0.03	0.064	18.87
Muscle Ca^{45}, 10^2 × % dose/gm	1.58	0.495	−0.29[4]	0.02	0.02	0.142	0.05	0.09	−0.03	0.169	0.06	−0.08	−0.11	0.200	38.43
Muscle P^{32}, % dose/gm	0.19	0.063	0.02[3]	−0.03[4]	0.00	0.055	0.03	0.02[3]	0.02	0.020	−0.01	0.00	0.01	0.025	28.24
Femur Ca^{45}, % dose/femur	2.99	0.516	−0.56[4]	−0.15[4]	0.02	0.145	0.03	0.07	0.00	0.177	0.11	−0.02	−0.23	0.207	20.24
Femur P^{32}, % dose/femur	1.68	0.454	0.04	−0.18[4]	−0.03	0.131	0.14	0.06[3]	0.06	0.155	0.03	0.06	0.01	0.182	15.94
Bone ash, %, dry-matter basis	49.23	2.397	2.07[4]	0.09	−0.15	0.680	−1.48	−0.65[4]	0.07	0.817	0.99[3]	−0.56	−0.82	0.961	6.69
Bone ash, %, wet basis	24.16	1.384	1.60[4]	0.31	−0.07	0.392	−1.01	−0.33[4]	0.27	0.471	0.57[3]	−0.32	−0.54	0.555	8.19
Growth, gm/week	32.56	4.133	−1.48[4]	−1.68[4]	0.63	1.172	−1.62	−1.24[4]	0.31	1.408	1.06	0.41	−0.43	1.656	15.45

[1] Response equation $Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3$.

[2] The quadratic terms are given a group significance since an orthogonal break out of individual components is not possible.

[3] $P < 0.05$.

[4] $P < 0.01$.

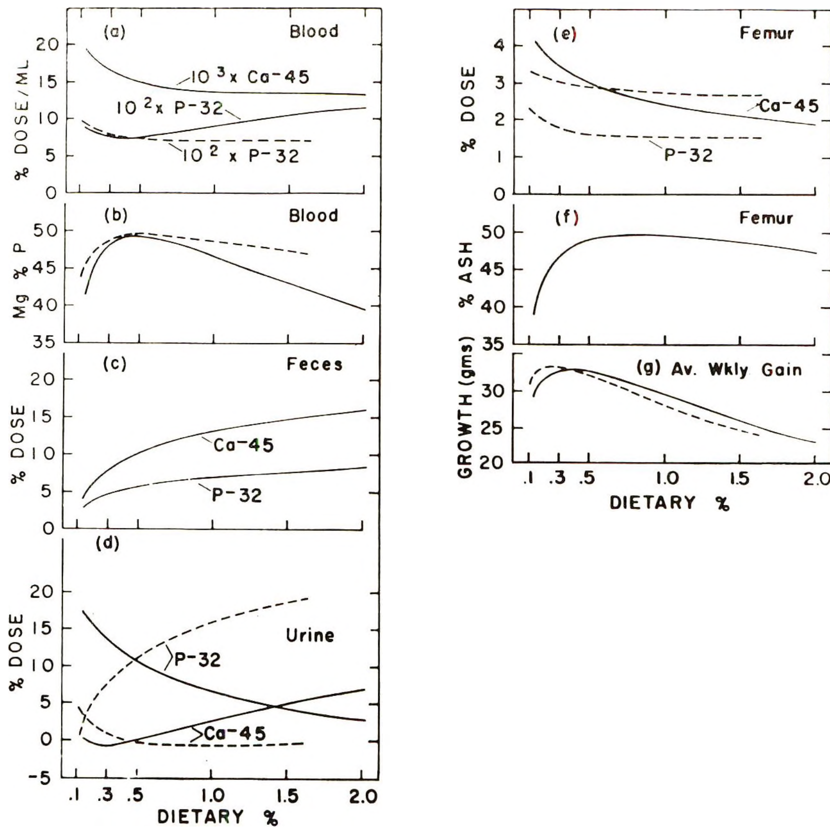

Fig. 1 Predicted relationships derived through the use of response equations. Dietary vitamin D_3 was held at 3×10^3 IU/kg of feed while the effects of the other variables were observed. Dietary calcium or phosphorus was held at 0.5 or 0.4%, respectively, while the other was varied. Legend: ---- indicates dietary phosphorus; ——— indicates dietary calcium.

at the higher levels. Increased calcium causes an increase in blood P^{32}. However, at levels lower than 0.5%, the reverse of this is observed. Increased dietary phosphorus causes a decline in blood P^{32} at lower dietary levels with no response noted at levels above 0.8%. The effects of dietary calcium and phosphorus upon blood phosphorus are illustrated in figure 1b. At higher dietary levels of calcium and phosphorus, calcium expresses the greatest depressing effect.

The effects of dietary calcium upon fecal elimination are demonstrated in figure 1c. As dietary calcium increases, both Ca^{45} and P^{32} elimination increases. Figure 1d demonstrates the dietary effects upon urinary excretion. The urinary excretion for Ca^{45} is much lower than P^{32} at normal dietary levels. At higher dietary levels of calcium, the urinary excretion of Ca^{45} and P^{32} is in the same range. The urinary excretion of Ca^{45} is complex and can only be described through linear, quadratic, and interaction response equation terms involving dietary calcium and phosphorus. The slightly negative values for Ca^{45} excretion are equation prediction errors. The results for these ranges should be considered as zero excretion.

The increase of dietary calcium and phosphorus from the lower experimental levels to higher levels caused a decrease in the amount of Ca^{45} deposited in the femur as shown in figure 1e. Increased dietary calcium causes the greatest decrease on the bone deposition of Ca^{45}. Only dietary phosphorus had an effect on P^{32} deposition. This effect was expressed only at low dietary levels with the Ca/P ratio

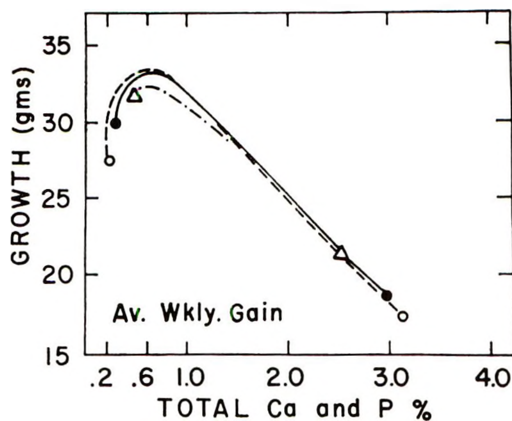

Fig. 2 Predicted effects of Ca/P ratio and total intake upon growth. Dietary vitamin D_2 was held at 3×10^3 IU/kg of feed. Both calcium and phosphorus were varied so that intake and ratio could be studied. Legend: △·–·–·△ indicates Ca/P 0.6; ○– – –○ indicates Ca/P 1.25; ●——● indicates Ca/P 2.0.

< 1. Analysis of stable calcium and phosphorus in bone ash showed no significant difference due to treatment. The dietary effects of dietary calcium on bone ash are shown in figure 1f. Dietary phosphorus has no significant effect upon the percentage of ash in bone. However, there is a rapid increase in bone ash as the calcium level approaches 0.5% or as the Ca/P ratio approaches 1.25. The maximal percentage of bone ash was calculated to occur when dietary calcium and phosphorus levels were 0.67 and 0.5%, respectively, or a ratio of 1.3.

Dietary calcium and dietary phosphorus express a similar effect on growth (fig. 1g). It is indicated by the regression coefficients in table 4 that calcium and phosphorus will be solved for a maximum response and vitamin D_3 for a minimum response of Y at the stationary point. The combination of calcium and phosphorus at this point results in an estimated maximal growth response. Changes in the amount of vitamin D_3, higher or lower than 348 IU, results in a slight increase in growth which was found to be nonsignificant. Maximal growth was obtained with 0.27% of calcium and 0.21% of phosphorus in the diet or a Ca/P ratio of 1.3. The value of Y at these combinations gave an estimated response of 34 gm. Figure 2 demonstrates the effects of the total intake of calcium and phosphorus as well as the ratio of the two dietary variables. As the total intake of calcium and phosphorus increases above 0.9%, a sharp decrease in growth is observed. This is similar to the effects observed in figure 1g where only dietary calcium or phosphorus was varied. The Ca/P ratio is of minor importance in comparison with the total amount of salt.

An increase of vitamin D_3 in the diet had a negative effect upon blood Ca^{45}, stable blood phosphorus and on liver uptake of Ca^{45}. Interactions between vitamin $D_3 \times$ calcium and vitamin $D_3 \times$ phosphorus resulted in negative effects upon stable blood phosphorus. An interaction between vitamin $D_3 \times$ phosphorus resulted in a negative effect on femur Ca^{45} uptake. No other significant effects of dietary vitamin D_3 were noted.

DISCUSSION

The responses to dietary calcium, phosphorus and vitamin D are not the result of the actual amount of diet consumed. All diets were fed ad libitum and it was observed that dietary intake was reduced when rations contained a high level of calcium and/or phosphorus. A decrease in dietary intake is a factor in the results expressed in figure 2.

The effects expressed by dietary calcium upon blood phosphorus cannot readily be explained. An explanation for the decreased stable phosphorus and increased P^{32} uptake when animals were fed high calcium levels may be through a competition between the cations (Ca^{++}) on the mucosal side of the gut with anions on the serosal side, one of these being ($PO_4^{=}$). Shelling ('32) showed that excess calcium is excreted through the kidneys until its concentration in the urine reaches a maximum and the remainder is excreted through the bowel as the insoluble phosphate salt, thus depleting the body of salt. Excess phosphorus follows a similar path, first through the kidneys, which are able to excrete phosphorus to a limited concentration, and then the remainder through the bowel as the calcium salt, thus removing calcium from the body. The results reported in this paper agree with those of Shelling ('32) with respect to calcium. However excess phosphorus does

not seem to affect the fecal elimination of Ca^{45}. As indicated in figure 1c dietary calcium has similar effects on Ca^{45} and P^{32}.

The results of this investigation support the conclusions of Wasserman ('60) that total calcium and phosphorus in the diet, as well as ratio must be taken into consideration. The ratio is of less importance when the total amount of calcium and phosphorus is increased to higher levels (fig. 2).

The effects of dietary vitamin D_3 on most of the responses were very low. This appears to be due to the conditions under which the experiment was conducted rather than a contradiction of other work. The animals were not depleted of body stores of vitamin D and they were exposed to fluorescent light for 12 hours each day. In addition, the small effects shown by vitamin D_3 can be explained by a low requirement at normal calcium and phosphorus levels. Wasserman ('60) reported that a Ca/P ratio close to unity with adequate calcium and phosphorus was satisfactory for growth and calcification to proceed in the absence of vitamin D.

One of the difficulties in biological research is the number of experimental points that have traditionally been conducted to describe responses involving more than one independent variable over wide ranges of each variable. In the experiment reported in this paper, methods are described to surmont this difficulty and to place the biologist in a more flexible position for making predictions and secondary calculations. An example of this system in use is a comparison of the urinary results observed in this study and those reported by Hansard and Plumlee ('54). They observed P^{32} urinary excretion values of 24 and 11% of the dose with diets of 0.013 and 0.5%, respectively, for calcium and 0.4% phosphorus. Responses calculated from the response equation for 0.125 (lower experimental limit) and 0.5%, respectively, for calcium and 0.4% phosphorus gave values of 17 and 10% of dose. Mathematical statements are the most precise means of stating these relationships and a systematic approach can add much to our understanding of biological processes.

In this experiment, the experimental error between duplicate samples within rats expressed as a coefficient of variation was approximately 5%. A comparison of this value with the coefficients of variation for the various measurements between rats readily gives an indication of the magnitude of the variation associated with a measurement exclusive of analytical variation. This variation is an important clue as to the nature of the system being measured. For example, the measurement of the percentage of bone ash has a coefficient of variation between rats of 6.7%. The mechanism involved can be thought of as a direct one not influenced appreciably by other mechanisms. However, in the case of Ca^{45} deposition in the kidney, where a coefficient of variation of 119%, exists, it is obvious that alternate metabolic possibilities exist (Box, '57).

SUMMARY

Ninety wealing male rats were divided into 15 groups and fed diets, ad libitum, containing varying levels of calcium (0.125 to 2.0%), phosphorus (0.1 to 1.6%), and vitamin D_3 (30 to 300,000 IU/kg of diet). The experimental design was a three dimensional central composite. Multiple regression equations were derived for 18 responses to diet. Actualy data were presented as well as values predicted by use of regression equations. A dietary content of calcium and phosphorus from 1.0 to 2.5% expresses a greater effect upon growth than ratios from 0.6 to 2.0. Estimated maximal growth was obtained with 0.27% of calcium and 0.21% of phosphorus in the diet or a Ca/P ratio of 1.3.

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Effect of Diet Fed During the Postnatal Period on Developing Rat Molars[1]

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There are at least two possible means whereby dietary alterations may influence the susceptibility of teeth to caries. One of these involves the influence of the diet (including water) in altering the composition of the teeth. The other covers the influence of the diet on the environment of the teeth including the oral flora as well as physico-chemical changes in the saliva. Admittedly both effects may occur simultaneously.

Presumably if the first of the preceding mechanisms is operative, one would ultimately expect to find analytical differences between the teeth of animals fed a cariogenic ration when compared with teeth of rats fed a noncariogenic ration. There are considerable data demonstrating that caries susceptibility in experimental animals can be influenced by the type of prenatal or early postnatal diets; however, there is no indication as to the effect of these diets upon the composition of the teeth.

The work of Sognnaes ('48, '55), McClure ('45), and numerous other investigators has repeatedly demonstrated that caries susceptibility in experimental animals can be significantly influenced by the type of prenatal or early postnatal diets. Mainly purified diets, high in mono- or disaccharides have been associated with the effects observed. In addition to the well documented literature, a number of review articles are available on the relationship between diet and caries susceptibility (Sognnaes, '48, '55; Shaw et al., '61; Shaw, '52). It is also generally accepted that dietary protein (Bavetta and McClure, '57) and certain mineral components have essential roles in the cariogenicity of experimental animal diets. It is thus evident that nutrition is a prime factor in the precipitation of dental caries in experimental animals but the mechanism is not clear.

Teeth are composed of both an inorganic mineral phase as well as an organic matrix. During the period of tooth development minerals are precipitated in the organic matrix to form the calcified structures of teeth. It is during this developmental period that the teeth would be most susceptible to nutritional factors. Sobel and Hanok ('48, '58) were able to vary the mineral composition of teeth during the period of active calcification by drastically altering the calcium and phosphorus ratio of the diet. Losee et al., ('57) reported that the level of dietary casein influenced both the weight of rat incisors and to a slight extent also their nitrogen content. Bhussry ('58) reported that the bucco-lingual enamel surfaces of sound human teeth had a higher density and lower nitrogen content than enamel from the mesiodistal surfaces which is more susceptible to caries.

The protein portion of the organic matrix of teeth has been reported by numerous investigators (Geller, '58) to belong to the collagen class. Histochemical and biochemical methods have also demonstrated the intimate association of mucopolysaccharides (ground substance) with this collagen.

In the present investigation a study was made of the influence of diet on the collagen, ground substance, hexosamine/collagen ratio, as well as total hexose in the teeth of rats offered a purified high carbohydrate ration as compared with

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those offered a natural diet from birth. The object of this work was to investigate the mechanism by which diet may influence susceptibility to caries. The data presented in this paper were from a second experiment, using a large number of animals, that confirmed the observations of an earlier pilot experiment.

EXPERIMENTAL

Experimental plan. Sixty litters of Holtzman rats were raised for this investigation. During breeding and prior to delivery all of the dams were fed a ground stock ration. At birth one-half of the litters were supplied with a purified ration complete in all known nutritional requirements and the other half of the litters continued to be fed the ground natural stock ration.[2] The purified ration had the following percentage composition: Casein,[3] 18.0; sucrose, 71.5; cottonseed oil, 5.0; HMW salt mix (Hubbel et al., '37), 5.0; choline chloride, 0.2; and L-cystine, 9.3. To each kilogram were added the following crystalline vitamins: (in milligrams) thiamine·HCl, 20; riboflavin, 20; pyridoxine ·HCl, 20; Ca pantothenate, 60; nicotinic acid, 100; ascorbic acid, 200; biotin, 4; folic acid, 10.0; *p*-aminobenzoic acid, 400; inositol, 800.0; α-tocopheryl acetate, 100; 2-methylnaphthoquinone, 5; vitamin B_{12}, 150 μg; vitamin A, 5,000 USP units; and vitamin D_2, 500 USP units. Animals kept on experiment longer than three weeks were weaned at 21 days of age and placed in individual wire-bottom cages. Records of litter weight were kept from birth so that the rate of growth of the two groups could be compared.

Animals from each group were killed at 21, 28, 35, and 42 days of age. After being sacrificed the molar tooth buds from each animal were thoroughly cleaned and then extracted with a dental explorer. All 4 first molars, all 4 second molars and all 4 third molars from each animal were pooled and analyzed. The teeth were dried to a constant weight and weighed to the nearest 0.1 mg.

Analytical techniques. The collagen of the teeth was determined after decalcification and hydrolysis by the method of Neuman and Logan ('50). The rate of glycine incorporation into the tooth collagen of the two groups was measured by the daily injections of 1 μc of glycine-C^{14} from 14 through 21 days of age. All animals were killed at the end of three weeks and their molars cleaned and extracted. They were then dried, weighed, and decalcified in 10% EDTA at pH 7.5 for 48 hours. The decalcified molars were then converted to gelatin by autoclaving them with water for 6 hours at 15 p.s.i. A portion of the gelatin solutions was plated on aluminum planchets and radioactivity was determined.

The total hexosamine content of the tooth buds of the two experimental groups was determined at the 28- and 42-day levels. A modification of the Rondle and Morgan procedure ('55) was used for the estimation of hexosamine. In addition the total hexose content was determined by the modified orcinol method of Bruchner ('55).

RESULTS

Growth. No significant difference in the growth of the weanling rats fed the two experimental diets was observed. The 6-week weight increment of the animals fed the purified diet was 155 gm, whereas those fed the natural ration gained 143 gm during the same period. The animals in both experimental groups gained between 35 and 40 gm the second week after weaning and between 35 and 45 gm the third week after weaning. This similarity in growth is of interest in view of the well-documented difference in caries susceptibility of the two groups and the differences in composition of the molar teeth reported in this paper. It thus appears that adequate growth is not necessarily a measure of dental health.

Tooth growth and development. The growth rate of the individual molar teeth as reflected by weight from the two experimental groups is shown in table 1. At 28 days of age all three molars from animals fed the purified diet were statistically heavier ($P = 0.001$) than the corresponding molars of those fed the natural diet. Although the actual differences in weight of the molars from the two groups

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

[3] Vitamin-free Test Casein, General Biochemicals, Chagrin Falls, Ohio.

was small, the differences observed were highly significant and were of interest in view of no significant differences in the body weights of the two groups. However, at 42 days of age this relationship was reversed and all three molars of the animals fed the natural diet were statistically heavier than the corresponding molars of those maintained fed the purified diet.

Collagen content. There is no significant difference between the collagen content of the first molars of the two dietary groups at 21, 28 and 35 days of age (table 2). The first molars of the animals fed the purified diet at 42 days of age contained slightly more collagen than those receiving the natural diet. This may be considered as being of borderline sig-

TABLE 1

Mean dry tooth bud weights of weanling rats at different age levels offered a purified high carbohydrate diet in comparison with those offered a natural stock ration from birth[1]

Days of age	Tooth bud	Purified ration	Natural diet	P value
		mg	*mg*	
21[2]	1st molar	37.1 ± 0.4[3]	35.8 ± 0.3	$<0.01>0.001$
	2nd molar	25.3 ± 0.2	24.5 ± 0.3	$<0.05>0.02$
	3rd molar	9.6 ± 0.4	8.3 ± 0.4	$<0.05>0.02$
28[4]	1st molar	44.2 ± 0.4	42.0 ± 0.2	0.001
	2nd molar	30.2 ± 0.3	28.8 ± 0.2	0.001
	3rd molar	13.3 ± 0.2	11.6 ± 0.2	0.001
35[5]	1st molar	45.5 ± 0.7	44.2 ± 0.6	n.s.
	2nd molar	31.7 ± 0.4	30.3 ± 0.2	0.02
	3rd molar	17.5 ± 0.2	15.1 ± 0.3	0.001
42[6]	1st molar	49.4 ± 0.5	51.6 ± 0.3	0.001
	2nd molar	33.6 ± 0.3	34.8 ± 0.2	$<0.01>0.001$
	3rd molar	20.0 ± 0.2	20.9 ± 0.4	<0.05

[1] Values are the mean dry weight of the 4 individual molars/rat in each group.
[2] Includes 30 animals fed purified ration and 29 animals fed the natural diet.
[3] Standard error of the mean.
[4] Includes 15 animals fed purified ration and 18 animals fed the natural diet.
[5] Includes 23 animals fed purified ration and 25 animals fed the natural diet.
[6] Includes 24 animals fed purified ration and 23 animals fed the natural diet.

TABLE 2

Collagen content in the tooth buds of weanling rats at different age levels, offered a purified high carbohydrate ration in comparison with those offered a natural stock ration from birth

Days of age	Tooth bud	Purified ration	Natural diet	P value
		mg collagen/100 mg dried tooth		
21[1]	1st molar	8.9 ± 0.2[2]	8.8 ± 0.2	$>0.1<0.5$
	2nd molar	8.5 ± 0.2	8.7 ± 0.2	0.5
	3rd molar	11.9 ± 0.2	10.7 ± 0.3	0.001
28[3]	1st molar	9.1 ± 0.3	8.7 ± 0.1	$>0.1<0.5$
	2nd molar	9.0 ± 0.2	8.8 ± 0.3	0.5
	3rd molar	8.7 ± 0.2	7.6 ± 0.3	0.001
35[4]	1st molar	9.7 ± 0.1	9.6 ± 0.1	0.5
	2nd molar	10.1 ± 0.1	9.9 ± 0.2	0.5
	3rd molar	10.8 ± 0.2	9.2 ± 0.2	0.001
42[5]	1st molar	11.5 ± 0.1	11.0 ± 0.1	$<0.02>0.01$
	2nd molar	9.8 ± 0.2	9.8 ± 0.2	n.s.
	3rd molar	9.3 ± 0.1	9.1 ± 0.1	$<0.5>0.1$

[1] Includes 13 animals fed purified ration and 13 animals fed the natural diet.
[2] Standard error of the mean.
[3] Includes 14 animals fed purified ration and 15 animals fed the natural diet.
[4] Includes 23 animals fed purified ration and 25 animals fed the natural diet.
[5] Includes 24 animals fed purified ration and 22 animals fed the natural diet.

nificance. There was no significant difference between the collagen content of the second molars of the two dietary groups at any age level investigated.

However, in contrast with the observations for the first and second molars there is a highly significant difference in the collagen content of the third molars of the two groups at 21, 28 and 35 days of age. The third molars of the animals fed the purified diet contained a statistically greater amount of collagen than those fed the natural diet. This may be related to the reported greater susceptibility of the third molars to decay (Shaw et al., '44).

Hexosamine and hexose content of tooth buds. All three molars of the animals fed the natural diet at 28 days of age contained a statistically greater amount of hexosamine than the molars of the animals supplied with the purified diet (table 3). At 42 days of age there was no significant difference in the hexosamine content of the first and second molars of the two groups; however, the third molars of the animals fed the purified diet contained statistically more hexosamine than the third molars of the animals supplied with the natural stock ration. The hexosamine findings at 28 days of age showed the reverse of the collagen picture at the same age level. A significant decrease was observed in the hexosamine content of the tooth buds at 42 days of age in comparison with the 28-day level. There appeared to be a greater relative loss of hexosamine in the molars of the animals fed the purified diet from 28 to 42 days of age. The results of the total hexose study indicated that the hexosamine content of the tooth buds accounts for only 40 to 50% of the carbohydrate present. The nature of this carbohydrate is under investigation.

Hexosamine/collagen ratio. The hexosamine/collagen ratio of the tooth buds at 28 and 42 days of age is shown in table 4 along with statistical analysis. All the hexosamine/collagen ratios are essentially the same at the 28-day level except for the increased ratio of the third molar of the natural diet group. A sharp decrease in the hexosamine/collagen ratio of the tooth buds from the twenty-eighth to the forty-second day was observed. This decreased ratio is apparently primarily due to the decline of the hexosamine content of the teeth as was indicated in table 3. The work of Sobel ('58) indicates that the decline of the hexosamine/collagen ratio can be equated with biological aging.

Radioglycine incorporation into tooth bud collagen. The rate of incorporation of glycine-C^{14} into tooth bud collagen at 21 days of age is shown in table 5. There was no significant difference between the incorporation of glycine-C^{14} into the tooth bud collagen of the first two molars of the two dietary groups. However, there was a significantly greater incorporation of glycine into the tooth bud collagen of the third molars of the animals fed the purified diet in comparison with the animals fed the natural diet. The increased incorporation of glycine into the collagen of the third molar of the purified diet group supports the increased collagen content of the third molar previously indicated.

TABLE 3

Hexosamine content of tooth buds at 28 and 42 days of age from rats offered a purified high carbohydrate ration in comparison with those offered a natural stock ration from birth

Days of age	Tooth bud	Purified ration	Natural diet	P value
		μg hexosamine/100 mg teeth		
28[1]	1st molar	244 ± 2.1[2]	255 ± 4.2	0.02
	2nd molar	225 ± 2.3	244 ± 4.7	0.02
	3rd molar	217 ± 2.9	252 ± 2.3	0.001
42[3]	1st molar	148 ± 2.6	144 ± 1.8	$<0.5>0.1$
	2nd molar	146 ± 2.3	141 ± 2.5	$<0.5>0.1$
	3rd molar	154 ± 3.1	130 ± 3.8	0.001

[1] Includes 16 animals fed purified ration and 16 animals fed the natural diet.
[2] Standard error of the mean.
[3] Includes 12 animals fed purified ration and 12 animals fed the natural diet.

TABLE 4

Hexosamine collagen ratios at 28 and 42 days of age in the molar tooth buds of weanling rats raised with a purified ration in comparison with those offered a natural stock ration from birth

Days of age	Tooth bud	Purified ration	Natural diet	P value
28[1]	1st molar	0.023 ± 0.001[2]	0.025 ± 0.001	0.5
	2nd molar	0.024 ± 0.001	0.025 ± 0.001	0.5
	3rd molar	0.023 ± 0.0003	0.029 ± 0.0002	0.001
42[3]	1st molar	0.013 ± 0.003	0.012 ± 0.005	$< 0.5 > 0.1$
	2nd molar	0.015 ± 0.005	0.014 ± 0.002	$< 0.5 > 0.1$
	3rd molar	0.017 ± 0.0003	0.014 ± 0.0004	0.001

[1] Includes 15 animals fed purified ration and 16 animals fed the natural diet.
[2] Standard error of the mean.
[3] Includes 18 animals fed purified ration and 18 animals fed the natural diet.

TABLE 5

The incorporation of glycine C^{14} into tooth bud collagen of animals raised with a purified high carbohydrate ration in comparison with those offered a natural stock ration

Tooth buds[1]	Purified ration	Natural ration	P value
	count/min./dry tooth weight		
1st molar	31.8 ± 4.12[2]	31.8 ± 2.08	1.0
2nd molar	36.4 ± 1.61	32.8 ± 1.23	< 0.1
3rd molar	112.8 ± 5.53	93.4 ± 5.80	< 0.02

[1] Includes 18 animals fed purified ration and 18 animals fed the natural diet.
[2] Standard error of the mean.

DISCUSSION

The results of this study indicate that the diet of the postnatal period affects the rate of growth as well as the chemical composition of the developing rat molar teeth.

McCance et al. ('61) found undernutrition to have profound effects on the growth and development of the skull, jaws, and teeth in pigs. Comparable effects upon tooth size have been observed in rats whose mothers were maintained with diets partially deficient in protein throughout pregnancy and lactation (Holloway et al., '61).

The process of calcification in teeth as well as in other calcifying tissues is intimately associated with both collagen and ground substance. Collagen and ground substance may function in these tissues by providing sites for the initiation and control of calcification (Glimchee et al., '60; Glimcher et al., '61). Neither the precise manner by which certain regions of the collagen fibrils act as centers for apatite nucleation nor the exact sequence of the nucleation process itself is known; however, it is reasonable to postulate that any alteration in the organic matrix of developing teeth could influence their subsequent mineralization.

The organic matrix of the developing third molar teeth of animals offered the purified high carbohydrate ration from birth differed from that in animals offered a natural stock ration in having a lower hexosamine/collagen ratio.

This study showed that the developing teeth of rats offered a natural stock ration from birth develop at a slower rate than those from animals offered a purified high carbohydrate ration from birth. This difference in growth was reflected by the weight of the developing teeth as well as a difference in collagen content and hexosamine/collagen ratio.

It is conceivable that the faster growth rate of the developing teeth of animals fed the purified high carbohydrate diet may have adversely affected the quality of the teeth. The significance of these findings cannot be stated until the completion of current investigations. The work of McCay ('55) as well as Silverberg and Silverberg ('55) indicates an inverse relationship between growth rate and longevity. It is conceivable that the same

relationship may play a role in dental health.

The present studies indicate that dietary changes during the formative period of tooth development may affect the collagen as well as the ground substance of developing rat teeth. It is conceivable that this alteration of the organic matrix would influence the process of mineralization of the teeth.

This difference in the organic matrix is of importance in view of the well documented differences in caries susceptibility produced in rats by the two diets; also because of the intimate relationship between the organic matrix and calcification. The differences in caries susceptibility are ultimately related to the difference in the composition of the two diets. The composition of the purified high carbohydrate diet is reported. The composition of the natural stock ration is available from the manufacturers. However, this information would not make it possible for us to relate the differences observed in the teeth to specific components of the diets. The first objective of these experiments was to see whether there was a difference in developing teeth as a result of feeding different diets. Future work has to determine what dietary factors may account for these differences.

SUMMARY

A study was made of the effect of postnatal diets on the rate of molar development and the composition of the organic matrix of rat molar teeth. The molars of animals offered from birth a purified 70% sucrose diet grew initially at an accelerated rate, but at 42 days of age the molars of the animals offered stock ration were heavier. There was a statistically greater amount of collagen in the third molars of the group fed the purified diet in comparison with those fed the natural diet at 3, 4 and 5 weeks of age. At 4 weeks of age all three molars of the animals fed the stock diet contained statistically more ground substance than the molars of those fed the purified diet, resulting in a higher hexosamine/collagen ratio. The hexosamine content of the molars from both experimental groups decreased rapidly from 4 to 6 weeks of age. The relationship between longevity and rapid development was discussed in reference to dental health.

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Biological Assay of Milk and Whey Protein Compositions for Infant Feeding[1]

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The generally accepted concept, that the concentrations of nutrients in human milk represent the ideal pattern to emulate in the preparation of formulas for infant feeding, has served as the basis for various modifications of cow's milk. Usually these modifications include the reduction of protein and mineral content by dilution and the addition of carbohydrate. In several proprietary formulas, the carbohydrate added is lactose and the butterfat is replaced with a mixture of animal and vegetable fats to supply a fatty acid composition closer to that of human milk fat.

Reduction of the protein content of cow's milk by dilution does not alter the qualitative composition of the protein complex which differs markedly from that of human milk. In human milk 35% of the total nitrogen is present in casein, 44% in whey protein and 21% in the nonprotein fraction (Morrison, '51), whereas cow's milk contains 82% of casein N, 13% of whey protein N and 5% of nonprotein nitrogen (Jenness and Patton, '59). These differences in the relative proportion of the protein constituents result in differences in the essential and semi-essential amino acid patterns, particularly in the content of the sulfur-containing amino acid cystine. The lower concentration of total amino acid sulfur in cow's milk protein is a consequence of the larger proportion of casein, a protein with a low cystine content.

The adjustment of the casein-whey protein ratio of cow's milk to that of human milk has been advocated since the earliest days of artificial feeding (Beidert, 1869). Use of unprocessed whey in infant formulas is unsatifactory because the high ash content of whey results in an unphysiologically high level of inorganic elements. Separation of protein from the ash in whey is difficult and has only recently been accomplished on a commercial scale. Recent advances in the technology of electrodialysis have allowed economical large-scale production of whey with a salt content low enough to permit preparation of an infant formula[2] with a casein-whey protein ratio and total ash content similar to those found in human milk.

The present communication reports the results of biological assays comparing the nutritional quality of cow's milk protein, whey protein and the combination of the two in amounts to simulate the casein-whey protein ratio of human milk.

EXPERIMENTAL METHODS AND RESULTS

The use of the rat in the biological assay of the nutritive quality of the protein of an infant formula with a lactose content similar to that of human milk is hampered by the limited tolerance of the rat to high concentrations of this sugar. As in previous studies with human milk (Tomarelli et al., '59), this difficulty was circumvented by conducting the assays on isolated protein or by the use of animals that had acquired a tolerance to high levels of dietary lactose; this adaptation was accomplished by feeding a high-lactose diet for several weeks before the start of the assay.

The following methods were used for the determination of the nutritive value of the protein:

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[2] SMA-Formula S 26, Wyeth Laboratories, Radnor, Pennsylvania.

Protein efficiency ratio (PER) =

$$\frac{\text{weight gain}}{\text{protein consumed}}.$$

Net protein utilization (NPU) =

$$\frac{\text{carcass N increase} + \text{nonprotein carcass N loss}}{\text{food N}} \times 100 \text{ (Miller and Bender, '55).}$$

Protein retention efficiency (PRE) =

$$\frac{\text{weight gain} + \text{weight loss of nonprotein group}}{\text{protein consumed}} \times 16 \text{ (Bender and Doell, '57).}$$

Biological value (BV) =

$$\frac{\text{food N} - (\text{fecal N} - \text{metabolic N}) - (\text{urine N} - \text{endogenous N})}{\text{food N} - (\text{fecal N} - \text{metabolic N})} \times 100 \text{ (Mitchell, '23–'24).}$$

True digestibility (TD) =

$$\frac{\text{food N} - (\text{fecal N} - \text{metabolic N})}{\text{food N}} \times 100 \text{ (Mitchell, '23–'24).}$$

Net protein utilization (NPU) =

$$\text{biological value} \times \text{true digestibility (Mitchell, '23–'24).}$$

Male Sprague-Dawley rats were used in all experiments. The rats were caged individually in a room of constant temperature and humidity. Food and water were given ad libitum. The composition of the various diets is listed in table 1.

Experiment 1. The nutritional quality of milk protein and whey protein were compared, using samples of skim milk and electrodialyzed whey that had been freed of lactose by prolonged dialysis. This treatment also removed the nonprotein nitrogen fraction. Three groups of 10 rats were fed diets A, B, and C (table 1) for two weeks. Individual food consumption was recorded. The weight gains and the food consumed over the two-week period permitted the calculation of protein efficiency ratio and protein retention efficiency. For the determination of biological value, each rat was placed in a metabolism cage for 7 days. Urine and feces

TABLE 1

Composition of diets[1]

	A	B	C	D	E	F	G	H	I
					gm/kg diet				
Glucose	810	650	677	285	185	185	185	183	171
Lactose	—	—	—	450	422	396	409	—	—
Salt mixture[2]	40	40	40	40	35	40	37	16	15
Hydrogenated cottonseed oil[3]	150	150	150	—	—	—	—	—	—
Fat mixture[4]	—	—	—	225	225	225	225	—	—
Milk protein preparation[5]	—	—	133	—	133	—	67	—	—
Whey protein preparation[6]	—	160	—	—	—	154	77	—	—
Infant formula — milk protein[7]	—	—	—	—	—	—	—	801	—
Infant formula — milk + whey protein[8]	—	—	—	—	—	—	—	—	814

[1] Content of protein was 10% (1.57% N × 6.38). Diets D through I contained 45% lactose and 22.5% fat, including that supplied with the test preparations. Each kilogram of diet was fortified with 1 gm of choline chloride, 5 mg each of thiamine·HCl, riboflavin, pyridoxine·HCl and 2-methyl-naphthoquinone; 50 mg each of niacin and Ca pantothenate; 100 mg each of inositol and p-amino-benzoic acid; 2 mg of folic acid, 0.5 mg of biotin and 50 μg of vitamin B_{12}. Fat-soluble vitamins were given by dropper, 2 drops twice weekly: 30 mg of mixed tocopherols, 4400 units of vitamin A and 600 units of vitamin D/gm of corn oil.

[2] Hubbel et al. ('37).

[3] Crisco, Procter and Gamble Company, Cincinnati.

[4] Corn, soybean, coconut and oleo.

[5] Dialyzed skim milk, 11.8% N.

[6] Dialyzed electrodialyzed whey; 9.9% N in the preparation of diet B, 10.2% N in the preparation of diets F and G.

[7] SMA, Wyeth Laboratories, Radnor, Pennsylvania, 1.96% N.

[8] SMA-S 26, Wyeth Laboratories, Radnor, Pennsylvania, 1.93% N.

were collected during the last 4 days of this period. The amount of urinary and fecal nitrogen excreted by the rats fed the protein-free diet, diet A, was taken as a measure of the endogenous urinary and fecal nitrogen excretion of the groups fed the protein samples. At the end of the second week, the rats were killed and the carcasses dried for 48 hours at 100°C in a vacuum oven. The dried brittle carcasses were ground in a plate grinder and nitrogen determined for the calculation of body protein deposition. A more detailed description of methods has been published previously (Tomarelli et al., '59).

The results of experiment 1 are presented in tables 2, 3 and 4. The nutritional value of whey protein was found to be higher than that of milk protein by all 4 indices of nutritional quality. The differences between the two proteins in biological value and net protein utilization were of a high statistical significance (tables 2 and 3). The differences in protein efficiency ratio and in protein retention efficiency were of probable significance (table 4) ($P = 0.05$ or less considered significant). Net protein utilization, calculated as biological value multiplied by true digestibility, yielded values of 88.5 and 80.0 for whey and milk protein, respectively, which agreed well with the values of 85.7 and 78.8 for net protein utilization determined by direct measurement of carcass protein deposition (table 3).

Experiment 2. Biological value was determined on dialyzed samples of milk protein, whey protein and an equal mixture of these two proteins. Comparisons were also made of two samples of infant food of identical composition, except that one contained milk protein, the other a mixture of milk and whey proteins with a casein-whey protein ratio similar to that of human milk. This experiment differed from the first in that older rats were used and the diets contained lactose. The use of older rats permitted the feeding of the protein-free diet and the test diet on alternate weeks, thus, each animal served as its own control for endogenous urinary and fecal nitrogen excretion.

TABLE 2

Biological value and true digestibility of whey and milk protein[1]

Protein	Food N	Fecal N	Urinary N	Biological value	True digestibility
	mg	*mg*	*mg*	%	%
Whey	690.8	73.8	124.4	95.4	92.9
	675.1	46.5	107.3	94.9	96.9
	596.6	62.4	112.0	92.4	93.6
	675.1	53.7	114.3	93.2	95.6
	643.7	61.5	69.5	99.9	94.3
	800.7	77.7	145.8	92.6	94.2
	863.5	68.4	151.5	93.0	95.8
	690.8	67.3	199.3	84.3	94.9
	471.0	51.6	lost	—	94.8
	533.8	61.1	118.5	94.6	94.5
			Average ± SE	93.4 ± 2.1	94.8
Milk[2]	675.1	52.3	159.0	87.0	96.1
	659.4	58.6	156.5	86.5	96.4
	643.7	69.5	110.9	92.5	92.8
	596.6	49.5	210.0	75.9	95.9
	796.3	87.8	248.3	80.0	92.7
	690.8	50.7	125.9	91.9	96.3
	612.3	52.9	139.5	87.0	95.0
	847.8	74.6	253.8	79.9	94.4
	879.2	64.3	257.5	80.2	96.2
	1004.8	87.6	282.5	79.7	94.9
			Average ± SE	84.1 ± 1.4	95.1

[1] Experiment 1. Corrections for endogeneous fecal and urinary nitrogen were 0.276 mg/gm and 0.798 mg/gm respectively. Statistical probability of the difference between biological values resulting by chance was < 0.001 (t test).

[2] Here and in the subsequent tables, milk protein refers to the nondialyzable complete protein complex of cow's milk.

TABLE 3
Net protein utilization of whey and milk protein[1]

	Carcass N	Carcass N increase	Food N	Net protein utilization
	%	mg	mg	mg
Whey protein	2.85	1340	2402	85.5
	2.80	1344	2512	84.7
	2.88	1325	2265	90.4
	2.94	1294	2355	87.3
	2.54	1168	2371	82.6
	2.82	1382	2371	87.8
	2.87	1193	2182	86.3
	3.06	1040	2355	77.1
	3.09	1082	2261	75.1
	2.67	1789	2481	99.7
			Average ± SE	85.7 ± 2.1
Milk protein	2.93	1127	2434	76.3
	2.73	1227	2449	79.6
	2.96	1332	2481	79.7
	3.00	900	1758	84.9
	2.77	942	2386	72.0
	2.90	1392	2419	86.8
	2.99	1076	2292	75.1
	2.79	1311	2700	77.3
	2.90	1160	2591	74.4
	2.90	1450	2685	81.4
			Average ± SE	78.8 ± 2.2

[1] Experiment 1. Maintenance nitrogen, derived from carcass nitrogen loss of protein-free group, was 7.68 mg/gm. Statistical probability of difference resulting by chance was 0.015 (*t* test).

TABLE 4
Protein efficiency ratio and protein retention efficiency of whey and milk protein[1]

Protein-free	Whey protein				Milk protein			
Weight gain	Weight gain	Protein eaten	Protein efficiency ratio	Protein retention efficiency	Weight gain	Protein eaten	Protein efficiency ratio	Protein retention efficiency
gm	gm	gm	gm/gm	%	gm	gm	gm/gm	%
−20	47	15.3	3.07	65.9	39	15.5	2.52	56.8
−13	48	16.0	3.00	64.0	45	15.6	2.88	62.6
−12	46	14.5	3.17	68.5	45	15.8	2.85	61.8
−22	44	15.0	2.93	64.0	30	11.2	4.11	65.7
−20	46	15.1	3.05	65.8	34	15.2	2.24	52.6
−17	49	15.1	3.25	68.8	48	15.4	3.12	66.6
−12	39	13.9	2.81	63.4	36	14.6	2.47	57.0
−21	34	15.0	2.27	53.3	47	17.2	2.73	58.6
− 9	35	14.4	2.43	56.6	40	16.5	2.42	54.2
−14	67	15.8	4.24	85.1	50	17.1	2.92	61.8
Average ± SE								
−16	46		3.02 ± 0.17	65.5 ± 2.7	41		2.68 ± 0.08	59.8 ± 1.6

[1] Experiment 1. Fourteen days experiment. Statistical probability of difference resulting by chance (*t* test); protein efficiency ratio = 0.08; protein retention efficiency = 0.07.

Male rats, weighing an average of 200 gm, were adapted by feeding an adeqaute diet containing 45% of lactose for two weeks preceding the test proper. They then received the protein-free diet, diet D (table 1) for 7 days. The rats were divided on the basis of weight into 5 groups of 7 rats each and fed diets E, F, G, H and I (table 1) for 7 days. Urine and feces were collected on the last 4 days. The rats were then fed protein-free diet D for 7 days; excreta collected on the last 4 days was analyzed for estimation of endogenous urinary and fecal nitrogen. The

results are presented in table 5. The biological values of 94.4 and 83.4%, obtained for whey and milk protein, respectively, are in good agreement with those obtained in the first experiment. The mixture of the two proteins yielded an intermediate biological value, 92.4%. The biological value of the protein of the infant food containing the mixture of whey and milk proteins was higher than that of the food containing milk protein, 84.9% vs. 79.4%.

Experiment 3. At the conclusion of the protein-free period of experiment 2, the rats were redivided by weight into groups of 7 and the biological value of the same samples tested in experiment 2 were estimated a second time. The results obtained were: for milk protein 84.7 ± 3.3, whey protein 92.7 ± 3.0, equal mixture of the two proteins 90.8 ± 2.4, infant food with milk protein 76.1 ± 2.8 and infant food with the protein mixture 88.1 ± 2.9.

The biological values obtained for the isolated milk and whey proteins in all three tests agree: milk protein 84.7, 84.1 and 83.4%, whey protein 92.7, 93.4 and 94.4%. The biological value of the infant food containing the milk and whey protein mixture was higher in both tests, 84.9 and 88.1%, than that of the food containing milk protein alone, 79.4 and 76.1%.

The value obtained for the protein of each of the infant foods was lower than that found for the corresponding protein mixture tested in the isolated state. The lower values for the infant foods are explained by the presence of nonprotein nitrogen in these samples, 5% in the food with milk protein alone and 15% in the food containing additional whey protein. These foods were compared on an isonitrogeneous basis with the isolated proteins, which were essentially free of nonprotein nitrogen. Recalculation of biological value in terms of true protein nitrogen is not possible since it is not known to what extent the nonprotein nitrogen is utilized under these dietary conditions.

No significant differences were found in the average true digestibilities of the proteins in any of the samples; all values were in the range of 90 to 95%.

DISCUSSION

The results of these animal assays demonstrate clearly that the nutritional value of the mixture of whey and milk protein in the proportion present in human milk is higher than that of milk protein alone. These results are in accord with numerous published reports on the greater nutritional adequacy of lactalbumin (heat-coagulated whey protein) as compared with casein (Henry, '57). The improvement in protein quality with an increase in the ratio of whey protein to casein is commensurate with the increased amounts of sulfur-containing amino acids, since these are the amino acids present in milk in amounts limiting for the growth and maintenance of the rat. The mixture of milk and whey protein contains 2.5% of methionine and 2.3% of cystine for a

TABLE 5

Biological value of whey and milk proteins[1]

	Milk	Whey	Whey + milk	Milk in infant food	Whey + milk in infant food
Diet	E	F	G	H	I
	86.2	95.4	95.3	82.7	88.0
	89.3	100.0	94.4	82.8	87.4
	82.6	93.8	93.4	80.2	86.6
	81.8	96.1	91.3	72.9	82.2
	78.8	87.0	95.4	77.5	79.6
	81.1	93.0	89.7	84.3	91.2
	83.3	95.6	89.6	75.5	79.3
Average ± SE	83.4 ± 1.3	94.4 ± 1.3	92.4 ± 0.9	79.4 ± 1.5	84.9 ± 1.7

[1] Experiment 2. Statistical probability of difference resulting by chance (*t* test); whey vs. milk, $P = < 0.001$; whey + milk vs. milk, $P = < 0.01$; milk food vs. milk + whey food, $P = < 0.001$.

total of 4.8%, whereas, in milk protein there is 2.7% of methionine, 0.8% of cystine, or a total of 3.5% (Jenness and Patton, '59). In comparison, human milk protein contains 2.0% of methionine and 2.3% of cystine (Soupart et al., '54). From the results of the present study, with an increased amount of a protein containing a high level of cystine, a semi-essential amino acid, a definite increase in nutritional quality is obtained.

Good correlation has been observed between the nutritive values of proteins determined on adult man and on the growing rat (Mitchell, '54). Because of the scarcity of data concerning the biological value of proteins for infants, the results of the animal bioassay can only serve as a guide in the assessment of a protein for the infant. This is particularly so when the content of sulfur-containing amino acids is involved, since the extent to which cystine can replace methionine for the human infant has not been as clearly delineated as it has been for the rat. The infant's daily requirement for methionine estimated by Holt and Sryderman ('61) at 45 mg/kg was determined in studies in which the basal formula contained the other amino acids, including cystine, in amounts present in human milk. A higher requirement for methionine would be expected if less cystine had been available; for example, in a basal diet with an amino acid content patterned after that of cow's milk protein. The contribution of cystine in protein nutrition is often overlooked, an oversight contributed to by the classification of this compound as a semi-essential amino acid. In studies with adult men, Rose and Wixom ('55) found that cystine is capable of replacing 80 to 89% of the minimal methionine requirement and it is reasonable to assume that the high content of cystine in human milk contributes equally with methionine in satisfying the infant's requirement for sulfur amino acids.

Cow's milk formulas in current use are invariably higher in protein content than human milk, and supply an adequate amount of all essential amino acids for

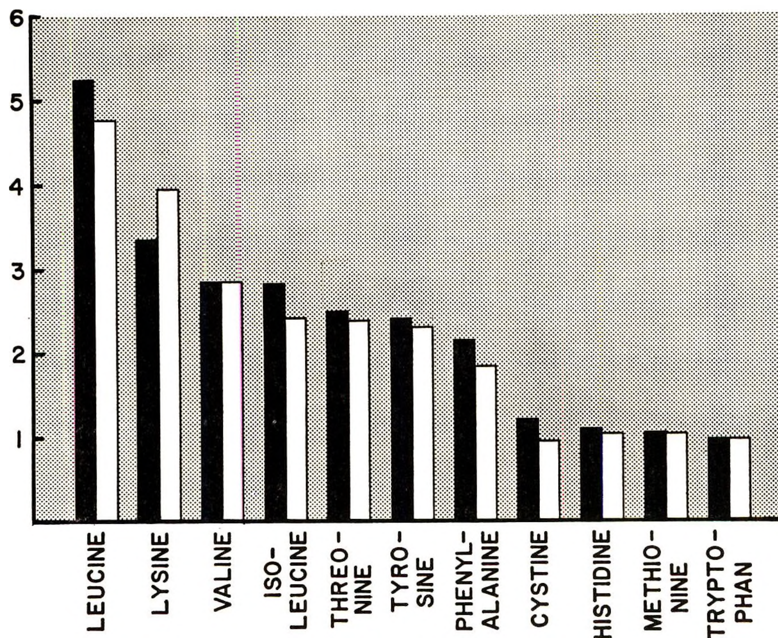

Fig. 1 Proportions by weight of essential and semi-essential amino acids, based on tryptophan as unity, in proteins of human milk (solid bars) and infant formula (see footnote 2 in text) containing milk and whey proteins (open bars). Human milk values from Soupart et al. ('54). Values for infant formula based on data from Jenness and Patton ('59).

infant nutrition according to present estimates of the amino acid content of proteins and infant requirements. Until we are more informed on such problems as amino acid imbalance, the influence of an excess of one amino acid on the requirement of another, the extent or rate at which cystine can be formed from methionine, it would appear desirable in preparing food for infants to imitate as closely as possible the amino acid pattern of human milk. The preparation of a formula with equal amounts of nitrogen from electrodialyzed whey and from milk, yields an infant food with a protein distribution similar to that of human milk and, as demonstrated in figure 1, supplies an essential amino acid pattern closely similar to that of human milk.

SUMMARY

The nutritional values of the protein of milk, of whey and of a mixture of the two in amounts to simulate the casein-whey protein ratio of human milk were determined by several rat assay procedures. Whey protein and the mixture of whey and milk protein were found to be superior to milk protein alone. Tests of commercial infant foods containing either milk protein alone or the whey-milk protein mixture also yielded results showing a nutritional superiority for the protein mixture. The higher biological activity of the protein mixture simulating the casein-whey protein ratio of human milk was attributed to the higher content of sulfur-containing amino acids.

ACKNOWLEDGMENT

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Studies on the Role of Manganese in Bone Formation

I. EFFECT UPON THE MUCOPOLYSACCHARIDE CONTENT OF CHICK BONE

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Wilgus and associates ('36, '37) demonstrated that manganese is essential for the development of normal bones and the prevention of perosis in the chick. Lack of manganese resulted in an enlargement of the intertarsal joint, either twisting or shortening of the leg bones or both, and in some instances slipping of the tendon of the gastrocnemius muscle out of the intercondyloid groove. Creek and associates ('60) showed that the severity of this abnormality is influenced by the weight of the chick.

Although the function of manganese in the prevention of this syndrome remains obscure, some information is available concerning the changes that take place in bone as a result of maganese deficiency. Wiese and co-workers ('39) have reported that bones from manganese-deficient chicks exhibit reduced alkaline phosphatase activity. Investigations by Gallup and Norris ('38) and Caskey et al. ('39) revealed that the bones from manganese-deficient chicks were thickened and shortened with a slight but significant reduction in ash content. However, calcification appeared to be normal, based on x-ray examination and silver nitrate staining. Further studies by Parker and associates ('55) indicated that the level of manganese in the diet does not significantly affect the amount or location of deposition of Ca^{45} and P^{32} in the tibiae.

Wolbach and Hegsted ('53) reported that distinct histological changes can be observed in the epiphyseal cartilage of bones from chicks suffering from perosis resulting from a deficiency of manganese or choline. The authors suggested that the skeletal manifestations of perosis are the result of retardation or suppression of epiphyseal cartilage sequences including matrix formation and cell proliferation, growth and maturation.

Since these observations suggested a defect in bone formation other than the calcification process per se, Leach[1] studied the effect of perosis upon the chemical composition of epiphyseal cartilage. The results indicated that manganese deficiency altered the mucopolysaccharide content of this tissue. This report presents data that confirm these preliminary results and provide further information dealing with the specificity of this observation.

EXPERIMENTAL PROCEDURE

The composition of the basal diet used in this investigation is presented in table 1. This diet contained 2.4 mg Mn/kg of diet by analysis. Duplicate or triplicate lots of 10 to 15 one-day-old White Plymouth Rock males were used in these experiments. The chicks were placed in electrically heated battery brooders with raised wire floors and were supplied the experimental diets and demineralized water ad libitum.

At the conclusion of each experiment, the legs of the chicks were examined for the incidence and severity of hock enlargement and the incidence of slipped tendon. The degree of enlargement was estimated and given scores of 1, 2 and 3 for increasing severity. The severity index represents the sum of these values divided by the number of chicks afflicted.

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[1] Leach, R. M., Jr. 1960 The effect of manganese, zinc, choline and folic acid deficiencies on the composition of epiphyseal cartilage. Ph. S. Thesis, Cornell University, Ithaca, New York.

TABLE 1
Composition of basal diet[1]

	gm/100 gm
Glucose	58.95
Isolated soybean protein	27.00
Corn oil	3.00
Cellulose	3.00
Vitamin mixture[2]	1.22
Mineral mixture[3]	5.83
Glycine	0.30
DL-Methionine	0.70
Protein (N × 6.25), %	24.86
Metabolizable energy (ME), Cal./gm	3.35

[1] Contains 2.4 mg Mn/kg of diet by analysis.
[2] Supplies the following/100 gm of diet: inositol, 25.0 mg; niacin, 5.0 mg; Ca pantothenate, 2.0 mg; pyridoxine·HCl, 0.45 mg; folic acid, 0.40 mg; menadione sodium bisulfite, 0.152 mg; biotin, 0.02 mg; vitamin B_{12}, 5.4 μg; α-tocopheryl acetate, 6.6 mg; vitamin A, 540 IU, vitamin D_3, 98 ICU, thiamine·HCl, 1.1 mg; riboflavin, 1.1 mg; and choline chloride, 154 mg.
[3] Supplies the following/100 gm of diet: $CaHPO_4$, 1.70 gm; $CaCO_3$, 1.83 gm; KH_2PO_4, 1.38 gm; NaCl, 0.60 gm; $MgSO_4$, 0.25 gm; $FeSO_4 \cdot 7H_2O$, 33.3 mg; KI, 0.26 mg; $CuSO_4 \cdot 5H_2O$, 1.67 mg; $CoCl_2 \cdot 6H_2O$, 0.17 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.83 mg; and ZnO, 7.47 mg.

Epiphyseal cartilage was obtained from the proximal and distal ends of the tibiotarsus and the proximal end of the tarsometatarsus. Dry ice was used for freezing the tissue upon removal. Following collection, the samples were homogenized in a small volume of water and lypophilized. The dried powder was extracted with a 2:1 mixture of chloroform : methanol, dried and ground. Other tissues studied were treated in a similar manner.

For the estimation of mucopolysaccharides, the lipid-free dry residue was hydrolyzed in sealed tubes according to the procedure of Anastassiadis and Common ('58). The method described by Dische ('47) was used for the estimation of hexuronic acid. Total hexosamine was determined by the method of Boas ('53). Glucosamine and galactosamine were estimated using the procedure of Roseman and Daffner ('56) as modified by Levvy and McAllan ('59). A modification of the column chromatographic procedure of Gardell ('53) was also used in the separation and analysis of hexosamines.

Because of the small amounts of tissue involved, most of the chemical analyses were performed in quadruplicate on pooled samples. Consequently, statistical analysis was not applied to the data obtained. However, with the exception of those presented in table 5, the results of chemical analyses are representative of results obtained in two or more experiments.

In the experiments using radiosulfate, the dose (20 μc/100 gm body weight) was administered intraperitoneally. The chicks were killed 24 hours later and the epiphyseal cartilage of the tibiotarsus removed for assay of radioactivity according to the method of Everett and Simmons ('52). The results were subjected to analysis of variance according to the procedure of Snedecor ('56).

RESULTS AND DISCUSSION

From the results of experiment 1 (table 2) it is apparent that the omission of manganese from the basal diet resulted in a depressed growth rate and an increase in the incidence and severity of bone abnormalities. In addition to the above effects, manganese appeared to exert an effect upon mucopolysaccharide content of the epiphyseal cartilage. This is evidenced by the lowered radiosulfate uptake by the cartilage and the reduction in total hexosamine and hexuronic acid content. Furthermore, the reduction in total hexosamine content occurred primarily in the galactosamine fraction, suggesting a decrease in the mucopolysaccharides which contain this hexosamine.

In this experiment and subsequent experiments, there was a low incidence of bone abnormalities in the control groups (100 mg Mn/kg diet). The occurrence of these abnormalities did not appear to be associated with a lack of any of the known nutritional factors. Furthermore, similar observations were made with this strain of chicks fed a wide variety of purified and practical chick diets. Thus, an explanation for these observations is unavailable at the present time.

To obtain further information dealing with the relationship of dietary manganese to cartilage mucopolysaccharides, an experiment was conducted in which graded levels of manganese were fed. The results of this experiment (table 3) show a direct relationship between the level of dietary manganese and the mucopolysaccharide content of the epiphyseal cartilage. As in the previous experiment, the reduction in galactosamine content ac-

TABLE 2

Effect of manganese deficiency upon growth rate, bone formation and the mucopolysaccharide content of epiphyseal cartilage

Observation	Level of added Mn	
	0	100
	mg/kg	*mg/kg*
4-Week weight, gm	388(29)[1]	589(30)
Enlarged hock, %	100	10
Severity index	2.66	1.00
$S^{35}O_4$ Uptake[2] (% dose/100 mg dry epiphyseal cartilage)	0.21[3]	0.39
Hexosamine (% of dry, lipid-free epiphyseal cartilage)		
Total	1.64	3.93
Glucosamine	0.69	1.08
Galactosamine	0.95	2.85
Total hexuronic acid (mg/100 mg dry lipid-free epiphyseal cartilage)	1.64	4.18

[1] Mean and number of survivors from duplicate lots of 15 male chicks.
[2] Average of 8 chicks selected at random from the treatment.
[3] Significantly different from the control, $P = 0.05$.

TABLE 3

Effect of graded levels of manganese upon growth rate, bone formation and the mucopolysaccharide content of epiphyseal cartilage

Level of added Mn	4-Week weight	Enlarged hock	Severity index	Hexosamine content % of dry, lipid-free epiphyseal cartilage		
				Total	Glucos-amine	Galactos-amine
mg/kg	*gm*	%				
0	369(28)[1]	100	2.78	1.98	0.72	1.26
10	471(29)	93	1.89	3.64	1.02	2.62
20	547(28)	39	1.54	4.16	1.02	3.14
100	562(28)	14	1.00	4.51	0.88	3.63

[1] Mean and number of survivors from triplicate lots of 10 male chicks.

counted for the major portion of the changes that were observed. The validity of these observations appears to be clearly established, since similar observations have been obtained in a total of 5 experiments.

In addition to the experiments described above, several experiments were conducted which dealt with the specificity of these observations. Some data on tissue specificity are presented in table 4. From the results it is apparent that manganese influences the mucopolysaccharide content of other osseous tissues such as the articular cartilage and decalcified diaphysis of the tibiotarsus and the epiphyseal cartilage of the humerus. With these tissues, normal levels of mucopolysaccharides were obtained with the addition of 10 mg/kg of manganese to the basal diet.

The effect of food intake has also been investigated. The data from a paired-feeding experiment are presented in table 5. Since the results are very similar to those obtained previously with ad libitum food intake, it appears that food intake is not an important factor in the results obtained.

The possibility that the alterations in mucopolysaccharides might be explained on the basis of the tissue histological changes has also been investigated. As mentioned previously, Wolbach and Hegsted ('53) reported that choline and manganese deficiencies resulted in distinct histological changes in the epiphyseal cartilage.

TABLE 4

The effect of manganese upon the mucopolysaccharide content of other bone tissue

Level of added Mn	Total hexosamine content, % of dry, lipid-free dry matter		
	Tibiotarsus		Humerus epiphysis
	Articular cartilage	Decalcified diaphysis	
mg/kg			
0	3.16(20)[1]	0.55(6)[1]	1.28(20)[1]
10	4.08	0.90	2.71
20	—	—	2.80
25	3.86	0.92	—
100	4.08	0.82	3.19

[1] Number of chicks involved per treatment.

TABLE 5

Effect of manganese upon growth rate, bone formation, and the mucopolysaccharide content of epiphyseal cartilage under conditions of paired feeding

Observation[2]	Level of added Mn	
	0	100[1]
	mg/kg	*mg/kg*
4-Week weight, gm	294	279
Enlarged hcck, %	100	0
Severity index	2.60	0
Hexosamine content (% of dry, lipid-free epiphyseal cartilage)		
Total	1.36	3.49
Glucosamine	0.54	0.95
Galactosamine	0.82	2.54
Hexuronic acid content — total (% of dry, lipid-free epiphyseal cartilage)	1.34	3.40

[1] Pair-fed to those receiving zero mg/kg Mn.
[2] Average for one lot of 20 male chicks (20 survivors/lot).

TABLE 6

Effect of choline deficiency upon growth rate, bone formation and the mucopolysaccharide content of epiphyseal cartilage

Oservation	Choline deficient basal[3]	Basal + 0.15% choline Cl
4-Week weight, gm	184(27)[1]	514(29)
Enlarged hock, %	100	7
Severity index	2.59	1.00
$S^{35}O_4$ Uptake[2] (% dose/100 mg dry epiphyseal cartilage)	0.34[4]	0.42
Hexosamine content (% of dry lipid-free epiphyseal cartilage)		
Total	3.83	3.88
Glucosamine	1.08	1.33
Galactosamine	2.75	2.55

[1] Mean and number of survivors from triplicate lots of 10 male chicks.
[2] Average of eight chicks selected at random from the treatment.
[3] The basal diet presented in Table 1 was modified as follows:
(a) 27% crude casein, 1.2% L-arginine·HCl, 1.0% glycine and 0.30% DL-methionine were substituted for the isolated soybean protein and amino acid supplements.
(b) Minus choline chloride plus 100 mg/kg of Mn from $MnSO_4 \cdot H_2O$.
[4] Significantly different from the control, P = 0.05.

These changes could be characterized as a failure of the cartilage cells to mature, the presence of an excess of a typical matrix in the zone of growth, and an excess of matrix with a reduction in mitoses in the zone of proliferation. Furthermore, tunneling of the cartilage is retarded or completely suppressed. Since choline and manganese deficiencies produce similar histological lesions, the effect of choline deficiency upon cartilage mucopolysaccharides was investigated. The results of this experiment are presented in table 6. Choline deficiency did not result in any substantial change in the mucopolysaccharide content of the epiphyseal cartilage. Thus, it appears that the gross histological changes in the tissue do not account for the changes in mucopolysaccharide that are observed with manganese deficiency. However, choline deficiency did not result in a decreased radiosulfate uptake by the epiphyseal cartilage, suggesting an alteration in the metabolism of mucopolysaccharides.

These experiments demonstrate, therefore, that manganese is necessary for the maintenance of proper amounts of mucopolysaccharides in the epiphyseal cartilage and other bone tissues. However, information dealing with the function of manganese in mucopolysaccharide metabolism is unavailable. Nutritional attempts to correct manganese deficiency (other than by the use of manganese) have been unsuccessful. For example, the feeding of substantial quantities of glucosamine, glucuronic acid, galactose and ascorbic acid has had no effect upon the course of manganese deficiency in the chick.[2]

The importance of mucopolysaccharides in the maintenance of the structural integrity of connective tissue is suggested by the studies of Thomas ('56), McCluskey and Thomas ('58) and Bryant and associates ('58). These investigators observed that the injection of young rabbits with papain resulted in a loss of ear rigidity. The loss of rigidity was accompanied by decreases in mucopolysaccharide content as evidenced by histochemical staining and chemical analysis. The effect of papain was thought to be due to a direct enzymatic action on chondromucoprotein, which resulted in a release of chondroitin sulfate from the cartilage. Thus, the effect of manganese upon the mucopolysaccharide content of bone provides a possible explanation for the occurrence of bone abnormalities in manganese deficient chicks.

SUMMARY

A study of the effect of manganese deficiency on the composition of chick epiphyseal cartilage revealed that this deficiency results in a severe reduction in mucopolysaccharide content. A reduction in mucopolysaccharides which contain galactosamine accounted for most of the changes observed. Further studies indicated that manganese had similar effects upon the mucopolysaccharide content of the organic matrix of other portions of the bone. The changes in mucopolysaccharides appeared to be a specific effect of manganese, since they were not related to alterations in food intake or to the choline content of the diet.

ACKNOWLEDGMENT

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"Unessential" Nitrogen: A Limiting Factor for Human Growth[1,2]

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That the nutritional quality of a protein was dependent upon its amino acid composition became apparent early in the present century with the discovery of the essential amino acids. In 1914 Mendel formulated the concept that the law of the minimum applied to the amino acids: "Growth," he stated, "is limited by the supply of each essential amino acid. It matters not whether this is exhibited as such or in the guise of protein; in either event the 'law of the minimum' is exemplified."

The view that only the *essential* amino acids are limiting factors in protein quality has persisted up to the present time. With the advent of accurate analytical data on the amino acid composition of proteins the possibility of evaluating protein quality from the content of the essential amino acids suggested itself. Mitchell and Block ('46) showed that a correlation existed between the biological value of a protein and the deficiency of the most limiting amino acid as compared with a standard reference protein, the protein of whole egg. A modification of their procedure for evaluating dietary protein was adopted by the FAO Committee on Protein Requirements ('57). In place of whole egg as a standard of reference they used an amino acid pattern based on available information of the minimal requirements of humans for the essential amino acids. The "protein score" consisted of the percentage of the most limiting amino acid as compared with the reference standard. Several modifications of the protein score have also been suggested (Oser, '51; Mitchell, '54) in which some weight was given to deficits of other essential amino acids as well as the most limiting one. Despite these refinements the correlation between the score and the biological value remains a rough one. In none of the proposed methods of scoring from composition are the unessential amino acids taken into consideration with the exception of cystine; this, together with methionine, is regarded as a potential limiting unit.

It has been generally appreciated that the diet had to provide sufficient nitrogen for the synthesis of the unessential amino acids. The assumption has been commonly made, however, that natural food proteins provided sufficient "unessential" nitrogen for that purpose. With the advent of quantitative data for the essential amino acid requirements of man, nutritionists have, with few exceptions, concerned themselves with the problem of providing the essentials in quantities sufficient to meet these requirements in the belief that this would guarantee an adequate protein intake.

We were led to question the concept that only the essential amino acids were limiting, when we compared data on the minimal requirements of adults for particular proteins with their minimal requirements for essential amino acids as determined by Rose et al. ('55) and the teams sponsored by the Department of Agriculture (Leverton et al., '56a, b, c, d, e; Swendseid et al., '56a, b; Jones et al., '56).

In the case of cow's milk (table 1) it appears that an intake of 0.20 gm of protein/kg will meet the requirements of the human adult for all the essential amino

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[1] A preliminary report of this work was presented in Some Aspects of Amino Acid Metabolism ed., (W. H. Cole, 1956). Rutgers University Press, New Brunswick, New Jersey.

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[3] (Snyderman, Holt, Dancis, Boyer and Roitman.)

[4] (Balis.)

acids. However, when adults are studied, using a diet in which cow's milk is the only source of protein, as was done by Bricker et al. ('45), it appears that approximately 0.45 gm of protein/kg are required to maintain nitrogen balance. Unpublished experiments carried out in our laboratory[5] confirmed these results on 6 adult subjects who were maintained for 3 to 4 weeks with a diet of cow's milk alone, nitrogen equilibrium being obtained with intakes of 0.50, 0.50, 0.40, 0.55, 0.45 and 0.50 gm of protein per kg per day.

[5] Snyderman, S. E., A. Boyer and L. E. Holt, Jr., unpublished observations.

TABLE 1

Quantities of milk and corn proteins required to provide minimal requirements of essential amino acids

	Essential amino acid requirements of adults[1]	Essential amino acids provided by	
		Milk protein[2] at 0.20 gm/kg/day	Corn protein[3] at 0.42 gm/kg/day
	mg/kg/day	*mg/kg/day*	*mg/kg/day*
Isoleucine	7.8	12.8	19.3
Leucine	8.5	21.6	55.4
Lysine	6.1	15.6	12.2
Methionine (in presence of cystine)	4.5	5.2	8.0
Total sulfur amino acids	7.0	7.0	13.0
Phenylalanine (in presence of tyrosine)	3.1	10.4	18.9
Threonine	5.0	5.2	16.8
Tryptophan	2.5	3.0	2.5
Valine	9.0	13.8	21.8

[1] The figures represent a composite of the observations of Rose et al. for males and of the teams sponsored by the Department of Agriculture for females, the assumption being made that the differences in these studies represented individual variations rather than sex differences. An average weight of 70 kg was assumed for males and of 55 kg for females.

[2] The figures are based on analyses of cow's milk protein given by Macy et al. ('53), The Composition of Milks, pub. 254, National Research Council — National Academy of Sciences, Washington, D. C.

[3] The data are based on analyses of corn meal given by Orr and Watt ('56), Amino Acid Composition of Foods, U. S. Department of Agriculture, Washington, D. C.

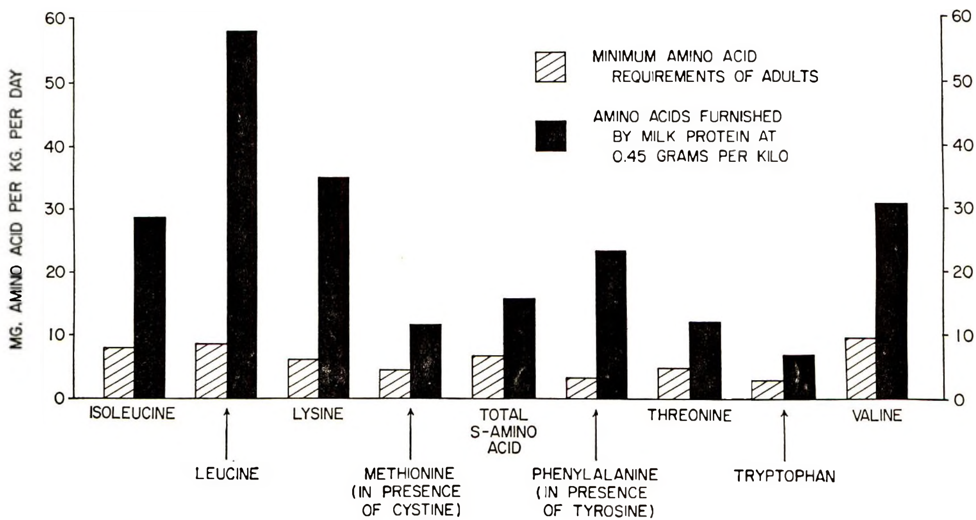

Fig. 1 Relation between minimum requirement of adults for essential amino acids and quantities of amino acids supplied by milk when fed at the minimal level (0.45 gm/kg) that will maintain nitrogen equilibrium.

In figure 1 are shown graphically the quantities of essential amino acids furnished by the minimal amount of milk protein that will maintain nitrogen equilibrium as compared with the minimal requirements of these amino acids as determined for adults. The discrepancy is striking. If a similar calculation is made in the case of corn (table 1) it appears that an intake of 0.42 gm of protein/kg will meet the requirements of all the essential amino acids as determined for adults. Nevertheless it has not been possible to maintain adults in nitrogen equilibrium with such low intakes. In a carefully studied adult, Truswell and Brock ('62) reported negative nitrogen balance in an adult with an intake of 0.47 gm/kg and positive balance at an intake of 0.84 gm/kg. Interpolation of their data indicates that 0.56 gm/kg is needed for nitrogen equilibrium, a figure one-third more than that which will provide the essential amino acid requirements.

In searching for an explanation of the inability of diets of natural proteins to maintain nitrogen equilibrium even though meeting the requirements for essential amino acids, only one explanation seemed possible, viz., that the limiting factor was "unessential nitrogen"[6] rather than essential amino acids. In the studies of Rose and those of teams sponsored by the Department of Agriculture, a considerable excess of unessential nitrogen was supplied beyond what would be provided by a natural protein. Therefore, if the first limiting nitrogen moiety of a natural protein were the unessential nitrogen moiety this would have been concealed by the design of their studies which was such that only the limiting essential amino acid was demonstrated.

Also, if unessential nitrogen is indeed the first limiting factor in many diets a number of experiments in which conclusions have been drawn from supplements of DL- amino acids will have to be reevaluated, since the supplement provided unessential nitrogen in the form of the unnatural isomer.

To ascertain what was indeed the first limiting nitrogenous moiety for man in a natural protein diet, we undertook to study this question in growing infants. The general plan of the study was to reduce the protein intake in a stepwise manner, keeping the calories constant, until evidence of inadequacy was obtained in the form of arrested weight gain or subnormal nitrogen retention and then supplementing this with some form of unessential nitrogen to see whether these shortcomings were overcome. Such studies were conducted on 4 infants, using a milk diet and they indicate that the most limiting nitrogenous moiety is unessential nitrogen rather than an essential amino acid. A supplement of glycine or of urea sufficed to restore normal weight gain and nitrogen retention to normal levels after these had been impaired by reducing the protein intake.

To demonstrate that the supplement of unessential nitrogen was actually contributing to protein synthesis, two studies were carried out in which labeled unessential nitrogen was given: one in which it was given as urea and in the other as ammonium chloride. Both of these studies showed a substantial incorporation of labeled nitrogen into the plasma protein and the hemoglobin of the infant.

EXPERIMENTAL

Subjects. The subjects were 4 normal infants who remained in the hospital for social reasons. They varied in age from three weeks to 5 months and in weight from 2.5 to 5.5 kg at the beginning of the study. Three were male and one was female.

Diets. All formulae were prepared from evaporated cow's milk. As the amount of milk in the formula was reduced, the intake was kept isocaloric by the addition of carbohydrate in the form of maltose and dextrins[7] and of fat as butterfat. Vitamin B and mineral intake were kept constant by the use of a vitamin B mixture[8] and a mineral mixture.[9]

[6] The term "unessential nitrogen" is applied to the moiety consisting of unessential amino acids, urea and ammonium compounds.

[7] Dextri-Maltose, Mead Johnson and Company, Evansville, Indiana.

[8] The composition of the B vitamin mixture was: thiamine, 0.38; riboflavin, 2.0; nicotinamide, 0.85; Ca pantothenate, 3.5; pyridoxine, 0.67; *i*-inositol, 180.; *p*-aminobenzoic acid, 0.5; folic acid, 0.05; choline chloride, 147.; biotin, 0.03; cyanocobalamin, 0.015 mg.

[9] The percentage composition of the mineral mixture was: NaCl, 18.9; $CaHPO_4$, 25.4; $MgSO_4$, 6.8; $KHCO_3$, 44.4; KCl, 2.88; Fe_3 citrate, 2.21; $CuSO_4$, 0.24; $MnSO_4$, 0.15; KI, 0.15; NaF, 0.03.

Vitamins A, C and D were provided daily as 0.6 ml of a propprietary preparation.[10] The composition of the formulae used is listed in table 2.

Metabolic periods. All metabolic periods were of 4 days duration. Separate collection of urine and stool were made possible by the use of metabolic beds (Hoag, '32). Nitrogen determinations were performed daily, in duplicate, on the 24-hour urine collection. The beginning and end of the fecal collection periods were demarcated by carmine and charcoal markers. Triplicate nitrogen determinations were performed on the complete stool collection.

Analytical procedures. Total nitrogen in urine and stool was determined by the Kjeldahl procedure as modified by Hiller et al. ('48). Plasma proteins were determined by the arginine method (Albanese et al., '42). The procedure of Bonsnes and Taussky ('45) was used for the determination of creatinine.

Quantitative analyses of the free amino acids of the plasma were performed by ion exchange column chromatography (Moore and Stein, '51).

Isotope measurements. The N^{15} ammonium chloride with an enrichment of 96.8% N^{15} was obtained commercially.[11] From this, labeled urea containing 10.2 atom % excess N^{15} was prepared by the method of Cavalieri et al. ('48). In one study, labeled urea, so prepared, was incorporated into the diet and in the other, labeled ammonium chloride was fed. The incorporation of N^{15} into plasma protein and into hemoglobin was measured as follows: The blood was collected in citrate and the plasma separated by centrifugation; 0.5 ml of plasma was incubated with urease at 37°C overnight. Air was then bubbled through for two hours and a urea solution containing 100 mg/100 ml was added. Proteins were precipitated by adding an equal volume of 10% trichloroacetic acid, the precipitate being washed three times with 5% trichloroacetic acid and then subjected to Kjeldahl distillation. The distillate was collected in 1 N HCl and then the N^{15} content was determined. For the determination of N^{15} in hemoglobin the red cells were washed three times with normal saline and then refrigerated overnight with 4 volumes of distilled water and 0.5 volume of toluene. The next morning the hemoglobin solution was treated in the same fashion as the plasma. A consolidated Nier model no. 21-201 ratio mass spectrometer was used to determine the N^{15} content of all the samples.

The extent of incorporation of unessential nitrogen into new protein was estimated as follows: In the case of plasma protein the total amount formed each day was calculated from the half-life of the labeled protein as shown graphically in figures 2 and 3. The ratio of labeled-to-total plasma protein gave an approximation of the percentage of new plasma protein derived from unessential nitrogen. In

[10] Trivisol, supplied by Mead Johnson and Company, Evansville, Indiana.

[11] Purchased from Isomet Corporation, Palisades Park, New Jersey.

TABLE 2

Composition of diets in which protein was fed at different levels

Evaporated milk, ml	57	36	28.4	23.6	19	17.3	15.2	13	11.4
Maltose and dextrins,[2] gm	8.1	10.5	11.9	12.7	13.5	14.0	14.4	14.7	15.2
Butterfat, gm	0	2.0	2.7	3.1	3.52	3.64	3.8	3.98	4.06
Calories/kg	110	110	110	110	112	112	112	112	112
Protein, gm/kg	*4.0*	2.5	2.0	*1.65*	*1.33*	*1.22*	*1.10*	*0.91*	0.82
Protein, % of Cal.	*14.5*	*9.1*	7.0	6.0	4.8	4.35	3.8	3.2	2.8
Carbohydrate, gm/kg	13.7	14.0	14.8	15.02	15.5	15.56	15.9	15.9	16.3
Carbohydrate, % of Cal.	49.5	51.0	53	54.5	55	56.6	56.2	56.8	58
Butterfat, gm/kg	4.5	4.84	4.95	4.96	5.03	5.0	5.0	5.0	4.96
Butterfat, % of Cal.	36	39.8	40	40.6	40.2	40	40	40	39.2

[1] The constituents listed were diluted to a volume of 130 cc, and this amount was fed per kilogram per day.

[2] Dextri-Maltose, Mead Johnson, Evansville, Indiana.

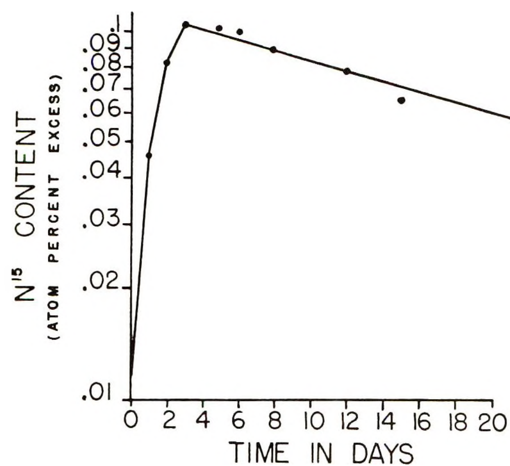

Fig. 2 Incorporation of N^{15} into plasma protein from N^{15} urea; subject W.H.

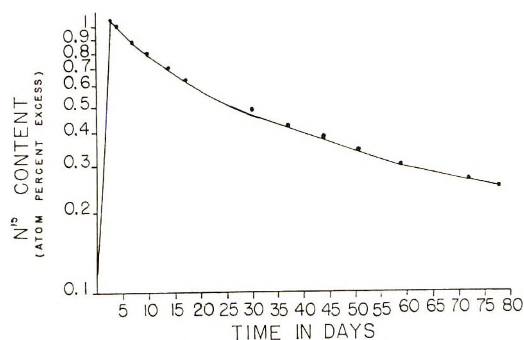

Fig. 3 Incorporation of N^{15} into plasma protein from N^{15} ammonium chloride; subject R.I.

the case of hemoglobin the accepted figure of 0.83% daily replacement was used to make a similar estimation.

RESULTS

Individual protocols

1. *Girl W.H.* (fig. 4), birth weight 2.25 kg, was one month old and weighed 2.55 kg when she became a subject in the study. Her protein intake was reduced from 5.05 gm/kg to 2.6, and then to 2.0 gm at which point a decreased weight gain was noticeable. Weight gain ceased entirely when she was given 1.65 gm of protein/kg. When glycine was added to the diet at this point, gain in weight was resumed at the original rate. This supplement permitted a further reduction of the protein intake to 1.32 gm/kg without alteration in the rate of weight gain. However, when the protein intake was reduced to 1.0 gm/kg, weight gain ceased for a second time. Presumably another limiting factor in the form of an essential amino acid was encountered. The intake was then returned to 1.32 gm of protein/kg and weight gain continued at the same rate for the entire three-week trial period. At this point, urea was substituted for the glycine and the good weight gain continued for the next two weeks.

2. *Boy W.H.* (fig. 5, table 3) was the fraternal twin of girl W.H. His birth weight was 2.35 kg. He was three months old and weighed 4.3 kg when he was first included in the study. When provided with 1.93 gm of protein/kg he gained weight at the same rate as he had during the pre-study periods which had provided 4.0 gm of protein/kg. But, when his protein intake was reduced to 1.51 gm/kg, weight gain declined. Supplementation of the diet with glycine allowed resumption of the normal rate of weight gain and with this supplement the protein intake was further reduced to 1.22 gm/kg without any effect on the weight gain. It was only when the protein intake was reduced to 0.91 gm/kg that the weight gain virtually ceased. The protein intake was then returned to 1.22 gm/kg and various supplements were tried. The substitution of an isonitrogenous amount of urea for the glycine allowed weight gain of equal magnitude except during a short period when the child had an intercurrent upper respiratory tract infection. Nitrogen retentions, first obtained at this time, demonstrated retentions of the same magnitude as in infants of comparable ages who received the same amount of nitrogen as whole protein. A supplement of glucose was then substituted for the urea. With this the weight gain was a little inferior to that with the urea supplement; however, the nitrogen retention decreased sharply. Another trial of urea supplementation was then instituted and continued for over a month; this resulted in good weight gain except during an intercurrent respiratory infection; the nitrogen retention remained at a high level.

To determine whether the nitrogen given to the patient was being used to form new tissue, labeled urea was given

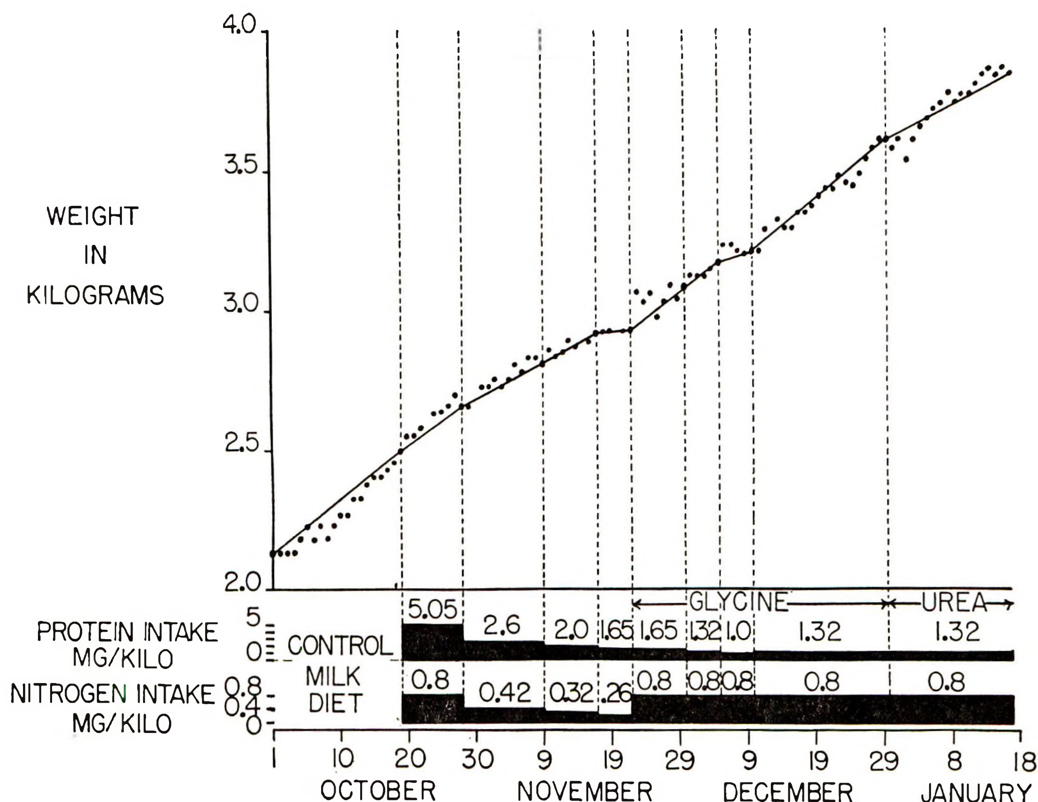

Fig. 4 Girl baby W.H.

for a three-day period, 18.6 gm of 10.2 atom % excess N^{15} urea being substituted for the usual urea supplement. Blood was drawn every 24 hours for the first three days and every two-to-three days for the next two weeks. Determination of the N^{15} content of the plasma protein and the hemoglobin demonstrated that the urea was being used in significant amounts to produce new proteins. These results are recorded graphically in figures 2 and 6, from which it is calculated that *13%* of the nitrogen of the plasma protein formed during the period of N^{15} administration and *4.9%* of the nitrogen of the hemoglobin formed at this time were derived from the labeled urea.

3. *Boy R.I.* (fig. 7, table 4), birth weight 3.5 kg, was three weeks old and weighed 4.2 kg when he became a subject in the study. He gained weight well with 3.1 gm of protein/kg, and somewhat less well with 1.43 gm/kg. When the protein intake was decreased to 1.1 gm/kg performance was further impaired and with 0.82 gm/kg, weight gain and nitrogen retention virtually ceased. At this point a urea supplement was tried for several days, but weight gain was not resumed. However, when the protein intake was increased to 1.1 gm/kg the urea supplement was effective in permitting a good rate of weight gain and adequate nitrogen retention. When urea was withdrawn for a 4-day period, weight gain fell off immediately and nitrogen retention was greatly impaired. Replacing the urea resulted in immediate weight gain and improved nitrogen retention. The child's weight curve remained very close to the seventy-fifth percentile curve throughout the study period.

While receiving a urea supplement he was given 0.96 gm of N^{15} ammonium chloride over a three-day period to demonstrate its rate of incorporation into

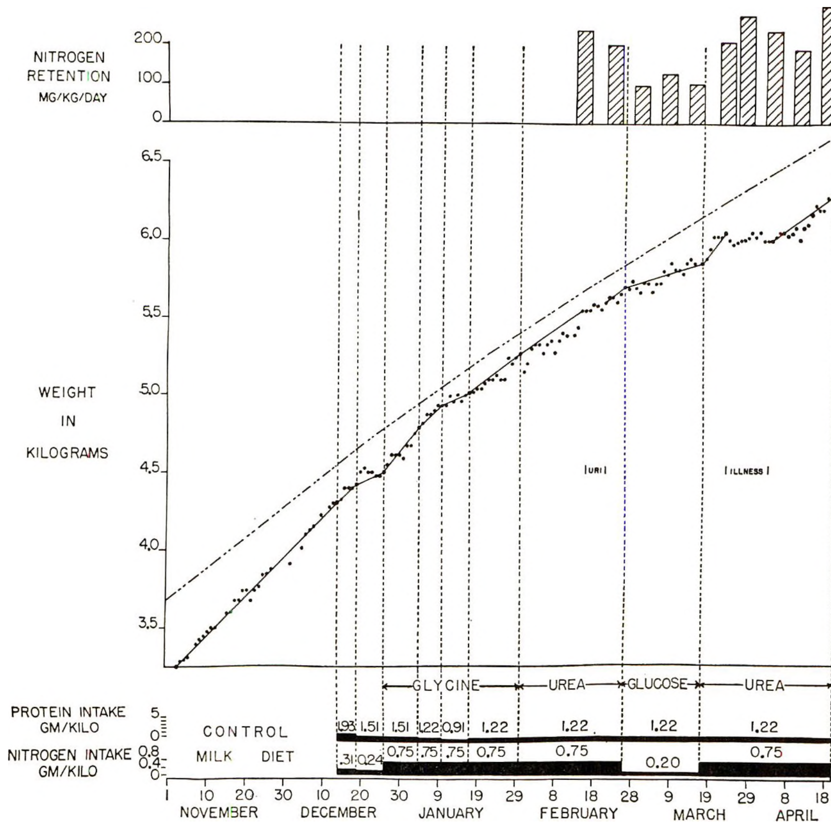

Fig. 5 Boy baby W.H.; dashed line indicates the third percentile of normal growth.

plasma protein and hemoglobin. Blood was drawn at intervals over the next month. Figures 3 and 8 are graphic demonstrations of the extent of such incorporation. Calculations indicate that *14%* of the nitrogen of the plasma protein synthesized during this period and 3.5% of the nitrogen of the newly formed hemoglobin were derived from the ammonium chloride supplement.

4. *Baby C.O.* (fig. 9, table 5), birth weight 3.48 kg, was three weeks old and had attained a weight of 4.0 kg when he was included in the study. A protein intake of 1.75 gm per kg per day allowed good weight gain; this was less than satisfactory when the intake was reduced to 1.5 and 1.1 gm/kg. The nitrogen retentions showed the expected decrease as the nitrogen intake was reduced. The addition of urea to the diet at this point resulted in improved weight gain and a

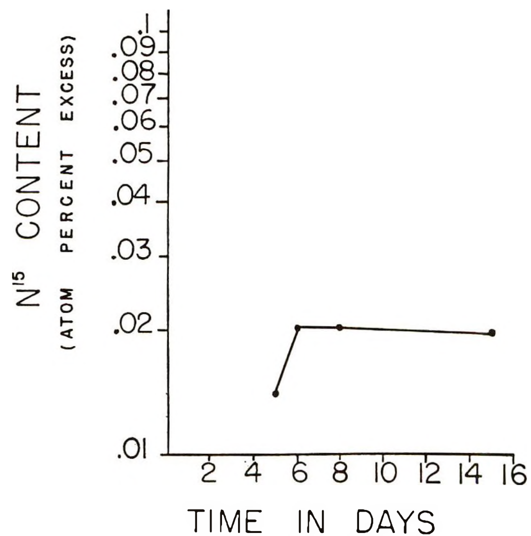

Fig. 6 Incorporation of N^{15} into hemoglobin from N^{15} urea; subject W.H.

TABLE 3

Metabolic data on boy W. H.

Date	2/13–18	2/20–23	2/27–3/1	3/5–8	3/12–15	3/21–24	3/26–29	4/2–5	4/9–12	4/16–19
Avg wt., kg	5.57	5.61	5.69	5.75	5.85	6.05	6.01	6.02	6.07	6.24
Avg wt. gain/ day, gm	56	21	0	42	28	28	28	17.5	14	24
Protein intake, gm/kg	1.22	1.22	1.22	1.22	1.22	1.22	1.22	1.22	1.22	1.22
Supplement	urea	urea	glucose	glucose	glucose	urea	urea	urea	urea	urea
Nitrogen intake, gm/day	3.95	4.12	1.06	1.19	1.12	3.98	4.08	4.19	4.10	4.40
Urine nitrogen, gm/day	2.42	2.73	0.36	0.32	0.30	2.49	2.09	2.45	2.76	2.27
Stool nitrogen, gm/day	0.18	0.23	0.13	0.14	0.19	0.18	0.31	0.29	0.21	0.22
Nitrogen retention, mg/kg/day	242	206	101	127	108	217	279	241	137	306
Plasma protein, gm/100 ml	5.01		5.34	5.92				5.54	5.48	
Albumin, gm/ 100 ml	2.93		3.02	3.54				3.24	3.10	
Globulin, gm/ 100 ml	2.08		2.32	2.38				2.30	2.38	
Nonprotein nitrogen, mg/100 ml	44							29.8		
Urine creatinine mg/kg/day	12.8	13.0	11.5	11.8	11.0					11.0

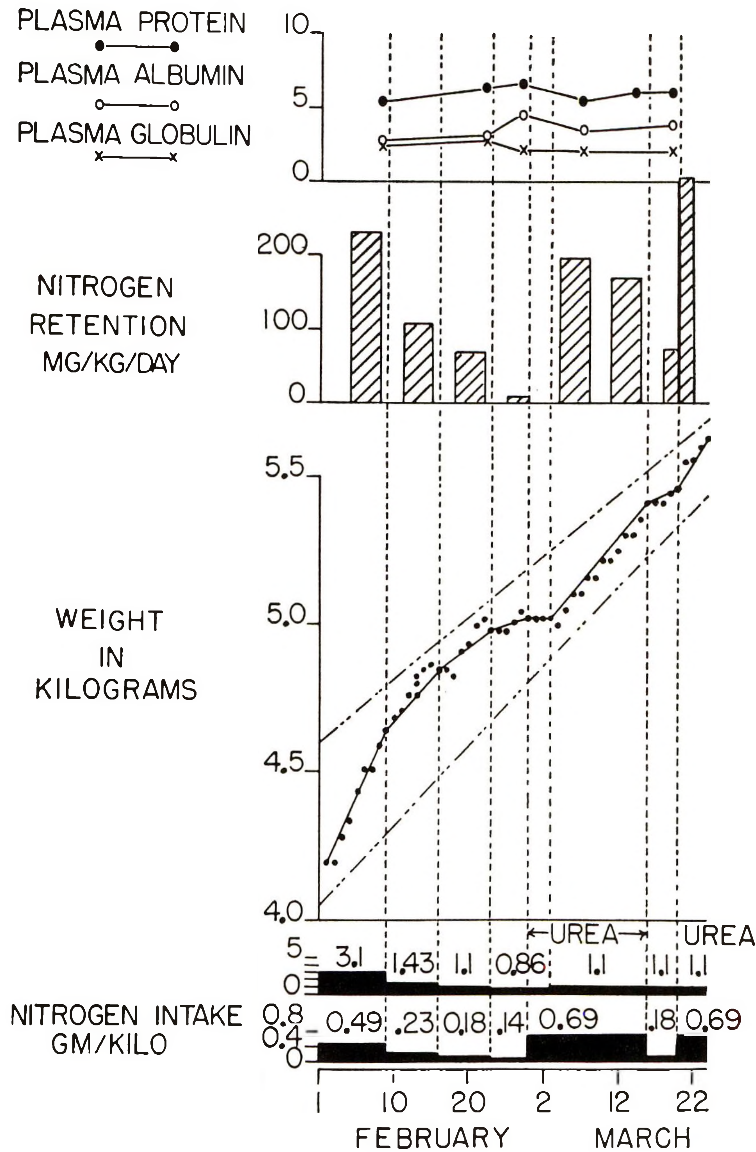

Fig. 7 Baby R.I.; dashed lines indicate the fiftieth and seventy-fifth percentile curves of weight gain.

sharp increase in the amount of nitrogen retained. Performance was, however, further improved when the protein intake was increased to 1.35 gm/kg. This continued with an intake of 1.1 gm of protein/kg supplemented with urea which allowed weight gain and nitrogen retention to proceed at their previous rates. This better performance on the second trial with this diet is probably related to the increasing age of the infant.

At this point an attempt was made to determine the second most limiting nitrogen component of a milk diet. The urea supplement was continued and the protein further reduced to 0.82 gm/kg. This resulted in a depression of the rate of weight gain and of the amount of nitro-

TABLE 4

Metabolic data on boy R. I.

Date	2/4–7	2/11–14	2/18–21	2/25–28	3/4–7	3/11–14	3/18–19	3/20–21
Avg wt., kg	4.44	4.78	4.91	5.0	5.06	5.25	5.44	5.51
Avg wt. gain/day, gm	63	35	35	14	35	42	14	56
Protein intake, gm/kg	3.1	1.43	1.1	0.86	1.1	1.1	1.1	1.1
Supplement					urea	urea		urea
Nitrogen intake, gm/day	2.14	1.04	0.88	0.68	3.48	3.49	0.96	3.52
Urine nitrogen, gm/day	0.77	0.31	0.31	0.30	2.28	2.38	0.39	1.67
Stool nitrogen, gm/day	0.35	0.23	0.24	0.23	0.20	0.19	0.17	0.17
Nitrogen retention, mg/kg/day	230	105	67	3	198	175	74	304
Plasma protein gm/100 ml	5.59		6.34	6.86	5.50	6.04	5.94	
Albumin, gm/100 ml	2.80		3.12	4.72	3.38		3.87	
Globulin, gm/100 ml	2.79		3.22	2.14	2.12		2.07	
Urine creatinine mg/kg/day	14.4	13.6	13.6	13.1	13.5	13.7	13.3	13.9

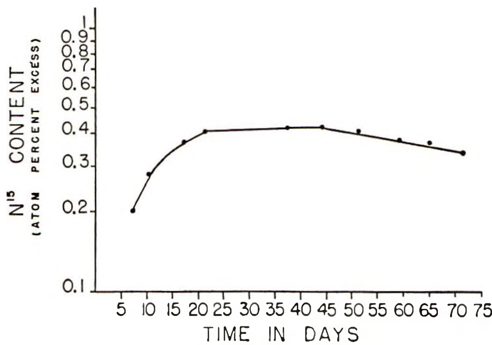

Fig. 8 Incorporation of N^{15} into hemoglobin from N^{15} ammonium chloride; subject R.I.

gen retained. The attempt was then made to supplement the diet with the amino acid that has commonly been regarded as the most limiting in milk, namely, methionine. When an added suppement of 50 mg of methionine/kg was given at this point, weight gain and nitrogen retention were much improved. The withdrawal of methionine resulted in an immediate reversal of this improvement. This observation, which requires further study, suggests that methionine is the second limiting nitrogenous component of milk diet.

Metabolic data

Metabolic data from the three male subjects in this study are presented in tables 3, 4 and 5. In general, the urinary excretion of nitrogen varied with the nitrogen intake and the stool nitrogen remained constant. This is in keeping with observations made by numerous other investigators who have carried out nitrogen balance studies in growing infants.

There was some fluctuation in the plasma protein levels which could not be related to the dietary changes. On two occasions, determinations of the plasma nonprotein nitrogen were normal despite the prolonged high intake of urea.

Analyses of the free amino acids of the plasma of boy R.I. were performed on three occasions of low-protein regimens, on one of which a urea supplement was given. Borderline levels were found for all the essential amino acids except for phenylalanine, threonine and methionine. Leucine, valine and lysine were slightly below our previous normal figures. With the exception of tyrosine, the unessential amino acids tended to remain at normal levels. The levels of alanine and glycine

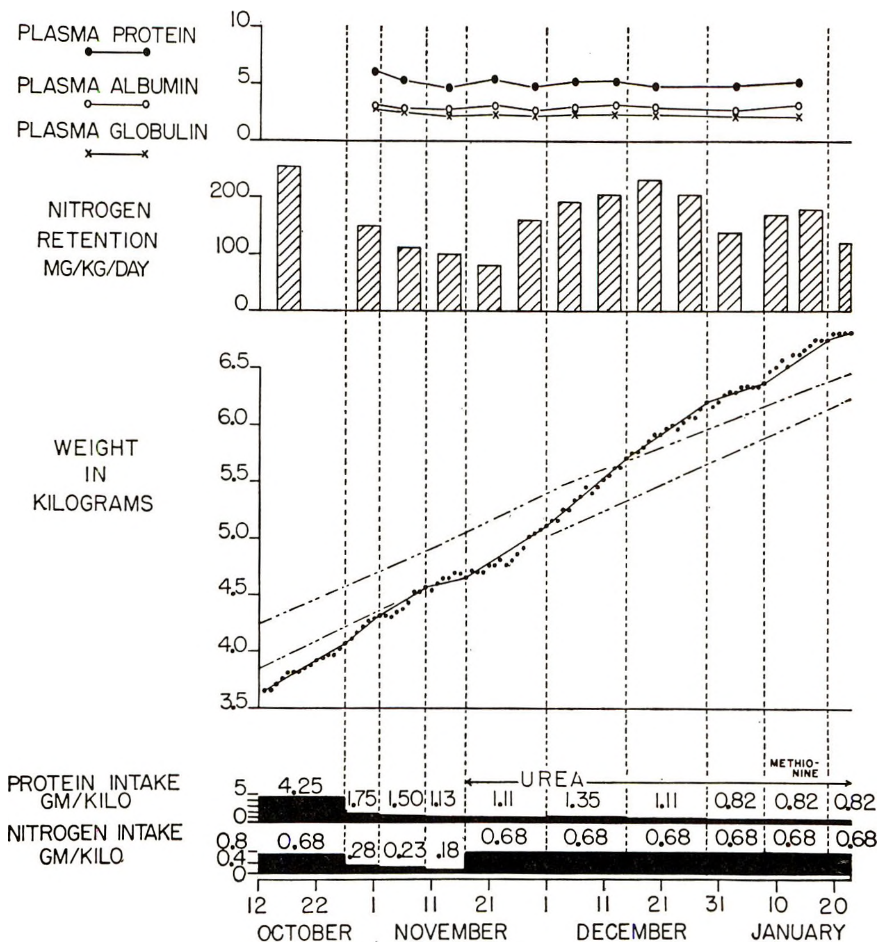

Fig. 9 Baby C.O.; dashed lines indicate the fiftieth and seventy-fifth percentile curves of weight gain.

were consistently higher than normal. The depressed amino acids increased somewhat during the period when 1.1 gm of protein supplemented with urea were fed (this was adequate to maintain normal weight gain and nitrogen retention). One determination of the plasma amino acid levels of baby C.O. when receiving 0.82 gm of protein/kg supplemented with urea which was clearly inadequate for him demonstrated similar but more marked alterations. These data are listed in table 6.

The administration of tagged unessential nitrogen demonstrated that such nitrogen was being incorporated into plasma protein and hemoglobin. An effort was made to determine into which amino acids such nitrogen was being incorporated by fractionating plasma protein obtained from subject R. I. on the day the N^{15} incorporated was at its peak. The protein obtained from 10 ml of plasma was hydrolyzed and 6 amino acid fractions were separated by column chromatography.[12] The amino acids and their content of N^{15} are listed in table 7. Heavy labeling was found only in the fractions containing unessential amino acids.

The data suggest that this nitrogen was incorporated into the unessential amino acids of plasma protein.

[12] We should like to express our thanks to Roland G. Westall for performing this separation.

TABLE 5

Metabolic data on baby C. O.

Date	10/29–11/1	11/5–8	11/12–15	11/19–22	11/26–29	12/3–6	12/10–13	12/17–20	12/24–27	12/31–1/3	1/7–1/10	1/14–17	1/21–22
Avg wt., kg	4.29	4.46	4.61	4.72	5.02	5.24	5.54	5.83	6.02	6.27	6.41	6.64	6.82
Avg wt. gain/day, gm	42	49	42	49	63	51	49	43	49	28	42	42	42
Protein intake, gm/kg	1.75	1.50	1.13	1.11	1.11	1.35	1.35	1.11	1.11	0.82	0.82	0.82	0.82
Supplement				urea	urea	urea	urea	urea	urea	urea	urea + methionine	urea + methionine	urea
Nitrogen intake, gm/day	1.13	1.04	0.82	3.16	3.26	3.31	3.57	3.80	4.0	4.04	4.26	4.37	4.48
Urine nitrogen, gm/day	0.27	0.29	0.19	2.58	2.21	2.08	2.23	2.21	2.57	2.90	2.94	2.99	3.52
Stool nitrogen gm/day	0.20	0.26	0.16	0.22	0.23	0.22	0.20	0.22	0.19	0.24	0.22	0.18	0.21
Nitrogen retention, mg/kg/day	154	110	101	77	160	193	206	228	206	143	171	181	110
Plasma protein, gm/100 ml	5.98	5.29	4.68	5.32	4.72	5.12	5.23	4.91		4.76			
Albumin, gm/100 ml	3.02	2.60	2.52	3.04	2.52	2.92	3.07	2.83		2.55			
Globulin, gm/100 ml	2.96	2.69	2.16	2.28	2.02	2.20	2.16	2.08		2.21			
Urine creatinine, mg/kg/day	12.8	15.1	14.1	13.9	12.9	15.5	13.3	10.4	12.4	12.1	11.5	11.4	12.2

TABLE 6
Plasma amino acid levels

	Boy R. I.			Baby C. O.	Normal range
Date	3/1	3/14	3/20	1/23	
Protein intake, gm/kg	0.86	1.1 +urea	1.1	0.82 +urea	
Alanine	3.3	5.8	4.5	4.6	1.4 –3.5
Arginine	0.5	0.7			0.6 –1.3
Aspartic acid	0.1		0.2	0	0 –0.3
α-NH_2-Butyric acid	0	0.4	0	0.1	0 –0.5
Glutamic acid	0.9	1.6	1.4	2.2	0.89–6.54
Glycine	2.2	2.5	2.1	2.0	0.9 –1.76
Histidine	0.9	0.7	0.6	0.6	0.64–1.3
Isoleucine	0.4	0.9	0.6	0.6	0.52–1.9
Leucine	0.6	1.0	0.9	0.7	0.98–3.0
Lysine	0.8	1.0	0.9	0.5	1.2 –3.5
Methionine	0.3	0.6	0.3	0.4	0.27–0.71
Ornithine	0.5	0.8	0.5	0.6	0.4 –0.9
Phenylalanine	0.8	0.5	0.7	0.5	0.4 –1.9
Proline	1.3	2.0	1.3	1.7	1.39–4.8
OH proline	1.7	0.9	0.7	0	
Serine	1.4	1.7	1.9	1.7	0.98–2.4
Taurine	0.7	0.9	1.2	1.4	0.16–1.3
Threonine	1.0	1.4	1.2	0.9	0.78–2.5
Tyrosine	0.5	0.6	0.5	0.6	1.0 –4.5
Valine	0.9	1.3	1.2	1.1	1.46–4.7

TABLE 7
Incorporation of N^{15} into various amino acid fractions of plasma protein

Amino acid fraction	N^{15} content
	atom % excess
Aspartic and glutamic acids	2.28
Proline	0.37
Serine and threonine	1.145
Ammonia	0.507
Lysine	0.06
Leucine and isoleucine	0.427

DISCUSSION

The results of this study show clearly that the essential amino acids are not the first limiting nitrogenous factor in milk protein, and indirect evidence indicates that this is true of other proteins also. This conclusion is at variance with the commonly accepted view, although it has been suggested by a few investigators. Thus Allison ('58) stated that "feeding sufficient egg protein to meet the requirements for maintenance supplies at least twice the minimum requirements for amino acids. . . . It may be possible to reduce the amount of egg protein below so-called maintenance requirements if some form of non-essential nitrogen is added to supply the deficit in nitrogen. It might even be said that the primary need is for nitrogen." He goes on to stress the (presumably secondary) need for a desirable pattern of essential amino acids. The practical implications of this are obvious. One is not justified in concluding that because minimal requirements for all essential amino acids are met the diet necessarily provides an adequate protein intake. The protein requirements may actually be two or three times this figure. It is perhaps fortunate that Rose suggested doubling his minimal requirement figures to obtain "safe practical allowances." His minimal requirement figures and those of others who have determined the requirements of adults (Leverton et al., '56a,b,c,d,e; Swendseid et al., '56, '61; Jones et al., '56) were determined under experimental conditions in which a large excess of unessential nitrogen was provided in the diet, far more than would be provided by any natural diet. In the case of the studies of amino acid requirements of infants made by the New York University group (Snyderman et al., '61), a marked excess of unessential nitrogen was avoided and the amino acid requirement figures, although they

do not represent true absolute minima, have more practical significance.

The observations in the present study have a definite bearing on the problem of evaluating protein quality. The desirability of an accurate method of scoring protein based on composition is obvious, but, as has been pointed out, presently available procedures give only a crude correlation between calcualted score and nutritional value as determined by nitrogen balance techniques. A more refined scoring procedure is needed. If, as it now appears, the first limiting factor in many proteins is unessential nitrogen, it follows that this moiety must be taken into account in any scoring procedure. Not only the quantity, but the pattern of the unessential amino acids, appears to be important. A number of recent studies (Frame, '58; Frost and Sandy, '51; Rechcigl et al., '57; Birnbaum et al., '57) indicate that unessential nitrogen compounds are not all alike in their ability to provide for the unessential nitrogen requirements of the body. Recent reports by Gitler[13] and by Thiessen[14] indicate that the pattern of unessential amino acids in whole egg is superior to that of casein. It is undeniable that the pattern of the essential amino acids exercises an important influence on the nutritional quality of the protein even though all of the amino acids are provided in quantities well beyond minimal requirements. How this effect is exerted is not altogether clear, but one reasonable explanation is that the total quantity of amino acid does not represent the quantity available for metabolic purposes. An excess of one amino acid may competitively interfere with the utilization of another in some metabolic process. The best known example of such amino acid antagonism is that of leucine and isoleucine (Harper et al., '55), but a number of others are coming to light (Snyderman et al., '60). With our present limited knowledge of the effectiveness of various unessential nitrogen compounds and our incomplete information about amino acid antagonisms which may reduce the availability of amino acids, it does not seem profitable to attempt to formulate a new system of scoring that takes the unessential amino acids into consideration. This is a task for the future.

The practical value of using simple nitrogenous compounds to supplement protein-deficient diets also comes up for consideration. Up to the present time the emphasis has been on supplementation with essential amino acids, since these were supposed to be the first limiting factors. It now appears that there is an area for effective supplementation with the relatively inexpensive unessential amino acids as well as with other simple nitrogenous compounds such as urea and ammonium salts that needs to be further explored. The value of urea as a source of nitrogen for ruminant animals is well established (Watson, '49; Read, '53) but its utilization by nonruminants has been a controversial matter, variable results being reported. One cause for these discrepancies appears to be the variability in the need for such nitrogen under the experimental conditions. Foster et al. ('49) showed that when rats were placed under stress in two ways, incorporation of N^{15} ammonium into amino acids could be demonstrated readily; that administration of tagged ammonium citrate to rats maintained with a low-glycine diet supplemented with benzoic acid resulted in the appearance of substantial quantities of N^{15} in the urinary hippuric acid; and that with a low-protein diet, the tagged ammonium citrate was rapidly incorporated into tissue proteins. Rose et al. ('49) were able to make rats fed low-protein diets gain weight notably by supplements of urea, ammonia, glutamic acid or glycine. In later studies Rose and Dekker ('56) carried out studies with N^{15} urea in rats fed 8.8 and 18% of protein. Incorporation of N^{15} into the carcass proteins was minimal with the higher protein intake but was increased 15- to 20-fold with the low-protein diet, the labeled nitrogen being found almost exclusively in the unessential amino acids. Our studies indicate that the growing child, at least under conditions of protein shortage, is

[13] Gitler, C. Communication to the amino acid committee of the Food and Nutrition Board, February, 1962.

[14] Thiessen, R. Communication to the amino acid committee of the Food and Nutrition Board, February, 1962.

capable of utilizing urea and ammonium for protein synthesis.

SUMMARY

Four infants were fed decreasing quantities of milk protein with constant calories, in order to ascertain the most limiting amino acid of the diet. It was found that when weight gain and nitrogen retention eventually suffered, this could be restored to normal by the administration of unessential nitrogen in the from of glycine or urea. Thus unessential nitrogen appears to be the most limiting factor.

The incorporation of unessential nitrogen into the hemoglobin and plasma proteins of infants fed a low-protein intake was demonstrated by studies in which N^{15} urea and N^{15} ammonium chloride were given.

In one instance a further reduction of protein intake was attempted in order to discover the second most limiting nitrogenous factor. Some evidence was obtained that this was methionine.

The implications of these observations for the determination of protein requirements, for the evaluation of protein quality and for the practical problem of supplementation are discussed.

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Effect of Nitrate and Nitrite on Vitamin A Storage in the Rat[1]

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The high nitrate content of some forages has been suggested as a factor responsible for precipitating vitamin A deficiencies in livestock receiving levels of carotene thought to be adequate (Garner, '58). A microbial reduction of nitrate in the rumen with the subsequent accumulation of some of its reduction products, including nitrite, is well known. The feeding of 0.3% of potassium nitrite was reported by O'Dell et al.[2] to cause an increased rate of depletion of vitamin A stores in the liver of rats. Several other workers, among them Muhrer et al.,[3] Smith et al.[4] and Holst et al.,[5] have reported that the feeding of rations having a high nitrate content had an adverse effect upon the vitamin A status of animals.

The experiments reported herein were conducted to determine whether nitrate or its reduction product, nitrite, interferes with the storage of vitamin A in the liver, either from a preformed source or from carotene, under controlled experimental conditions.

EXPERIMENTAL

In each of three trials, Sprague-Dawley female rats were given a low vitamin A diet (table 1) for a period of two to three weeks to deplete liver vitamin A stores. After this time, the rats were divided into 9 groups of 8 rats each for each trial, and were fed the following diets: groups 1, 2 and 3, control (low vitamin A diet, table 1); groups 4, 5 and 6, control plus 3% of sodium nitrate; groups 7, 8 and 9, control plus 0.5% of sodium nitrite. These diets were fed for a period of 6 days in all instances. On the third and fourth days, vitamin A or carotene sources were administered. On the sixth day, the animals were killed and the livers were immediately frozen. Liver carotene or vitamin A, or both, were determined at a later date by the method of Johnson and Baumann ('47a). The vitamin A sources used in these studies were analyzed colorimetrically as described in an industrial bulletin[6] with the exception that the oil solution was analyzed without prior saponification. The procedure used in these experiments for measuring the storage of vitamin A is based on the liver storage test of Guggenheim and Koch as modified by Foy and Morgareidge ('48).

TABLE 1
Vitamin A depletion diet fed to rats

	% of diet
Cornstarch	65
Casein, vitamin-free[1]	18
Brewer's yeast	8
Salt mixture[2]	4
Fortified corn oil[3]	5

[1] "Vitamin Free" Casein, Nutritional Biochemicals Corporation, Cleveland.
[2] Philips and Hart ('35).
[3] Fortified corn oil contained approximately 40 IU vitamin D as irradiated ergosterol and 1 mg DL-α-tocopheryl acetate/gm. This supplied 2 IU vitamin D and 0.05 mg α-tocopheryl acetate/gm of complete diet.

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[1] Published with the approval of the Director of the South Dakota Agricultural Experiment Station as publication no. 546 of the journal series. The vitamin A sources were kindly supplied by Nopco Chemical Company, Newark, New Jersey.

[2] O'Dell, L. B., Z. Erek, L. Flynn, G. B. Garner and M. E. Muhrer 1960 Effects of nitrate containing rations in producing vitamin A and vitamin E deficiencies in rats. J. Animal Sci., *19*: 1280 (abstract).

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[5] Holst, W. O., L. M. Flynn, G. B. Garner and W. H. Pfander 1961 Dietary nitrite vs. sheep performance. Ibid., *20*: 936 (abstract).

[6] A colormetric assay for vitamin A. Technical Service Bureau Publication VA-84. Nopco Chemical Company, Harrison, New Jersey, 1958.

TABLE 2

Body and liver weights of rats receiving various dietary treatments[1]

Trial	Treatment	Control diet			3% $NaNO_3$ diet			0.5% $NaNO_2$ diet		
		Group no.	Final body wt	Liver wt	Group no.	Final body wt	Liver wt	Group no.	Final body wt	Liver wt
			gm	*gm*		*gm*	*gm*		*gm*	*gm*
1[2]	None	1	120	5.6	4	114	4.5	7	107	4.3
	Vitamin A, oral	2	124	6.0	5	124	5.6	8	115	4.4
	Vitamin A, injected	3	118	5.6	6	122	5.0	9	111	4.3
2[3]	None	1	132	5.8	4	124	5.4	7	116	4.8
	Vitamin A, oral	2	132	7.1	5	122	5.4	8	119	5.3
	Vitamin A, injected	3	135	6.8	6	128	5.5	9	119	5.4
3[4]	None	1	142	6.7	4	135	6.0	7	129	6.2
	Carotene, 1 mg/rat	2	144	7.5	5	138	6.9	8	129	6.4
	Carotene, 1.7 mg/rat	3	144	7.6	6	139	6.9	9	130	6.6

[1] Average body weights for rats prior to receiving the experimental diets were as follows: trial 1, 108; trial 2, 115; trial 3, 128.

[2] Vitamin A source for trial 1 was vitamin A palmitate dissolved in sesame oil. Total dosage was equivalent to 294 μg of vitamin A alcohol/rat.

[3] Vitamin A source for trial 2 was vitamin A palmitate in a carbohydrate base (Capstress, Nopco Chemical Company, Harrison, N. J.) dispersed in water. Total dosage was equivalent to 210 μg of vitamin A alcohol/rat.

[4] Carotene source was pure β-carotene dissolved in corn oil.

In view of the report that nitrate reduces feed consumption and lowers weight gain,[7] the length of time that the rats were fed diets with added nitrate or nitrite was held to a minimum, i.e. 6 days. Vavich and Kemmerer ('50) have shown that although the size of the rat is an important factor in influencing the amount of vitamin A stored (an inverse relationship), the amount of diet consumed has no effect on vitamin A storage, when intake of the vitamin is held constant.

Trial 1. Vitamin A palmitate dissolved in sesame oil (9800 IU/ml) was administered orally from a blunt pointed syringe at the rate of 0.05 ml/rat on each of two consecutive days (total dosage, 980 IU/rat) to groups 2, 5 and 8; and by subcutaneous injection to groups 3, 6 and 9. Groups 1, 4 and 7 remained untreated.

Trial 2. Water-dispersed vitamin A palmitate (3500 IU/ml) was administered orally at the rate of 0.1 ml/rat on each of two consecutive days (total dosage, 700 IU/rat) to groups 2, 5 and 8; and by subcutaneous injection to groups 3, 6 and 9. Groups 1, 4 and 7 remained untreated.

Trial 3. Pure β-carotene was dissolved in corn oil and administered by stomach tube at the rate of 1 mg of β-carotene (total dosage)/rat for groups 2, 5 and 8; and 1.7 mg/rat for groups 3, 6 and 9. In each instance, one-half of the carotene dosage was administered on two consecutive days in 0.5 ml of oil/day. As in the previous two trials, groups 1, 4 and 7 were not treated.

RESULTS AND DISCUSSION

Despite the relatively short experimental period, differences in average final weights (table 2) were apparent with the lower weights generally being associated with nitrate, and especially nitrite feeding. These weight differences were also reflected in the liver weights.

Data pertaining to the liver storage of vitamin A and carotene in the various trials are shown in table 3. In trial 1, oral administration of the oil solution of vitamin A resulted in an increase in liver

[7] Weichenthal, B. A., R. J. Emerick, L. B. Embry and F. W. Whetzal 1961 Influence of nitrate on performance and vitamin A status of fattening cattle. J. Animal Sci., 20: 955 (abstract).

TABLE 3

Average liver vitamin A and carotene levels of rats receiving nitrate or nitrite and given various vitamin A or carotene treatments

Trial	Treatment	Control diet			3% $NaNO_3$ diet			0.5% $NaNO_2$ diet		
		Group no.	Carotene	Vitamin A	Group no.	Carotene	Vitamin A	Group no.	Carotene	Vitamin A
			μg	μg		μg	μg		μg	μg
1[1]	None	1	—	2.6	4	—	1.8	7	—	2.7
	Vitamin A, oral	2	—	49.7	5	—	49.7	8	—	30.9[2]
	Vitamin A, injected	3	—	4.6	6	—	5.8	9	—	18.1[2]
2[3]	None	1	—	1	4	—	1	7	—	1
	Vitamin A, oral	2	—	107.2	5	—	104.0	8	—	86.8[2]
	Vitamin A, injected	3	—	20.8	6	—	17.3	9	—	23.3
3[4]	None	1	4.2	3.0	4	4.1	3.8	7	4.7	3.2
	Carotene, 1 mg/rat	2	7.8	98.1	5	5.7[5]	74.3[5]	8	7.6	64.6[2]
	Carotene, 1.7 mg/rat	3	13.2	152.9	6	9.6[2]	129.8[5]	9	10.4[2]	87.6[2]

[1] Vitamin A source for trial 1 was vitamin A palmitate dissolved in sesame oil. Total dosage was equivalent to 294 μg of vitamin A alcohol/rat.
[2] Significantly different from values obtained from rats receiving similar treatment in control diet ($P < 0.01$).
[3] Vitamin A source for trial 2 was vitamin A palmitate in a carbohydrate base (Capstress, Nopco Chemical Company) dispersed in water. Total dosage was equivalent to 210 μg of vitamin A alcohol/rat.
[4] Carotene source was pure β-carotene dissolved in corn oil.
[5] Significantly lower than values obtained from rats receiving similar treatment in control diet ($P < 0.05$).

vitamin A levels representing an average of 26.2 and 26.6% of the administered dose in the rats receiving the control and the nitrate diet, respectively. Vitamin A storage of the oral dose was significantly reduced ($P < 0.01$) in the nitrite-fed rats. In this instance, storage was reduced to 15.7% of the administered dose. Injection of the oil-vitamin A solution resulted in very small amounts of vitamin A appearing in the liver of the rats fed the control and nitrate diets. However, storage of the injected dose was significantly higher ($P < 0.01$) in the rats receiving nitrite. This variation remains unexplained at this time.

Oral administration of a water-dispersed source of vitamin A (trial 2) resulted in an increase in liver vitamin A levels for all groups. This amounted to 59.5, 57.8 and 48.2% of the administered dose in the control, nitrate and nitrite groups respectively (table 3). As with the orally administered oil solution in the previous trial, storage of the orally administered water-dispersed vitamin A was significantly reduced ($P < 0.01$) by nitrite, but not nitrate feeding. Liver storage of the injected water-dispersed vitamin A was considerably less than that obtained by oral administration, and amounted to only 9.9, 8.2 and 11.1% of the administered dose for groups 3, 6 and 9, respectively. Thus, neither nitrate nor nitrite had a significant effect on the liver storage of the injected vitamin A. The small increase in storage in the group fed nitrite (group 9), however, is consistent with the observations made in trial 1.

Results of trials 1 and 2 indicate that absorption and subsequent liver storage of vitamin A, during the short time allowed, was less when administered by injection than when administered orally. It must be recognized, however, that the data are not adequate for comparing the effectiveness of these two methods of administration in promoting growth or liver vitamin A storage, when longer periods of time are involved.

Administration of two levels of carotene to rats receiving the control diet (trial 3) resulted in increases of liver vitamin A levels equivalent to 9.5 and 8.8% of the respective 1.0- and 1.7-mg carotene dos-

ages (table 3). Liver storage of vitamin A in the rats dosed with carotene was significantly reduced by the feeding of nitrate ($P < 0.05$) and nitrite ($P < 0.01$). The respective increases in liver vitamin A levels resulting from the 1.0- and 1.7-mg carotene dosages amounted to 7.0 and 7.4% for the nitrate-fed groups (groups 5 and 6), and 6.1 and 5.0% for the nitrite-fed groups (groups 8 and 9). Liver accumulations of carotene in this trial were small, with both nitrate and nitrite causing significantly lower levels in most instances.

In these experiments, the feeding of nitrite, but not nitrate, resulted in a reduction in the amount of vitamin A stored in the liver when vitamin A was administered orally, but not when administered by subcutaneous injection. This suggests that the action of nitrite in reducing liver storage of the orally administered vitamin A was the result of either a decrease in absorption of the vitamin from the intestine, or an increase in its destruction within the digestive tract. A reduction in vitamin A absorption by nitrite has not been demonstrated. However, Olson et al.[8] observed that a destruction of the provitamin, carotene, occurred when nitrite was present under acid conditions. In this study, the decreased storage of vitamin A from orally administered carotene in the presence of dietary nitrite was of a magnitude that would suggest a possible implication of the same mechanisms whereby the detrimental action of nitrite on preformed vitamin A was accomplished. The effect of nitrate on vitamin A storage from a carotene source, however, cannot be explained on the same basis. Olson et al.[9] showed that nitrate itself caused no destruction of carotene under a variety of conditions. The finding that the nitrate ion has an effect on thyroid function (Wyngaarden et al., '52; Bloomfield et al., '61), and the observation of Johnson and Baumann ('47b) that a functioning thyroid gland is necessary for the conversion of carotene to vitamin A, suggest a possible mechanism for the effect of nitrate on vitamin A storage from a carotene source. The possibility, however, that the nitrate effect is the result of a lowered absorption of carotene should not be overlooked. The need for additional work in this area is indicated.

Although the data obtained in these experiments, indicating an effect of nitrate or nitrite on the vitamin A status of rats, differ from results reported in some cattle and sheep studies,[10,11,12] they are in agreement with the *in vitro* data obtained by Olson et al.[13] which showed that sodium nitrite mixed with acid stomach contents destroyed carotene rapidly. Since the reduction of nitrate and absorption of reduction products in the ruminant animal often occur under pH conditions approaching neutrality, the difference sometimes obtained between ruminants and nonruminants appears to be expected.

SUMMARY

Rats receiving diets with and without 3% of sodium nitrate or 0.5% of sodium nitrite were administered vitamin A palmitate in oil solution or in a water dispersion, either orally or by subcutaneous injection, or β-carotene in oil solution by stomach tube. In all instances, during the brief periods allowed in these studies, the water dispersed source of vitamin A contributed to liver storage to a much greater extent than the oil solution, and the orally administered sources to a much greater extent than the injected sources. The feeding of nitrite, but not nitrate, significantly lowered liver storage of vitamin A from orally administered sources of preformed vitamin A, but not from injected sources. Both nitrate and nitrite significantly lowered the liver storage of vitamin A from carotene with the greatest effect resulting from nitrite. Possible mechanisms were discussed.

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[9] See footnote 8.

[10] See footnote 7.

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Relationship Between Calcium in Sweat, Calcium Balance, and Calcium Requirements

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In a recent study (Paupe, '59) calcium secretion in the sweat was reported in 115 experiments on 98 subjects of various ages. The calcium concentration of sweat ranged from 30 to 300 mg/liter, with the conclusions that there were no relationships with age, or blood and urine levels. This report also cited an excellent review of the literature on dermal sweating by Mitchell and Hamilton ('49) who reported calcium sweat values ranging from 8 to 265 mg/liter. In their subsequent study Mitchell and Hamilton observed that under profuse sweating conditions, there was an average cutaneous loss of 20 mg of calcium/hour and under comfortable environmental conditions, the sweat calcium loss was reduced to 6.2 mg/hr. This meant that under high sweating conditions the sweat calcium accounted for almost 30% of the daily total body loss. These authors stressed the importance of the high errors that could be incurred in a balance experiment if the dermal losses (either emotional or thermogenic) were not included as a part of the balance losses and concluded that these losses were sufficiently important to warrant further studies.

These fairly high calcium excretion rates in the sweat indicate that the calcium requirements may be increased under profuse sweating conditions. Even though the report of Mitchell and Hamilton was published in 1949, neither the National Research Council ('58), nor the Food and Agriculture Organization (FAO) ('57) mention the possibility of increased requirements under these conditions. As a result, a series of studies were performed by this laboratory to determine the hourly and daily concentration of calcium in the sweat of men during rest, moderate and heavy physical activities while in both a temperate and an extremely hot environment. In a preliminary study at Yuma, sweat samples were collected in a study that was primarily designed to measure the energy requirements (Consolazio et al., '61). It was observed that the 8 test subjects had daily sweat rates ranging from 4,200 to 8,000 gm/day for a 20-day period. The calcium concentration of 66 sweat samples collected during rest and exercise averaged 20.6 ± 9.0 mg/100 ml with a range of 5.4 to 50.1 mg/100 ml. The sweat samples collected periodically over an 8-hour period indicated that the quantity of calcium excreted was very high, ranging from 0.79 to 1.95 gm/day. This rate of calcium excretion could possibly have been the result of the high daily calcium intake, which averaged 3.5 and 3.6 gm/day for the two periods. This was partially due to the milk consumption which averaged 1.5 quarts per man per day. The calcium balance was positive, averaging 0.41 gm/day for period 1 and 0.80 gm/day for period 2, where the sweat calcium values were included in the balance (table 1).

The questions to be answered in the present studies were (a) whether the calcium excretion rate in the sweat was a reflection of the level of calcium intake; (b) whether the calcium concentration of the sweat is decreased after acclimatization to the heat; (c) whether the calcium excretion rate is higher in the heat than during exercise in a temperate environment; and (d) whether the calcium requirements are increased.

In all, three additional studies were performed to evaluate the sweat excretion in

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TABLE 1

Calcium study, Yuma, 1959;[1] summary of calcium balance

Subjects	Period 1 — calcium excretion				Period 2 — calcium excretion			
	Urine	Feces	Sweat	Total output	Urine	Feces	Sweat	Total output
		avg mg/man/day				*avg mg/man/day*		
1	289	1112	1017	2418	393	877	1122	2392
2	320	1103	1170	2593	498	1166	885	2549
3	294	1552	1452	3298	403	1466	1115	2984
4	323	1400	1449	3172	286	1308	1049	2643
5	140	1081	1190	2411	265	876	967	2108
6	379	1343	1627	3349	399	1368	1095	2862
7	370	2160	1953	4483	383	1529	785	2697
8	435	1218	1884	3537	350	1990	1280	3620
Mean	319	1371	1468	3158	372	1323	1037	2732
Total daily intake, gm				3.630				3.530
Balance				0.472				0.798

[1] Periods were each for 10 days' duration.

relation to the total calcium excretion and balance. They included a 7-week study (1) in a temperate environment, in which 9 young men performed physical activities on a motor driven treadmill and a bicycle[1] for three hours daily. Two separate 4-day menus containing 1.566 and 1.669 gm of calcium/day were alternated. In study 2 the test periods were divided into three separate phases at temperatures of 70, 85 and 100°F, each of 4 days' duration. Each phase was repeated randomly 4 times for a total of 48 days. The test group consisted of 8 healthy men between the ages of 19 and 25. The activity level of the men during all the test periods was controlled at a constant daily rate. The physical activity included riding a bicycle ergometer for 50 minutes a day at a fairly high activity level (6 to 8 Cal./min) that produced 120 watts of work, and riding an exercycle for 50 minutes a day at a moderate rate (3 to 4.5 Cal./min). In this study the food intake was controlled to 3,182 Cal./day, and included 441 mg of calcium/day.

Study 3 was conducted for a total of 32 days on three healthy young men. This study consisted of two 4-day preliminary periods at 75°F and 30% relative humidity (RH), 4 periods at 100°F and 70% RH, and two periods of recovery or adjustment at 75°F and 70% RH. Morning, afternoon and the total daily sweat rates were computed from body weight changes during these periods. The controlled daily calcium intake averaged 581 mg/day with a constant food intake of 3,072 Cal./day. The daily activity consisted of 30 minutes of moderate activity on the bicycle, the remainder of the day being spent in sedentary type activities. The subjects exercised in a random order each day with the exercise periods alternating between mornings and afternoons. When the men left the test area (during the evenings) no moderate or heavy activities were permitted.

EXPERIMENTAL PROCEDURES

Studies 2 and 3 were conducted in an environmental chamber, so that the various temperatures and relative humidities could be controlled from period to period.

The food consisted of 4-day menus that were rotated during the experimental phase. Food was issued and consumed at each meal and no additional food or snacks were permitted after the last evening meal. Only distilled water was used for cooking purposes. In study 2 cool distilled water was supplied ad libitum for drinking purposes and the daily intake recorded, but in study 3 the men were permitted to drink weighed, cool tap water, which did not have the bland distilled water taste. The tap water was analyzed periodically for its calcium content.

Sweat rates, including water from the skin and lungs, were measured, using the method of Adolph et al. ('47). They were determined by measuring weight changes

[1] Exercycle, Exercycle Corporation, New York.

during the morning and afternoon periods. These differences in body weight were then corrected for water intake, urinary output and defecation. (Gaseous exchanges of respiration contribute a small quantity, which is usually less than 1% of the observed decrease in body weight.) Sweat samples taken daily throughout the studies, were collected in polyethylene bags or gloves that covered one whole arm, for one hour or more at a time. Both the arm and glove were washed with distilled water and dried before each collection period began. Each individual sweat sample was centrifuged, the clear supernatant fluid poured off into ampules which were immediately sealed and frozen for the subsequent analysis of calcium. Arm sweat has been used by many investigators including Kuno ('56) and Mitchell and Hamilton ('49) who believed arm sweat to be representative of the total body sweat.

All subjects were weighed in the nude state using the Plima balance, that has an accuracy of 10 gm. Other weighings for measuring sweat rates were done after breakfast at 7:45 A.M., before lunch at 11:45 A.M., after lunch at 12:45 P.M., and before the evening meal at 4:30 P.M. In some instances sweat rates were computed from 5:30 P.M. to 11:00 P.M. or from 11:00 P.M. to 7:00 A.M. Weighings were also taken between these periods.

Data on temperature and relative humidity were recorded daily in the environmental chamber at one hour intervals from 7:00 A.M. to 4:30 P.M. Urine and fecal collections were made coinciding with each collection period, so that calcium balance could be calculated. Chemical analysis of the food, urine, feces and sweat was performed, using the permanganate titration procedure (Consolazio and Johnson, '60).

RESULTS

Study 1. Temperate environment, three hours exercise daily. The daily urinary excretion of calcium averaged from 238 to 258 mg/day for the 9 men, which was fairly constant during the 7 test periods (table 2). The fecal calcium excretions per period were relatively constant except for period 3 when the excretion rate decreased to 429 mg per man per day. The other values ranged from 603 to 714 mg per man per day. The calcium balances both with and without the sweat excretion values are presented in table 2. The sweat calcium excretion rates per hour during the exercise periods and the total per day are shown in tables 2 and 3.

TABLE 3

Calcium in sweat;[1] temperate environment (study 1)

Weeks of study	Calcium/3 hours' exercise
	mg
1	—
2	72
3	55
4	74
5	65
6	64
7	54
Mean	63.9 or 21.3 mg/hour during exercise

[1] Average of 9 men.

TABLE 2

Calcium excretion and balance, temperate environment (study 1)[1,2]

	Periods						
	1	2	3	4	5	6	7
				mg/man/day			
Urinary excretion, mg	—	258	250	256	235	238	243
Fecal excretion, mg	—	662	429	603	714	655	660
Sweat excretion,[3] mg	—	147	164	160	159	147	155
Total excretion, all sources	—	1067	843	1019	1108	1040	1058
Balance, no sweat included		+704	+933	+703	+610	+713	+725
Balance, sweat included		+557	+769	+543	+451	+566	+570
Sweat as % of total excretion		13.8	19.5	15.7	14.3	14.1	14.7

[1] Each period of 7 days' duration.
[2] Mean for 9 men; calcium intake averaged 1.62 gm/day.
[3] Includes a factor of 3.0 mg/hour for calcium excretion during comfortable periods.

Study 2. Environmental temperatures of 70, 85 and 100°F, with one hour 40 minutes of exercise daily. The values for the various excretions of calcium are presented in table 4. Urinary calcium excretions in this study averaged from 125 to 220 mg per man per day for the 12 experimental periods with no significant difference between the 70, 85 and 100°F experimental phases, which averaged 199, 183 and 189 mg per man per day. Fecal excretion averaged 199, 226 and 216 mg per man per day for the same three temperature phases, respectively. The sweat calcium excretion increased with an increase in environmental temperatures, averaged 8.1, 11.6 and 20.2 mg/hr for the 7.5 hours spent daily in the environmental chamber (table 5). With an intake of 441 mg per man per day, the calcium balance exclusive of any sweat excretion values was positive averaging a +77, +66 and +68 mg/day for the 70, 85 and 100°F test periods. When the sweat calcium was included in the output, the balance was on the negative side averaging a −76, −103 and a −166 mg/day for the same respective periods (table 6).

Study 3. Seventy and 100°F periods with a minimal amount of exercise (30 minutes/day). The 4 menus contained 517, 751, 428 and 494 mg of calcium/day, averaging 547 mg for all. Since the men were allowed to drink tap water in this study, the actual total calcium intake was increased, based on chemical analysis of the water, to 557 mg during the cool periods and 581 mg/day for the extremely

TABLE 4

Calcium excretion in urine, feces and sweat[1] *(study 2)*

Subject	70°F.			85° F.			100° F.		
	Urine	Feces	Sweat	Urine	Feces	Sweat	Urine	Feces	Sweat
		mg/day			*mg/day*			*mg/day*	
A	248	186	111	235	219	142	252	198	260
B	230	195	119	190	216	138	183	226	214
C	278	142	105	242	192	118	264	164	143
D	145	249	114	118	220	138	141	217	194
F	134	215	121	157	252	161	134	245	208
G	128	186	110	131	267	129	140	246	176
H	228	222	100	205	212	132	208	218	210
Mean All	199	199	111	183	226	137	189	216	201

[1] Mean for 16 days; a value of 3 mg/hour was assumed for calcium excretion during the 16.5 hours outside the chamber.

TABLE 5

Sweat excretion of calcium while in chamber (study 2)[1,2]

Subject	Environmental temperature														
	70° F.					85° F.					100° F.				
	1	2	3	4	Mean[3]	1	2	3	4	Mean[3]	1	2	3	4	Mean[3]
			mg/period					*mg/period*					*mg/period*		
A	85	68	46	45	61	151	80	67	71	92	151	223	223	243	210
B	79	54	77	64	69	145	90	67	50	88	245	213	103	94	164
C	58	65	43	52	55	139	43	43	46	68	97	102	95	76	93
D	60	97	46	53	64	120	99	68	65	88	136	186	100	155	144
F	84	67	57	76	71	188	104	69	81	111	130	201	151	150	158
G	49	115	34	41	60	129	49	63	74	79	120	159	98	125	126
H	47	72	36	43	50	90	95	87	55	82	146	201	192	111	163
Mean	66	77	48	53	61	137	80	66	63	87	146	184	137	136	151
Mean/hour					8.1					11.6					20.2

[1] Period of 7.5 hours.
[2] Each period of 4 days' duration.
[3] Mean for 16 days at each temperature.

hot periods. Urinary calcium excretion averaged 346 mg for the preheat phase, 319 mg during the hot periods and 317 mg/day for the post-heat periods (table 7). Fecal excretions presented in the same table averaged 314, 341 and 415 mg/day for the same periods, respectively. During the 16-day hot period the sweat calcium showed a decrease with time, finally leveling off at 127 mg/day. Each successive 4-day period in the heat averaged 270, 153, 125 and 127 mg/day (table 8). The calcium in sweat during the cool periods averaged only 70 mg/day or 3 mg/hour.

The calcium balance, exclusive of sweat, averaged −93, −80 and −178 mg/day for the preheat, heat and post-heat periods, respectively (table 9), but when the sweat values were included, a greater negative balance occurred, averaging −163, −298 and −248 mg/day for the same periods, respectively. A summary of the daily variations in sweat calcium concentration in milligrams/100 ml and excretion in milligrams per hour is presented in tables 10 and 11. These values dropped consistently during the 16-day period. In this study of minimal exercise, the sweat calcium ac-

TABLE 6

Calcium intake, excretions, and balance; mean for all subjects (study 2)

	Environmental temperature											
	70° F.				85° F.				100° F.			
	1	2	3	4	1	2	3	4	1	2	3	4
	mg/man/day				*mg/man/day*				*mg/man/day*			
Intake	441	441	441	441	441	441	441	441	441	441	441	441
Output:												
In urine	197	215	214	168	125	183	220	202	176	201	195	183
In feces	216	183	210	188	308	163	222	209	289	222	160	194
Total in sweat Per day	116	127	98	103	187	130	116	113	196	234	187	186
Total output Omitting sweat	413	398	424	356	433	346	442	411	465	423	355	377
Balance	+61	+76	+50	+118	+41	+128	+32	+63	+9	+51	+119	+97
Balance Including sweat	−88	−84	−81	−18	−179	−35	−117	−83	−221	−216	−101	−122
Per cent of total excretion from sweat			21.8				25.1				33.2	

TABLE 7

Calcium excretion (study 3)[1]

Temperature °F.	Subject A				Subject B				Subject C			
	Urine	Feces	Sweat	Total	Urine	Feces	Sweat	Total	Urine	Feces	Sweat	Total
	avg mg/day				*avg mg/day*				*avg mg/day*			
75	406	304	70	780	169	289	70	528	442	213	70	725
75	479	271	70	820	172	403	70	645	408	344	70	822
100	391	359	321	1071	158	383	260	801	427	339	380	1146
100	451	519	196	1166	161	371	196	728	414	311	216	941
100	318	370	168	856	162	323	174	659	427	275	182	884
100	368	354	174	896	166	288	174	628	392	203	182	777
75	420	533	70	1023	190	385	70	645	428	402	70	900
75	357	396	70	823	150	355	70	575	357	420	70	847

[1] Mean for each 4-day period.

TABLE 8

Calcium excreted in sweat (study 2)[1]

Days at 100° F.	Calcium excreted during 7.5 hours in chamber				
	Subject			Mean all men	
	1	2	3		
	mg	*mg*	*mg*	*mg*	*mg Ca/hour*
1–4	271	210	330	270	36
5–8	146	146	166	153	20
9–12	118	124	132	125	17
13–16	124	124	132	127	17

[1] Average of 3 men; the calcium intake averaged 581 mg/day; men exercised for 30 minutes a day at a moderate rate.

counted for 9.6, 22.7 and 8.7% of the total calcium output for the pre-heat, heat and post-heat phases, respectively. Blood specimens were drawn for serum calcium analysis at the beginning, middle and end of the experiment. No significant change in the calcium levels were observed for any individual, with the values ranging from 10.3 to 12.7 mg/100 ml.

DISCUSSION

It has been observed for many years, by investigators in the field of mineral metabolism, that an appreciable quantity of calcium and other minerals are excreted in sweat. On the other hand, in a very recent review, Leitch and Aitken ('59) concluded that no more than 20 mg of calcium is lost in the sweat daily, in a temperate environment. These authors felt that even when sweating at a rate of 800 gm/hr for a 24-hour period, an individual could lose no more than 90 mg of calcium a day, which would not affect the calcium balance. Mitchell and Hamilton in 1949, thoroughly reviewed the literature and in their own subsequent studies concluded that dermal losses were quite appreciable and should be included as part of the total output in a balance study. That this in turn might affect the nutritional requirements was stressed by Comar ('59) who felt that adequate studies should be performed of the possible compensatory relationships between losses through the three major ex-

TABLE 9

Calcium balance (study 3)[1]

Temperature ° F.	Subject A	Subject B	Subject C	Mean all men (excludes sweat)	Mean all men (includes sweat)
	mg/man/day			*mg/man/day*	*mg/man/day*
75	−153	+100	−102	−52	−122
75	−188	−18	−196	−134	−204
100	−169	+40	−192	−107	−427
100	−383	+64	−145	−155	−357
100	−111	+90	−134	−52	−226
100	−134	+132	−17	−6	−183
75	−392	−19	−274	−228	−298
75	−197	+48	−230	−128	−198

[1] Each period was of 4 days' duration.

TABLE 10

Diurnal sweat calcium concentration (study 3)[1]

Time	Days in hot environment															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
hours	*mg/100 ml*					*mg/100 ml*				*mg/100 ml*				*mg/100 ml*		
0–1.5				10.7			11.3								6.0	
1.5–3.5	24.8	23.4	12.1	6.1	4.7	6.3	6.6	4.4	3.6	3.3	5.2	2.7	3.9	3.1		
3.5–6.5				3.8			5.1				2.7				2.4	
6.5–8.5	10.3	7.9	3.4	3.6	3.6	3.2	3.4	2.9	2.6	2.5	2.1	2.3	2.2	2.6	2.8	1.9
8.5–11							3.1				3.0				2.9	
11–13							2.1				2.1				1.9	
13–15							2.3				1.8				1.9	

[1] Average of 3 men.

TABLE 11

Diurnal sweat calcium excretion rate[1,2] *(study 3)*

Time	Days in hot environment															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
hours	*mg/hour*						*mg/hour*								*mg/hour*	
0–1.5				39			26				18				16	
1.5–3.5	56	73	25	31	13	13	15	18	9	12	14	11	9	12	16	13
3.5–6.5				12			22				13				17	
6.5–8.5	37	22	12	11	15	14	14	11	12	10	10	9	10	9	14	9
8.5–11							12				13				9	
11–13							9				8				6	
13–15							9				8				6	

[1] Sweat calcium excretion rates in successive periods of time in the hot environment, as influenced by number of days in the hot environment.
[2] Mean for 3 men.

cretory pathways, namely, skin, kidney and alimentary tract. In the evaluation of calcium requirements the sweat or dermal (sensible perspiration) calcium excretion should be seriously considered. Freyberg and Grant ('37) showed that insensible perspiration appears to be free of calcium. It should be stressed that a true calcium balance depends upon many variables including intake, growth and development, vitamin D nutriture, a number of hormones, physical exercise, balance of other minerals, excretion rate absorption and utilization of the food calcium ingested, and the presence of factors accelerating or depressing calcium utilization.

It has been shown by the Harvard group that a negative calcium balance will result from a low calcium intake (Bauer et al., '29), but on the other hand it has been stated that calcium equilibrium can be attained with as little as 3 mg/kg and as much as 10 mg/kg of body weight per day, the difference being interpreted as an adaptation to levels of the previous intake (NRC, '58). A high calcium intake can at first produce a high calcium balance and in turn, an increased deposition of calcium in the bones (NRC, '58) which will cease when an equilibrium is reached.

In the present studies, it should be stressed that an attempt was not made to attain a true calcium balance (equilibrium) since the time intervals were not long enough. It is known that in some instances it may take as long as 6 months to attain calcium equilibrium (Nicolaysen et al., '53). The calcium secretion in the sweat is primarily dependent upon two factors, environmental temperature and physical activity. To prevent an increase in body temperature, man then begins to dissipate heat through the sweating process. It has been shown that in a temperate environment, the calcium excretion in the sweat averaged 21.3 mg/hour during the three-hour exercising periods. It was practically impossible to collect arm sweat when the men were not exercising under these conditions, hence an assumed value of 3 mg/hour was used to calculate the calcium excretion due to sweating, during the remainder of the day.

During study 2 at three environmental temperatures when the men were working for one hour and 40 minutes each day, the hourly excretions of calcium during the time the men were in the chamber (7.5 hours) were 8.1, 11.6 and 20.2 mg/hour for the 70, 85 and 100°F phases (table 12). This leads one to believe that the calcium secretion in sweat is increased with an increase in sweat rate, as in the 100°F phase. Since the men had exposures of only 4 days at a time at each temperature, they may not have had the full effect of acclimatization. In this study (3), where of physical activity of only 30 minutes was performed daily, the calcium concentration in the sweat and the excretion rates decreased gradually for each 4-day period in the heat, the excretion falling from a mean of 36 to 17 mg/hour at the end of 16 days. Actually the decrease, if any, was very slight after the first 4 to 5 days' exposure to the heat. During heat exposure days 7, 11 and 15, comparisons of arm sweat calcium were made continuously for 15 hourly periods during the day. In day 7 the calcium concentrations in milli-

TABLE 12

Summary of 4 calcium studies

	Sweat rate	Calcium excreted in sweat[1]	Sweat calcium total daily as % of calcium output
	gm/man/day	*mg/hour*	
Yuma			
Period 1	6451	175	46.4
Period 2	4942	170	38.0
Study 1			
Period 1	1279	17	—
Period 2	1394	24	13.8
Period 3	1066	19	19.5
Period 4	1318	25	15.7
Period 5	1601	22	14.3
Period 6	1506	21	14.1
Period 7	708	21	14.7
Study 2			
70	812	8	21.8
85	1194	12	25.1
100	2248	20	33.2
Study 3			
75	510	3	9.6
100	2327	36	31.8
100	2629	20	21.5
100	2865	17	21.9
100	3079	17	23.1
75	508	3	8.7

[1] Study 1, average for 3 hours during exercise, study 2 and 3 average/hour for period spent in environmental chamber (7.5 hours). At Yuma the sweat was collected for periods of 8 hours daily.

grams/100 ml dropped from 11.3 to 2.3, day 11, 5.2 to 1.8, and in day 15 the concentrations decreased from 6.0 to 1.9 mg/100 ml. The same occurrence was also observed by Hopf ('59) and Mitchell and Hamilton ('49) who showed a high initial calcium concentration which decreased progressively during a single sweating period, from a range of 5.2 to 0.4 mg/100 ml. Once the men were exposed for a few hours to a lower ambient temperature, where sweating was not perceptible, the calcium concentration on sweating at the beginning of a new day was again at the higher level.

One questions whether the calcium concentration in the sweat would eventually drop to zero if profuse sweating continued for longer periods of time than the 16 days in this study. In a recent study in conjunction with a nutrition survey by the Interdepartmental Committee on Nutrition for National Defense (ICNND) in Thailand ('61) one- to two-hour arm sweat samples were collected from 30 young men who had been continuously exposed to extreme heat and humidity (in a tropical environment) almost all of their lives. The calcium concentrations in the sweat with an intake of from 0.3 to 0.8 gm of calcium/day ranged from 0.4 to 10.2 mg/100 ml, which when projected to a sweat rate of 2,000 gm/day, not uncommon in this country, amounted to 8 to 204 mg/day of calcium excreted a day from the sweat alone. In addition, Mitchell and Hamilton ('49) observed that even after 32 to 35 weeks of exposure to extreme heat, their subjects still excreted up to 20.2 mg of calcium/hour in the sweat. This sugggests that the calcium excretion in sweat does not become zero even with long acclimatization. There is also a possibility of adaptation of the calcium concentration in sweat to habitual low calcium intakes.

As in other studies, the urinary excretion of calcium was remarkably constant from period to period, regardless of whether the men were living in either a hot or cool environment. It has been shown (Knapp, '47) that the quantity of calcium in urine is dependent on an endogenous factor or factors presumably endocrine, and on calcium intake per kilogram of body weight. Hironaka et al. ('60) showed that the calcium excretion is inevitably increased in senescence, since senescence is essentially an atrophic process. But it was observed by Konishi ('57) and Shore et al. ('59) that the calcium excretion in urine decreases with exercise. This is one of the main reasons that, in these studies, the physical activity was kept at a constant daily level for each experiment.

The observations made in the present studies, are in agreement with the work of Mitchell and Hamilton ('49) even though these investigators did not calculate a calcium balance that included sweat calcium. These sweat calcium losses are very important, especially since no one to our knowledge has included a factor for calcium lost in sweat, when computing calcium balance. Under conditions of sweating under comfortable environmental conditions, Mitchell and Hamilton ('49) observed an excretion of 6.2 mg calcium/

hour which is equivalent to approximately 149 mg/day. It was shown in the present studies that sweat calcium values averaged 20.6% of the total output in a temperate environment, 36.7% during exercise in the heat and 22.7% with practically no exercise in the heat (table 12). Kuno ('56) as mentioned previously, and Mitchell and Hamilton ('49) observed values of 29.9% of the total output during profuse sweating and 14.4% of the total under nonperceptible sweating conditions.

In the two studies on low calcium intakes, 441 mg/day, in study 2 and 581 mg/day in study 3, the calcium balances were practically in equilibrium when the sweat calcium losses were not included in the balance, but when the calcium losses due to sweat were included, these values were highly negative especially in the studies in the heat. In study 2 the negative balance was −166 mg at 100°F, −103 mg at 85°F, and −76 mg/day at 70°F; and in study 3 the calcium balance for the 4 consecutive hot periods at 100°F were −427, −357, −226, and −183 mg/day.

Does this mean that all the calcium balance studies in the literature may be in error, since values for sweat calcium were not included as part of the daily output? Hegsted et al. ('52), in their study on the adjustment to a low calcium intake, reported the men to be in equilibrium after a considerable period of time, but one must realize that this study was made during a Peruvian summer on prisoners performing some type of physical labor. Even though these men may not have been sweating profusely it appears that they may have been actually in a daily negative balance of 100 to 150 mg during the equilibrium period, when they supposedly were in balance. This may also be true for many of the other calcium balance studies performed with low intakes that have been published in the literature. This leads one to believe that the calcium requirements may be increased under these conditions to attain equilibrium, since it may be very questionable whether an individual really does attain equilibrium with a low calcium intake, in the heat. On the other hand the fact should not be overlooked that a negative calcium balance does not necessarily mean an insufficient supply of calcium, since calcium balance may not be related to calcium requirement.

An individual losing calcium at the rate of 100 to 200 mg/day should exhibit severe osteoporosis within two to three years. Yet there is no evidence that this occurs in adult populations consuming low calcium intakes, living in tropical environments.

Another important question that remains to be considered is whether a low calcium intake, with a subsequent appreciable negative balance, due to sweat losses, can account for the smaller body builds of individuals throughout the world, especially in tropical or semi-tropical environments. Is there any truth in the idea that a low calcium intake, with a negative calcium balance due to calcium lost in sweat, will retard skeletal growth? This problem has been discussed for many years and recently Walker ('54) reviewed the subject, without taking the sweat calcium losses into consideration. He states that "it is widely accepted that in humans a low intake can prejudice the rate of attainment of height and makes for ultimate stuntedness." He observed that people in poor homes with low calcium intakes are shorter in height, when compared with children of a better class of the same race, in the same country. Walker ('54) especially mentions that native children from tropical or semi-tropical countries, who habitually have low calcium intakes, are shorter in height when compared with western children, but he also states that in neither case is there any evidence that differences in calcium intakes are specifically implicated. Our data suggest that sweat calcium losses may be an important factor and may be one of the "missing links" related to retarded growth or stunting.

SUMMARY

These data suggest that calcium may be lost during heavy sweating conditions (up to 20 mg calcium/hour) and that this loss should be considered in establishing recommended allowances for calcium.

It was observed that 7 men consuming 441 mg of calcium a day in a study extending for 48 days, excreted 8.1, 11.6 and 20.2 mg/hour of calcium when living at 70, 85 and 100°F. This accounted for 21.8,

25.1 and 33.2% of the total calcium excreted.

These observations are important since they show an additional calcium loss, which has not been reported in previous calcium balance studies in the literature. It is questionable whether an individual, consuming a low calcium diet, ever really attains calcium balance (equilibrium), under heavy sweating conditions.

It was observed that (a) the calcium excreted in sweat, in men working at a moderate rate in extreme heat (100°F), was still fairly high after acclimatization, averaging 17 mg/hour after the first 4 days, and (b) that the daily total calcium in sweat increased as the sweat rate increased. Therefore it appears that the calcium requirements may be increased under these conditions.

It was shown that even after acclimatization the urinary calcium did not decrease in compensation for the losses of calcium in sweat. It is recognized that changes in the urinary excretion of calcium in adjusting to different levels of dietary calcium and the various other metabolic factors, may require months to achieve.

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Studies on the Effect of Raw Soybean Meal on Fat Absorption in Young Chicks[1]

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In a recent paper from this laboratory, Brambila et al. ('61) reported that in chicks receiving diets containing raw soybean meal absorption of dietary fat was considerably lower than that in chicks receiving heated soybean meal. The poor fat absorbability in chicks fed raw soybean meal could be overcome by supplementing their diet with either a crude or a crystalline trypsin preparation. The studies reported in this paper provide further information on the effect of raw soybean meal on fat absorption in chicks.

EXPERIMENTAL

Male White Plymouth Rock or Rhode Island Red × Barred Plymouth Rock (RIR × BPR) chicks purchased from a commercial hatchery were used in all experiments. Chicks were housed in battery brooders with raised wire floors and thermostatically controlled heating units. Feed and water were supplied ad libitum and chicks started to be fed the experimental diets at one or two days of age. The composition of the experimental diet is shown in table 1. The soybean meal component of the diet was varied to be raw meal, heated meal or various proportions of raw to heated meal as indicated in various experiments. The raw soybean meal was obtained from a commercial soybean processor as extracted soybean flakes removed from the processing equipment prior to heating. Since Brambila et al. ('61) showed clearly that heat treatment overcame the effect of raw soybean meal on fat absorption, commercially produced 50% protein soybean meal was used in the heated meal treatments in these experiments. Degummed soybean oil additions to this diet were at the expense of an equal weight of glucose. Chromic oxide (0.3%) was incorporated in the diet as an index material to eliminate the need for quantitative collection of excreta (Dansky and Hill, '52) in determining metabolizable energy values and fat absorption for the diet.

TABLE 1
Basal diet

	%
Soybean meal[1]	50.0
Soybean oil	0.5
Glucose	40.85
Glycine	0.5
DL-Methionine	0.3
Mineral mixture[2]	5.63
Vitamin mixture[3]	1.22
Chromium premix[4]	1.00

[1] Varying proportions of raw or heated soybean meal as indicated in each experiment.
[2] Supplies in gm/kg diet: $CaHPO_4$, 20.7; $CaCO_3$, 14.8; KH_2PO_4, 10.0; KCl, 1.0; NaCl, 6.0; $MgSO_4$, 3.0; $FeSO_4 \cdot 7H_2O$, 0.333; $MnSO_4 \cdot H_2O$, 0.333; KI, 0.0026; $CuSO_4 \cdot 5H_2O$, 0.0167; ZnO, 0.062; $CoCl \cdot 6H_2O$, 0.0017; $NaMoO_4 \cdot 2H_2O$, 0.0083; Na_2SeO_3, 0.0001.
[3] Supplies in mg/kg diet: thiamine·HCl, 15.0; riboflavin, 15.0; niacin, 50.0; Ca pantothenate, 20.0; pyridoxine·HCl, 6.0; folic acid, 6.0; biotin, 0.6; menadione sodium bisulfite, 1.52; inositol, 250.0; BHT, 100; vitamin D_3, 4,500 ICU; vitamin A, 5,000 USP units, Vitamin E, 110 IU premixed in glucose.
[4] Contains 30% Cr_2O_3.

Fresh pancreas weights were determined at the end of the experiment and were adjusted by analysis of covariance (Steele and Torrie, '60) to eliminate effects due to differences in body weights in making comparisons of treatment effects. Pancreas weights adjusted to the same body weight are given in all tables.

Trypsin inhibitor assays were carried out on raw meal and purified inhibitor by the technique of Borchers et al. ('47). The activities of the test materials were compared to a standard sample of purified "Kunitz" trypsin inhibitor.

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Fat absorbability was determined by techniques described by Renner and Hill ('61); metabolizable energy was determined according to methods described by Hill and Anderson ('58). All values for fat absorbability were corrected for endogenous fat by including low-fat control diets in each experiment and correcting fat absorbability values for fat excreted by chicks fed the low-fat diet. Soybean oil fatty acids were prepared from degummed soybean oil by refluxing with KOH-ethanol for 5 hours, followed by acidification with H_2SO_4 and water washing.

RESULTS

Brambila et al. ('61) observed a greater depression of fat absorption in two-week old chicks fed raw soybean meal than in three-week old chicks, and almost no effect in 4-week old chicks. This suggested that raw meal influenced fat absorption only in very young chicks. An experiment (table 2) was conducted to study the effect of raw soybean meal on fat absorption in chicks at two and four weeks of age. In addition, two breeds of chicks were studied since some preliminary observations suggested that differences between breeds may exist. The influence of raw soybean meal on absorption of soybean oil or soybean fatty acids was studied to determine whether raw meal affected digestion of the fat (hydrolysis) or primarily absorption.

Each percentage value for fat absorbability was transformed to an arcsin value and analysis of variance carried out by procedures outlined by Steele and Torrie ('60). Raw soybean meal significantly depressed fat absorption in chicks two weeks of age ($P < 0.01$) but not four weeks of age. The absorption of both soybean fatty acids and soybean oil was depressed to the same extent by raw soybean meal. At two weeks of age chicks absorbed less soybean fatty acids than soybean oil when fed either raw or heated meal. Both breeds of chicks were affected by the raw meal. This experiment indicates that raw soybean meal does not inhibit fat absorption merely by inhibiting hydrolysis.

Pancreas weights, measured on the RIR × BPR chicks at the end of the experiment, showed the characteristic increase in weight in chicks fed the raw soybean meal. The pancreas weights were adjusted to the same body weight by analysis of covariance and differences among the adjusted treatment means determined by Duncan's multiple range test (Steele and

TABLE 2

Effect of age and type of fat on influence of raw soybean meal on fat absorption

Treatment	WPR chicks		RIR × BPR chicks		Pancreas weight (adjusted)
	Avg weight[1]	Fat absorbability	Avg weight	Fat absorbability	
	gm	%	*gm*	%	*gm*
2 Weeks of age					
Raw basal	163	—	136	—	—
+15% soybean oil	207	67	154	64	—
+15% soybean oil fatty acids	182	56	134	49	—
Heated basal	194	—	161	—	—
+15% soybean oil	220	88	188	93	—
+15% soybean oil fatty acids	221	75	181	85	—
4 Weeks of age					
Raw basal	510	—	388	—	2.18[2]
+15% soybean oil	609	95	423	97	1.87
+15% soybean oil fatty acids	547	88	392	93	1.61
Heated basal	547	—	425	—	1.33
+15% soybean oil	623	96	483	96	1.34
+15% soybean oil fatty acids	644	86	481	89	1.42

[1] All values for body weight and fat absorbability represent average for 3 groups of 8 chicks each.
[2] Nine individual determinations per treatment adjusted to same body weight by covariance analysis.

Torrie, '61). The difference between pancreas weight of chicks fed the raw soybean meal basal and those receiving soybean oil or soybean fatty acids was significantly different ($P < 0.01$). The pancreas of chicks fed diets containing raw soybean meal plus soybean oil or soybean fatty acids did not enlarge to the same degree as in chicks receiving the low-fat basal diet containing raw meal. The difference in pancreas weight between the chicks fed the low-fat diet containing raw soybean meal and those receiving raw meal plus fatty acids or soybean oil was statistically significant ($P < 0.05$).

The above experiment indicated that the effect of raw soybean meal on fat absorption was observed only in two-week old chicks. This could be a true age effect or it could be the result of adaptation to the raw meal during the feeding period. The experiment shown in table 3, conducted to determine whether fat absorbability would be depressed in 4-week old chicks fed raw meal only a short time before the collection period began, shows that fat absorbability was only slightly depressed in these chicks fed raw soybean meal either from hatching or for a week just prior to the collection period. This indicates that the change in effect of raw meal on fat absorption in chicks from two to four weeks of age is probably a true age effect and not merely an adaptation to raw meal feeding.

An additional experiment was conducted to determine the level of dietary raw soybean meal required to depress fat absorbability. The results of this experiment, shown in table 4, show that 10% of dietary raw soybean meal (5% dietary protein) causes nearly maximal effect on fat absorbability. Increasing levels of raw meal continued to depress growth further but had a relatively slight additional influence on fat absorption.

Apparently chicks fed raw soybean meal suffer from amino acid deficiencies due to the poor biological value of protein in raw meal. Supplementation of diets containing raw meal with amino acid mixtures (Fisher and Johnson, '58) has been reported

TABLE 3

Effect of length of feeding period on effect of raw soybean meal on fat absorbability

Treatment	Average weight[1]		Fat absorbability[1]	
	2-Week	4-Week	2-Week	4-Week
	gm	*gm*	%	%
Basal raw soybean meal	127	385	—	—
Basal raw soybean meal + 15% soybean oil	145	478	43	89
Basal heated soybean meal	192	550	—	—
Basal heated soybean meal + 15% soybean oil	211	664	84	94
Heated meal + 15% soybean oil for 2.5 weeks, then raw meal + soybean oil	213	545	87	89

[1] Each value represents average of determination on three groups of 8 WPR male chicks per treatment.

TABLE 4

Effect of dietary level of raw soybean meal on fat absorbability

Treatment[1]	Avg wt 2 weeks[2]	Fat absorbability
	gm	%
Basal heated soybean meal	192	—
+ 15% soybean oil	211	84
10% Raw meal + 15% soybean oil	189	51
20% Raw meal + 15% soybean oil	168	41
30% Raw meal + 15% soybean oil	151	43
50% Raw meal + 15% soybean oil	145	43
Basal raw soybean meal	127	—

[1] All diets contained 50% soybean meal, proportion of raw meal only indicated.
[2] Values for fat absorbability and body weight represent averages of three groups of 8 BPR ×RIR male chicks per treatment.

to overcome the growth depressing effects of the meal. Barnes et al. ('44) in studies with rats reported that fat absorption could be influenced by dietary protein level. An experiment (table 5) was conducted to determine whether changes in the protein level of the diet could influence the effect of raw soybean meal on fat absorption. Lowering the protein level in the heated control diet from 25 to 15% only slightly depressed fat absorption. Increasing the protein level in the diet containing 10% of raw meal to 35% by the addition of 20% of additional heated meal at the expense of glucose did not significantly improve fat absorption. A mixture of casein and gelatin added to the diet containing raw meal likewise had no statistically significant effect on fat absorption although the numerical value was slightly improved.

In the above experiments the effect of raw soybean meal on fat absorption was found (1) to occur only in very young chicks, (2) to require relatively small amounts of raw meal for maximum effect, and (3) to be independent of protein level of the diet.

This indicates that some specific substance present in raw meal is probably responsible for the effects on fat absorption observed. In our earlier studies (Brambila et al., '61) dietary trypsin was found to overcome the effect of raw soybean meal on fat absorption, suggesting that one of the trypsin inhibitors present in raw meal may be responsible for the observed effect. Rackis et al. ('58) reported isolation of two trypsin inhibitors from soybean whey proteins and designated them A_1 and A_2. One of these (A_2) was found to be identical with the inhibitor isolated and characterized by Kunitz ('45). An experiment (table 6) was conducted to determine whether a commercially available crystalline preparation of the "Kunitz" inhibitor would influence fat absorption in young chicks. The raw soybean meal (15%) and the purified trypsin inhibitor (0.3%) were fed at equivalent levels of total antitrypsin activity determined by assay of inhibitor and raw meal prior to the experiment. In the case of the raw meal, this activity was presumably due to two separate inhibitors (A_1 and A_2), whereas that of the purified trypsin inhibitor represented only one of the inhibitors (A_2) in the meal. The purified trypsin inhibitor was stable when mixed in the experimental diet, since assay of the diet remaining at the end of the experiment indicated that most of the activity still could be detected.

Chicks fed the purified inhibitor weighed significantly less at two weeks of age than chicks fed the comparable heated meal control diet without the inhibitor ($P < 0.05$). Chicks receiving raw meal at equivalent trypsin inhibitor activity were considerably smaller than those fed the purified trypsin inhibitor. The chicks receiving the purified inhibitor or raw soybean meal had considerably larger adjusted pancreas weights than chicks fed diets containing all heated meal ($P < 0.01$). The adjusted pancreas weights of chicks fed diets containing raw meal or the trypsin inhibitor were not significantly different.

Fat absorbability was not depressed in chicks receiving the purified inhibitor compared to chicks receiving the heated meal control diet alone, whereas chicks receiving only raw meal absorbed 43% of the dietary soybean oil.

TABLE 5

Independence of the effect of raw soybean meal on fat absorption from dietary protein level

Treatment	Avg wt[1] 2 weeks	Fat absorbability[1]
	gm	%
50% Heated soybean meal	141	—
+ 15% soybean oil	152	91
40% Heated meal + 10% raw meal + 15% soybean oil	126	49
30% Heated meal + 15% soybean oil	137	86
60% Heated meal + 10% raw meal + 15% soybean meal	121	53
40% Heated meal + 10% raw meal + 10% casein-gelatin mixture + 15% soybean meal	126	58

[1] Values for body weight and fat absorbability represent average of three groups of 8 RIR × BPR male chicks per treatment.

TABLE 6

Effect of feeding crystalline soybean trypsin inhibitor

Treatment	Avg wt 2 weeks[1]	Feed/gain	Adjusted pancreas wt[2]	Fat absorbability[1]	Nitrogen retention[1]	Metabolizable energy of diet[1]	Metabolizable energy adjusted to 100% fat absorbability[1]
	gm		*gm*	*%*	*% of intake*	*Cal./gm dry matter*	*Cal./gm dry matter*
Heated soybean meal basal	155	—	—	—	46.4	2.81	—
Heated soybean meal basal + 17.5% soybean oil	173	1.43	0.63	77	54.5	3.41	3.82
Heated soybean meal basal + 17.5% soybean oil + 0.30% SBTI[3]	155	1.73	0.83	78	42.4	3.15	3.57
Basal — 15% raw meal + 35% heated meal	107	—	—	—	29.3	2.30	—
Basal — 15% raw meal + 35% heated meal + 17.5% soybean oil	116	2.60	0.82	43	27.4	2.20	3.22

[1] Data represent average values of measurement on three groups of 8 RIR × BPR chicks per treatment.
[2] Pancreas of 24 chicks per treatment weighed.
[3] 3X Crystalline soybean trypsin inhibitor obtained from Gallard Schlesinger Chemical Corporation, Garden City, Long Island, New York.

The diets were mixed on a dry-matter basis and dietary metabolizable energy values determined among the various treatments (table 6). The observed feed/gain ratios reflected the different metabolizable energy values of the diets detected.

Values for the diets with added soybean oil were adjusted to 100% fat absorption so that the metabolizable energy values of the nonfat components of the diets could be compared. The metabolizable energy of the diet containing raw soybean meal was considerably lower than the diet containing heated meal. The diet containing heated meal plus the trypsin inhibitor had a significantly lower ($P < 0.01$) adjusted metabolizable energy value than the corresponding heated meal control diet as determined by analysis of variance and Duncan's multiple range test (Steele and Torrie, '60). However, the diet containing raw meal had an adjusted metabolizable energy value significantly lower ($P < 0.01$) than that of the other two diets.

DISCUSSION

These experiments indicate that consumption of raw soybean meal markedly depresses fat absorption in young chicks. This is apparently a specific effect of some component of raw meal and not merely a nonspecific result of its poor nutritive value.

The results of Brambila et al. ('61), who found that dietary trypsin reversed the effect of raw meal on fat absorption, suggest that one of the trypsin inhibitors in raw meal is responsible for the observed effects on fat absorption. These studies show that a highly active crystalline preparation of the "Kunitz" inhibitor does not influence fat absorption in the young chick. The crystalline inhibitor caused a marked pancreatic hypertrophy similar to that observed with raw soybean meal. This confirms the report of Lyman and Lepkovsky ('57) who observed an increase in pancreatic secretion in rats receiving purified trypsin inhibitor. The lack of effect of the purified "Kunitz" trypsin inhibitor on fat absorption serves to separate any effect raw soybeans have on the pancreas from the effect on fat absorption. Apparently the poor absorption of fat is not the result of an alteration of the intestinal environ-

ment brought about by pancreatic hyperfunction.

The possibility still exists that the A_1 trypsin inhibitor reported by Rackis et al. ('59) may influence fat absorption. This inhibitor is apparently not a contaminant of the commercial preparations of "Kunitz" (A_2) inhibitor. It is also possible that neither of the trypsin inhibitors of raw soybean meal may affect fat absorption and dietary trypsin supplements may overcome the depression of fat absorption in a manner unrelated to trypsin inhibitors in the meal.

The depression of metabolizable energy value of the nonfat components of the diet due to the feeding of the trypsin inhibitor indicates that these inhibitors of raw soybean meal are at least partially responsible for the low metabolizable energy value of raw meal (Renner and Hill, '58). The metabolizable energy of the diet containing an equivalent trypsin inhibitor activity from raw meal was considerably below that of the diet containing the inhibitor. Both of the trypsin inhibitors of raw soybean meal need to be evaluated for their effect on metabolizable energy value of the diet.

SUMMARY

A series of experiments indicated that dietary raw soybean meal depressed fat absorption in chicks of two breeds at two weeks of age but not at four weeks of age. Apparently fat absorption is affected by raw meal feeding rather than digestion since absorption of soybean oil or soybean fatty acid was equally affected.

As little as 5% of protein from raw meal in a diet containing 25% of total crude protein was sufficient to cause nearly maximal depression of fat absorption. Changes in protein level of the diet could not influence fat absorption to the same extent as raw meal.

A crystalline preparation of "Kunitz" soybean trypsin inhibitor did not affect fat absorption when fed to chicks. Chicks receiving a diet containing heated soybean meal plus the trypsin inhibitor had an enlarged pancreas, grew more slowly and metabolized the diet slightly less than chicks fed a comparable diet containing heated meal.

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Ability of the Chick to Utilize D- and Excess L-Indispensable Amino Acid Nitrogen in the Synthesis of Dispensable Amino Acids[1]

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The nitrogen from the D- isomer of a racemic mixture of amino acids has been shown to be a source of nitrogen for the synthesis of dispensable amino acids in rats. Phillips and Berg ('54) fed rats minimal levels of the indispensable amino acids and observed somewhat better growth when the poorly invertible group of indispensable amino acids (valine, leucine, isoleucine, lysine and threonine) was added in the DL- form at twice the L- level. They assumed that the D- forms of these amino acids provided nitrogen for limited synthesis of the dispensable amino acids. The D-amino acids were, however, considerably less efficient as a source of nitrogen than glycine or ammonium citrate.

Rechcigl et al. ('60) observed that the growth of rats fed a mixture of L-indispensable amino acids, calculated to just meet the requirements, was inferior to that of a comparable group receiving twice the level of a mixture of DL-indispensable amino acids. A higher level of L-indispensable amino acids also supported more rapid growth.

Wachter and Berg ('60) observed excellent growth of rats when an L-amino acid diet containing both the indispensable and the dispensable amino acids was fed. Replacement in this diet of the poorly invertible group of indispensable amino acids (isoleucine, leucine, lysine, threonine and valine) with twice the level of the DL-forms resulted in less rapid growth. This retardation of growth was magnified when the readily invertible indispensable amino acids (arginine, methionine, histidine, tryptophan and phenylalanine) were also added to the diet in the DL- forms at the L-level. Replacement of the L-dispensable amino acids in this diet with their DL-isomers improved the growth response. Excellent growth was also obtained when the dispensable amino acids were replaced by glycine and ammonium citrate indicating that the synthesis of the dispensable amino acids occurs readily.

The present paper reports studies on the ability of the chick to utilize the nitrogen from D- and excess L-indispensable amino acids for the synthesis of dispensable amino acids.

EXPERIMENTAL

One-day-old Cornish × White Rock chicks of both sexes were fed for one week a low-protein diet to eliminate any possible carry-over effect that the absorbed yolk might have exerted. The composition of the low-protein diet was as follows, in per cent: soybean protein,[4] 10.00; dextrin (moist cornstarch heated for two hours at 115°C in an autoclave, then dried and ground), 67.87; DL-methionine, 0.40; glycine, 0.75; soybean oil, 9.00; cellulose,[5] 3.00; mineral mix (Briggs et al., '43), 6.00; vitamin mixture (Adkins et al., '62), 0.50; α-tocopheryl acetate (10 mg/gm), 0.10; vitamin D_3 (1,500 ICU/gm), 0.08; vitamin A acetate (25,000 USP units/gm), 0.10; choline chloride (70% aqueous), 0.20; sodium bicarbonate, 1.00; magnesium trisilicate, 0.75; aluminum hydroxide, 0.25. At the end of one week, chicks were weighed, wing-banded and separated

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into uniform groups. Chicks were housed in an electrically heated battery with raised wire-mesh floors. Feed and water were supplied ad libitum.

The birds were weighed and the feed consumption determined at three-day intervals. Excreta were also collected at three-day intervals for nitrogen retention studies. The excreta were collected in 2% boric acid, homogenized in a Waring blender, made to volume and aliquots analyzed for nitrogen by the semi-micro-Kjeldahl method. The nitrogen content of the feed was determined by the same method.

The basal diet used in these studies was the same as the isolated-soybean-protein diet described previously with the exceptions that the amino acid mixtures shown in table 1 replaced the isolated soybean protein, methionine, and glycine, and the amount of dextrin in the diet was altered to bring the diet to 100%. The 1L-amino acid mixture contained the L- form of the indispensable amino acids, with the exception of DL-methionine, at levels which the National Research Council ('54) considers to be needed to satisfy the requirements of the chick. DL-Methionine was used in place of the L- form since the D- form is well utilized by the chick. The 1DL-amino acid mixture contained the DL- forms of the indispensable amino acids with the exception of L-arginine which was not replaced due to the high cost of DL-arginine. The 2L-amino acid mixture contained the same forms of indispensable amino acids used in the 1L-amino acid mixture. With the exceptions of arginine, methionine and glycine, the 1DL- and 2L-amino acid mixtures contained these acids at twice the levels used in the 1L-amino acid mixture. Since glycine is optically inactive, the same amount of this amino acid was used in all mixtures. The same amounts of L-arginine and DL-methionine were used in all mixtures since the same forms were used in all mixtures. No dispensable amino acids were added to any of the diets.

In those experiments in which the influence of the diet on the concentration of amino acids in the plasma was studied, blood was taken from each bird at the end of the experiment and the blood from the 5 birds in each pen pooled for analysis. Blood was taken by heart puncture with a syringe containing a drop of heparin. The blood was then centrifuged and the plasma obtained. Plasma was deproteinized by the addition of an equal volume of 10% perchloric acid. The deproteinized plasma was treated with 1-fluoro-2,4-dinitrobenzene forming dinitrophenylated amino acids which were separated by two dimensional paper chromatography according to the method of Peraino and Harper ('61). The dinitrophenylated amino acids were then eluted from the paper with 1% sodium bicarbonate solution and the optical densities determined in the Beckman spectrophotometer. The optical densities were determined at 360 mμ with the exception of the optical densities of proline and hydroxyproline which were determined at 385 mμ.

TABLE 1

Amino acid mixtures

Amino acid	1L-AA[1] Diet[2]		1DL-AA Diet[2]		2L-AA Diet	
	Form	*gm/kg*	Form	*gm/kg*	Form	*gm/kg*
Arginine·HCl	L-	15.0	L-	15.0	L-	15.0
Lysine·HCl	L-	12.5	DL-	25.0	L-	25.0
Histidine·HCl	L-	4.0	DL-	8.0	L-	8.0
Methionine	DL-	8.0	DL-	8.0	DL-	8.0
Tryptophan	L-	2.0	DL-	4.0	L-	4.0
Glycine	—	10.0	—	10.0	—	10.0
Phenylalanine	L-	7.0	DL-	14.0	L-	14.0
Leucine	L-	14.0	DL-	28.0	L-	28.0
Isoleucine	L-	6.0	DL-	12.0	L-	12.0
Threonine	L-	6.0	DL-	12.0	L-	12.0
Valine	L-	8.0	DL-	16.0	L-	16.0
Tyrosine	L-	7.0	DL-	14.0	L-	14.0

[1] AA indicates amino acid.

[2] In the first two experiments the amount of phenylalanine in the 1L- and 1DL-amino acid diets was doubled and no tyrosine was added.

RESULTS AND DISCUSSION

Growth, feed efficiency and nitrogen retention data

Growth and nitrogen retention data of chicks fed the 1L- and 1DL-amino acid diets in experiment 1 are shown in table 2. The values represent the total, or average as the case may be, of two pens of 5 chicks each. Chicks fed the 1DL-amino acid diet were observed to be somewhat heavier. They gained approximately 1.5 times the amount gained by chicks fed the 1L-amino acid diet. The weight gains of the two pens of chicks fed the 1L-amino acid diet were quite similar. One pen of chicks fed the 1DL-amino acid diet, however, was considerably heavier than the other. Chicks fed the 1DL-amino acid diet consumed approximately 1.75 times the amount of nitrogen than the chicks fed the 1L-diet consumed. Despite this fact, the percentage of nitrogen retention was only slightly less for the chicks fed the 1DL-amino acid diet compared with those fed the 1L-amino acid diet, 50.9% compared with 53.1%. The grams of weight gained per gram of nitrogen consumed by the chicks of one of the pens fed the 1DL-amino acid diet was approximately the same as that of chicks fed the 1L-amino acid diet.

The differences observed in experiment 1 were considered of sufficient magnitude to merit running the experiment again. The results of the two replicate groups of chicks per diet in experiment 2 (table 2) were quite uniform. The average weight of birds fed the 1L-amino acid diet was 101.1 gm compared with 140.0 gm for birds fed the 1DL-amino acid diet. The grams gain of birds fed the 1DL-amino acid diet was over twice that of birds fed the 1L-amino acid diet. It should be pointed out, however, that the growth rate of chicks fed the 1DL-diet was still suboptimal.

It is seen from results of the nitrogen retention studies that the amount of nitrogen consumed by the birds fed the 1DL-amino acid diet was about twice that consumed by birds fed the 1L-amino acid diet. Nevertheless, the percentage of nitrogen retained was only slightly lower for chicks fed the 1DL-amino acid diet compared with that of chicks fed the 1L-amino acid diet. The grams gain per gram of nitrogen consumed was higher for the chicks fed the

TABLE 2

Growth and nitrogen retention data of chicks fed D- and excess L- indispensable amino acids as the source of nitrogen for synthesis of dispensable amino acids

	Experiment 1[1]		Experiment 2[2]		Experiment 3[3]		
	1L-AA[4] Diet	1DL-AA Diet	1L-AA Diet	1DL-AA Diet	1L-AA Diet Pen 5	1DL-AA Diet Pen 4	2 L-AA Diet Pen 6
Average weight, gm[5]	99.2 ± 5.0	113.6 ± 6.5	101.1 ± 4.9	140.0 ± 4.5	101.6 ± 8.8	137.8 ± 6.9	155.2 ± 6.2
Average gain, gm[5]	30.3 ± 4.9	46.4 ± 6.4	30.6 ± 4.7	69.4 ± 4.3	38.2 ± 7.8	74.2 ± 6.5	91.8 ± 6.2
Feed consumed, gm	1331	1467	1474	1892	682	1014	997
Nitrogen consumed, gm	19.43	33.26	21.71	41.32	10.22	23.32	22.95
Nitrogen excreted, gm	9.12	16.32	10.30	20.72	3.79	8.84	7.52
Nitrogen retained, gm	10.31	16.93	11.41	20.60	6.43	14.48	15.43
Nitrogen retained, %	53.06	50.90	52.55	49.86	62.92	62.09	67.23
Gm gain/gm N consumed	15.59	13.95	14.09	16.80	18.69	15.91	20.00

[1] The values given are for two pens of 5 chicks each; 16-day experimental period.
[2] The values given are for two pens of 5 chicks each; 18-day experimental period.
[3] The values given are for one pen of 5 chicks; 17-day experimental period.
[4] AA indicates amino acid.
[5] The mean ± SE of the mean.

1DL-amino acid diet, 16.8, compared with 14.1 for chicks fed the 1L-amino acid diet.

The extent to which excess L- as well as D-indispensable amino acids may serve as a source of nitrogen for the synthesis of dispensable amino acids was studied in experiment 3. The 1L-, 1DL-, and 2L-amino acid mixtures shown in table 1 were added to the basal diet. The 1DL- and 2L-amino acid diets were isonitrogenous.

Growth and feed efficiency data of chicks fed the above mentioned diets are shown in table 3. The average gains of chicks fed the 2L- and 1DL-diets were approximately equal and were about double the average gains of chicks fed the 1L-basal diet. The results for the two replicate groups of chicks fed the 2L-diet were considerably less uniform than those for the two fed the 1L- or 1DL-diets. No explanation for these results can be offered. The feed efficiency of chicks fed the 2L- and 1DL-diets was markedly improved over that of chicks fed the 1L-basal diet. The 2L-diet appeared to elicit a somewhat greater improvement in efficiency than did the 1DL-diet.

The results of nitrogen retention studies on one of the two pens of chicks fed the various diets are shown in table 2. Even though the 2L- and 1DL-diets supplied over 1.5 times as much nitrogen as the 1L-basal diet (these two diets did not supply twice as much nitrogen as the 1L-diet, because the amounts of arginine, methionine and glycine were equal in all diets) the percentage of nitrogen retained by chicks fed these diets was as high as, or higher than, that of chicks fed the 1L-basal diet. From the results of this study it appeared that chicks fed the 2L-diet retained a higher amount of nitrogen than chicks fed the 1DL-diet. It should be remembered from the results shown in table 3 that chicks in pen 6 grew considerably more rapidly than those in pen 1. Whether this fact would have an influence on the nitrogen retention is unknown. The grams gain per gram of nitrogen consumed for chicks fed the 2L-diet was also higher than that of chicks fed the 1DL-diet. The grams gain per gram of nitrogen consumed for chicks fed the 1L-basal diet cannot accurately be compared with that of chicks fed the other two diets since the amount of nitrogen available for dispensable amino acid synthesis was severely limiting.

Plasma amino acid studies

The influence of these amino acid diets on the concentration of free amino acids in the plasma is shown in table 4. Since none of the diets contained dispensable amino acids the amounts found in the plasma are indicative of the amounts synthesized.

In experiment 2 the concentrations of dispensable amino acids in the plasma of chicks fed the 1DL-diet were markedly higher than those observed in chicks fed the 1L-diet, presumably due to the conversion of much of the extra nitrogen from the D-indispensable amino acids into dispensable amino acids.

Since the concentrations of serine, tyrosine and cystine in the plasma are influenced by the levels of glycine, phenylalanine and methionine, respectively, in the diet, these amino acids were not grouped with the dispensable amino acids. The serine and cystine concentrations in the

TABLE 3

Growth and feed efficiency data of chicks fed D- and excess L- indispensable amino acids as the source of nitrogen for the synthesis of dispensable amino acids

Diet	Pen	Avg weight[1]	Avg gain[1]	Gm feed/ gm gain
		gm	*gm*	
1L-AA[2]	3	103.6 ± 12.5	39.6 ± 11.3	3.26
	5	101.6 ± 8.8	38.2 ± 7.8	3.57
1DL-AA	2	136.0 ± 6.7	72.2 ± 7.1	2.43
	4	137.8 ± 6.9	74.2 ± 6.5	2.73
2L-AA	1	129.4 ± 9.1	66.4 ± 9.0	2.21
	6	155.2 ± 6.2	91.8 ± 6.2	2.17

[1] The mean ± SE of the mean of 5 chicks/pen; 17-day experimental period.
[2] AA indicates amino acid.

TABLE 4

Influence of diet on the concentrations of free amino acids in the plasma

	Experiment 2		Experiment 3		
	1 L-AA[1] Diet	1 DL-AA Diet	1 L-AA Diet	1 DL-AA Diet	2 L-AA Diet
	μmoles of amino acid/ml plasma		*μmoles of amino acid/ml plasma*		
Glutamic acid	0.18	0.25	0.05	0.06	0.09
Glutamine + asparagine	0.24	0.43	0.12	0.26	0.29
Alanine	0.15	0.36	0.10	0.22	0.15
Proline	0.09	0.13	0.10	0.17	0.18
Hydroxyproline	0.03	0.06	0.05	0.03	0.07
	0.69	1.23	0.42	0.74	0.78
Serine	0.72	0.70	0.47	0.30	0.48
Tyrosine	0.31	0.38	0.20	0.29	0.79
Cystine	0.04	0.04	0.01	—	0.02
	1.07	1.12	0.68	0.59	1.29
Glycine	1.19	0.88	0.77	0.67	0.58
Threonine	0.27	0.57	0.33	0.30	1.18
Lysine	0.33	0.62	0.23	0.21	0.23
Valine	0.19	0.55	0.24	0.40	0.25
Leucine + isoleucine	0.12	0.83	0.14	0.52	0.17
Phenylalanine + methionine	0.18	0.75	—	—	—
Phenylalanine	—	—	0.14	0.24	0.22
Methionine	—	—	0.07	0.06	—
	2.28	4.20	1.92	2.40	2.63
Total	4.04	6.55	3.02	3.73	4.70

[1] AA indicates amino acid.

plasma of chicks fed both diets were about the same that would be expected since the glycine and methionine levels of both diets were the same. The amounts of tyrosine formed by chicks fed the two diets differed only slightly.

The indispensable amino acids, with the exception of glycine, were observed to be present in considerably higher amounts in the plasma of chicks fed the 1DL- diet. A large part of this increase may consist of D-amino acids circulating in the blood. Since the separation of phenylalanine and methionine on the chromatogram was not complete, the values for these two amino acids were combined. Presumably the large value for chicks fed the 1DL-diet is due primarily to D-phenylalanine.

The concentrations of dispensable amino acids in the plasma of chicks fed the 2L- and 1DL-diets in experiment 3 were approximately double those of chicks fed the 1L-diet. This is a further indication that the nitrogen from the D- and excess L-indispensable amino acids was being utilized for the synthesis of dispensable amino acids.

Tyrosine was included in the diet in this experiment in contrast with the procedure followed in experiments 1 and 2. Since the amount of both L-tyrosine and DL-tyrosine in the 2L- and 1DL-diets, respectively, was twice the amount in the 1L-basal diet, no explanation can be offered for the wide differences in the amounts of tyrosine found in the plasma of chicks fed the 2L- and 1DL-diets, unless there are differences in the rates of absorption of the two isomers from the intestine.

With the exception of threonine, which was very high in the plasma of chicks fed the 2L-diet, the concentrations of indispensable amino acids in the plasma of chicks fed the 1L- and 2L-diets were quite similar. The concentrations of valine, leucine and isoleucine in the plasma of chicks fed the 1DL-diet were considerably higher than those observed in chicks fed the other two diets. The high values may have been due to the D- forms of these amino acids circulating in the blood. Similar values were obtained in the previous experiment.

The results of these studies indicate quite clearly that the nitrogen from D- and

excess L-indispensable amino acids in the diet was utilized for the synthesis of dispensable amino acids in chicks fed a diet devoid of dispensable amino acids. The data do not permit any conclusion as to whether the intestinal microflora played a part in this utilization. The findings of these studies are in agreement with those of Phillips and Berg ('54) who observed that the young rat was able to utilize D-indispensable amino acid nitrogen for the synthesis of dispensable amino acids for growth and with those of Rechcigl et al. ('60) who found that the young rat could utilize both excess L- and D-indispensable amino acid nitrogen for the synthesis of dispensable amino acids for growth. These results should not be construed to indicate that D- or excess L-indispensable amino acids can satisfy the dispensable amino acid requirements for maximal growth of the chick. Luckey et al. ('47) and Stucki and Harper ('61) showed that dispensable as well as indispensable amino acids must be included in the diet for maximum growth of chicks. Similar conclusions have been reached by other workers in studies with the rat.

The increased concentrations of valine, leucine and isoleucine observed in the plasma of chicks fed the DL-diets prompts one to comment on the work of Hedin and Schultze ('61) with rats. These workers found that as much as 65% of the administered D-isoleucine, 51% of D-valine and 62% of D-threonine was excreted in the urine, thus indicating that under the conditions of their experiment the D- isomers of these amino acids were poorly utilized. The high concentrations of these amino acids in the plasma of the chicks in these experiments might also indicate relatively poor utilization.

SUMMARY

Studies were conducted on the ability of the chick to utilize D- and excess L-indispensable amino acid nitrogen for the synthesis of dispensable amino acids. A purified type of diet containing only the essential amino acids was used in these studies. Growth, feed efficiency, nitrogen retention and plasma amino acid concentrations were the criteria used to determine the extent to which the D- or excess L-indispensable amino acid nitrogen was utilized. The nitrogen from D- and excess L-indispensable amino acids was shown to be utilized by the chick for the synthesis of dispensable amino acids. Growth of chicks fed diets containing D- or excess L-indispensable amino acids indicated that the two sources of nitrogen were utilized equally well. Feed efficiency and nitrogen retention data, however, indicated that the nitrogen from L-indispensable amino acids may have been somewhat more efficiently utilized than the nitrogen from D-indispensable amino acids. Marked increases in the concentrations of dispensable amino acids of plasma were observed as a result of the addition of D- or excess L-indispensable amino acids to the diet. The data do not permit any conclusion as to whether the intestinal microflora played a part in this utilization.

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Effect of Threonine-induced Amino Acid Imbalance on the Excretion of Tryptophan Metabolites by the Rat[1]

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Since the studies of Krehl and co-workers (Krehl et al., '45a, b) on the effect of the addition of corn grits to low-protein diets on rats, the phenomenon of amino acid imbalance has been investigated extensively. Imbalances have been produced by the addition of gelatin (Krehl et al., '46a; Briggs et al., '46; Salmon, '49), protein hydrolysate (Krehl, et al., '46a; Salmon, '49; Henderson et al., '47) or amino acids (Henderson et al., '47; Krehl et al., '46b; Singal et al., '47; Hankes et al., '48) to tryptophan-low diets. To explain this effect Salmon ('54) advanced the hypothesis that in the absence of enough tryptophan to permit utilization of the total ingested amino acids for the synthesis of tissue protein, the surplus amino acids are excreted or disposed of in some way, and that in this process there is a wasting of the tryptophan originally available. The present study was designed to test this hypothesis by comparing the excretion of radioactive tryptophan and certain of its metabolites by rats fed normal and "imbalanced" diets. If the hypothesis were correct, there should be an increased excretion of administered tryptophan or some of its metabolites by rats consuming imbalanced diets. The use of radioactive tryptophan permitted a simple estimation of the amount of tryptophan excreted or metabolized via the principal routes of excretion — carbon dioxide, urine and feces.

EXPERIMENTAL

Animals and animal diets. Since the conditions for the induction of amino acid imbalance in animals by the addition of a single amino acid to a low-protein diet are rather delicate (Henderson et al., '53; Morrison and Harper, '60), a preliminary experiment was conducted to establish the dietary pattern that consistently produced the desired results. The amount of threonine was determined that induces inhibition of growth in weanling Sprague-Dawley rats fed a hydrolyzed casein-basal diet and a specific amount of tryptophan. The amount of tryptophan was such that it permits reasonable growth when excess threonine was not added.

The basal diet had the following composition: 9% of acid-hydrolyzed salt-free vitamin-free casein,[4] 3% of cottonseed oil, 4% HMW salts (Hubbell et al., '37), 0.2% of cystine, and sucrose. Varying amounts of DL-tryptophan were incorporated into the basal control diet, and graded quantities of both DL-threonine and of DL-tryptophan were incorporated into the experimental rations. In some diets 2 mg of niacin were incorporated/100 gm of diet. Vitamins were included in the dry diet mixtures in the following amounts (mg/100 gm of diet): thiamine·HCl, 1.0; riboflavin, 1.0; pyridoxine, 1.0; Ca pantothenate, 2.0; choline chloride, 20.0; inositol, 10.0; biotin, 0.02; and pteroylglutamic acid, 0.2. Suitably fortified halibut liver oil was administered to the rats once each week to provide the following daily in-

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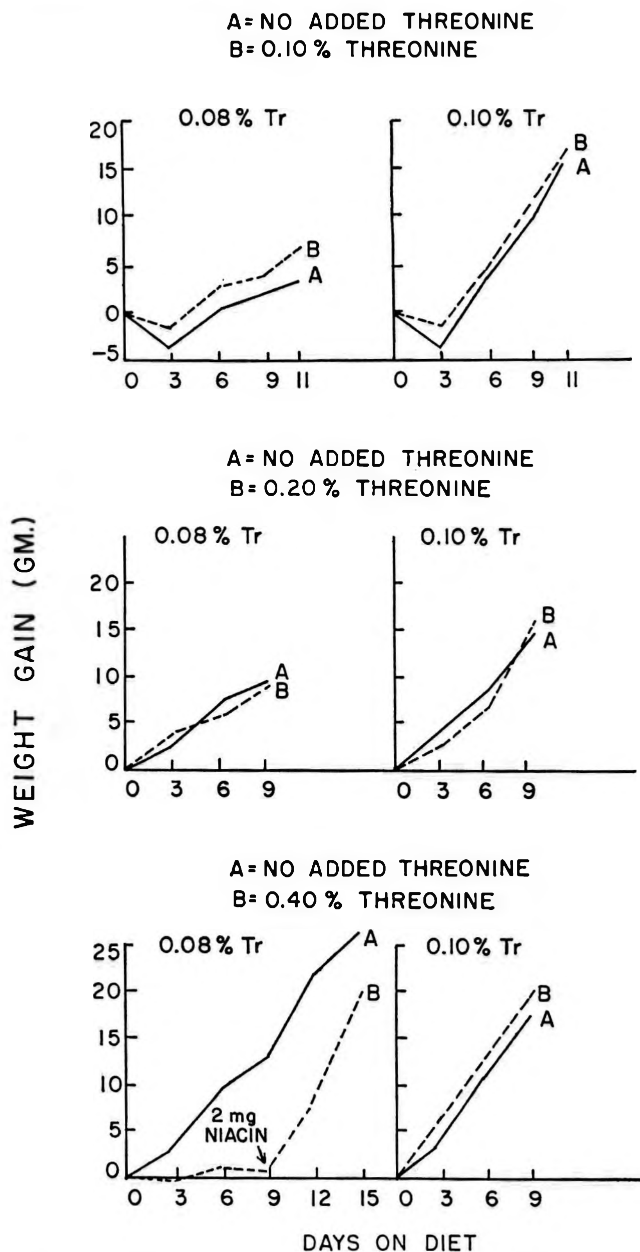

Fig. 1 Average weight gain of rats fed a 9% acid hydrolyzed casein diet containing 0.08 or 0.1% of tryptophan with or without various levels of threonine supplementation.

takes: vitamin A, 400 IU; vitamin D, 4 IU; 2-methyl 1,4-naphthoquinone, 0.06 mg; and α-tocopherol, 0.7 mg.

Groups of three or four weanling female Sprague-Dawley rats were randomly designated as experimental or as control groups. They were housed singly in wire cages for periods of from 9 to 15 days and provided ad libitum their respective diets and water. Each rat was weighed every three days. Neither 0.05 nor 0.06% of DL-tryptophan, when added alone to the basal diet, was

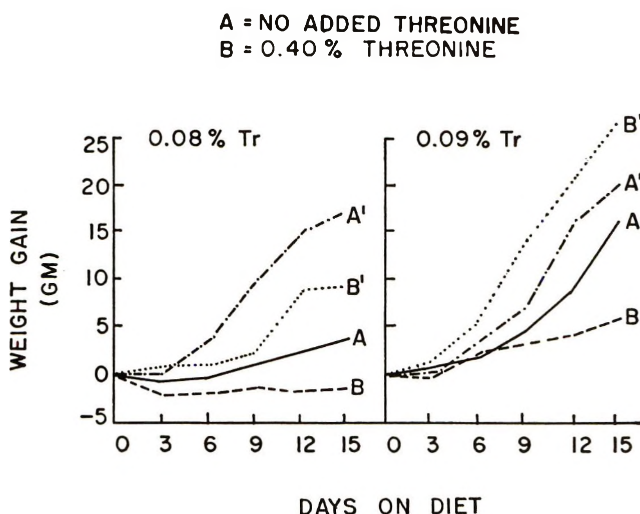

Fig. 2 Average weight gain of rats fed a 9% acid hydrolyzed casein diet containing 0.08 or 0.09% of tryptophan with or without threonine and niacin supplementation. A, no added threonine; B, 0.40% threonine added; A[1], 2 mg/100 gm of niacin added; B[1], 0.40% of threonine + 2 mg/100 gm of niacin added.

sufficient to support growth. The average weight changes of groups fed higher levels of DL-tryptophan are shown in figure 1. It is evident that 0.08% of tryptophan supported growth that varied considerably from experiment to experiment, whereas 0.10% of tryptophan permitted reasonable growth in all experiments. The addition of 0.10 or 0.20% of DL-threonine produced no growth inhibition in rats fed diets containing 0.08 or 0.10% of the DL-tryptophan. Supplementary DL-threonine at a level of 0.40% did not inhibit the growth of rats fed diets containing 0.10% of DL-tryptophan but retarded growth of rats fed diets containing 0.08% of DL-tryptophan. The addition of 2 mg of niacin/100 gm of diet corrected this growth inhibition. Growth curves obtained with tryptophan levels of 0.08 and 0.09% are compared in figure 2. The growth of rats fed the 0.09% tryptophan diets was also inhibited by the addition of 0.40% of DL-threonine and this was prevented by the addition of 2 mg of niacin/100 gm of diet. Because of the variable responses to 0.08% of dietary tryptophan, we used a 0.09% level in the radioisotope experiment and produced growth inhibition by the addition of 0.40% of DL-threonine.

In the main experiment, 4 experimental and control pairs of female weanling Sprague-Dawley rats were used. The rats were kept separately in ordinary wire cages for 12 days, fed their respective diets and given water ad libitum. They were inspected daily and food intake and body weights recorded every three days. On the thirteenth day, each rat was transferred to a specially constructed metabolism cage and kept in it for 48 hours. During the first 24 hours in the metabolism cage,[5] the rats were fed a diet of the same composition as previously except that the added DL-tryptophan was replaced by an equal amount of a mixture of DL-tryptophan-3-C^{14} and nonlabeled DL-tryptophan.[6] To reduce contamination of urine and feces with spilled food, the diet was placed in a small beaker inside a food cup provided with a wire mesh extending from the rim of the beaker to the rim of the food cup. Slightly more food (10 gm) was offered than the average daily intake during the first 12 days of the experiment. After the first 24 hours, the rats were given their original nonlabeled diet until killed at exactly 48 hours.

[5] An airtight plexiglass cage, with provision for separate carbon dioxide, urine and feces collection, so designed so that the rat had complete freedom of movement and could have food and water ad libitum.

[6] This was prepared by dissolving the labeled and nonlabeled tryptophan in 95% ethanol. This solution was then dried and the resulting powder incorporated into 50 gm of the appropriate diet by thorough blending in an electric mixer.

The activity of the tryptophan administered during the thirteenth day of the experiment was computed from the activity of the labeled tryptophan mixture and dietary consumption. An aliquot of the labeled tryptophan mixture was diluted with a known amount of nonradioactive DL-tryptophan, dissolved in concentrated ammonia, and two 0.1-ml aliquots counted in a Packard Tri-Carb liquid scintillation spectrometer using 2 ml of absolute ethanol and 7.9 ml of PPO-POPOP-toluene scintillator solution.[7] A correction for quenching was made. The activity of the original tryptophan mixture used was 926,300 count/min/mg. Similar aliquots were transferred to one-inch planchets, dried, weighed and counted in a windowless gas-flow counter. After correcting to a sample thickness of 10 mg/cm², the activity was 136,440 count/min/mg. Assay of the activity of the tryptophan was necessary by these two methods to permit comparison between the activity of (a) expired carbon dioxide, which was counted as dried $BaC^{14}O_3$ in a windowless counter, and (b) the urine and feces, which were assayed by liquid scintillation counting. A self-absorption curve for the labeled tryptophan mixture counted in the dry state in a windowless counter was similar to that of $BaC^{14}O_3$, allowing a direct comparison of the administered activity with the activity of the carbon dioxide subsequently collected.

Collection and analyses of samples. During the experiment carbon dioxide-free air was swept through the metabolic cage at a rate of 500 ml/min and the expired carbon dioxide was collected continuously by 12-hour periods during the two-day metabolic study. For the collection, 2.8 liters of carbon dioxide-free 5 N sodium hydroxide were used. The CO_2 contained in two 5-ml aliquots of the sodium hydroxide was precipitated as $BaCO_3$, filtered through tared sintered-glass crucibles, dried and weighed. Two other aliquots were similarly precipitated, centrifuged, washed three times with carbon dioxide-free water, and slurried in methanol. The slurry was plated to give a sample thickness of about 10 mg/cm², let stand overnight, dried, weighed and counted in the windowless gas-flow counter. Activity was corrected to a sample thickness of 10 mg/cm².

Urine was collected for each 24-hour period from each rat during the two days in the metabolic cage and frozen prior to analysis. After thawing, it was centrifuged, diluted to a known volume, and two 0.1-ml aliquots counted in the liquid scintillation spectrometer with 2 ml of absolute ethanol and 7.9 ml of PRO-POPOP-toluene scintillator solution. Corrections for quenching were made immediately.

Fecal collections were made during the last two days of the experiment and pooled with the well-formed feces from the lower intestinal tract obtained after sacrifice. The ground dried feces were subjected to continuous Soxhlet extraction for 12 hours with absolute ethanol. Further extraction for more than 12 hours did not show any additional increase in extractable activity. Two 2-ml aliquots were counted in the liquid scintillation spectrometer using 8 ml of the phosphor-toluene solution. Each sample was checked and corrected for quenching.

RESULTS

Data on weight gains and on food and tryptophan intakes are summarized in table 1. All of the experimental rats gained less weight than did their respective controls during the 14-day experimental period. The control rats gained an average of 19 gm, and the experimental rats gained an average of only 11 gm. All of the experimental animals ingested less diet than did their controls, both while in the ordinary wire cage and while in the metabolic cage.

On the average, the total respiratory CO_2 derived from labeled DL-tryptophan for the 48 hours was approximately the same for both the experimental and control rats (table 2). There was, however, a slight increase in the urinary and fecal excretions by the experimental animals over the controls. The exaggerated fecal excretion by the experimental rat in pair 1 is suspected to be due to contamination by a small amount of urine. No significant difference was found in the *average total excretion* by the experimental and control groups.

When the excretion of labeled tryptophan is related to the quantity of radiotryptophan ingested, however, differences

[7] 2,5-Diphenyloxazole, 4 gm; 1,4-bis-2(5-phenyloxazolyl)-benzene, 500 mg; toulene, to make 1 liter.

in excretion between the experimental and control rats become apparent (table 3). Expressed as percentage of intake, both the $C^{14}O_2$ and urinary radioactivity excretions of the experimental group were significantly higher than those of the controls ($P = 0.003$ and 0.044, respectively). The difference in fecal excretion was not significant, but since the extracted fecal residue was not counted, no statement can be made regarding digestibility.

When the total excretions are expressed as percentage of intake, the experimental rats excreted an average total of 12.6% of the ingested labeled tryptophan, whereas the controls excreted an average total of only 8.2%. The difference of 4.4%, representing an increase in excretion by

TABLE 1

Weights and dietary intakes of weanling rats fed a basal diet containing 9% of hydrolyzed casein and 0.09% of DL-tryptophan, without (C) or with (E) 0.40% of DL-threonine

Pair	Initial weight		Total 14-day weight gain		Total 14-day food intake		Metabolism cage food intake		Metabolism cage labeled tryptophan intake	
	C	E	C	E	C	E	C	E	C	E
	gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*mg*	*mg*
1	57	60	22	10	107.2	79.5	6.1	3.5	5.54	3.15
2	60	56	17	15	98.4	84.0	4.9	4.0	4.41	3.60
3	40	47	17	12	65.8	64.5	5.3	3.0	4.77	2.70
4	66	49	20	8	115.2	64.2	7.8	6.0	7.02	5.40
Average	56	53	19	11	96.7	73.1	6.0	4.1	5.44	3.71

TABLE 2

Two-day excretions of products of labeled DL-tryptophan, estimated in micrograms of tryptophan[1]

Pair	As $C^{14}O_2$		In urine		In feces		Total	
	C[2]	E[2]	C	E	C	E	C	E
1	372.8	307.7	168.5	132.3	2.2	6.9	543.5	446.9
2	226.7	308.6	110.6	105.3	4.5	1.9	341.8	415.8
3	216.7	196.8	96.8	115.6	2.9	1.9	316.4	314.3
4	442.2	443.8	162.0	247.8	5.2	6.5	609.4	698.1
Average	314.6	314.2	134.5	150.3	3.7	4.3	452.8	468.8

[1] C^{14} excreted as tryptophan in $\mu g = \frac{\text{total activity excreted}}{\text{activity/mg of tryptophan administered}} \times 1{,}000$.

Radioactivity of expired CO_2 was assayed as $BaCO_3$ at 10 mg/cm^2 thickness in windowless gas-flow counter; radioactivities of urine and feces were assayed in a Packard Tri-Carb liquid scintillation spectrometer.

[2] Given 9% hydrolyzed casein-basal diet and 0.09% of DL-tryptophan, without (C) or with (E) 0.40% of DL-threonine.

TABLE 3

Two-day excretions of products of labeled DL-tryptophan, estimated as percentage of amount ingested

Pair	As $C^{14}O_2$		In urine		In feces		Total	
	C[1]	E[1]	C	E	C	E	C	E
1	6.73	9.77	3.04	4.20	0.04	0.22	9.81	14.19
2	5.14	8.57	2.51	2.93	0.10	0.05	7.75	11.55
3	4.54	7.29	2.03	4.28	0.06	0.07	6.63	11.64
4	6.30	8.22	2.31	4.59	0.07	0.12	8.68	12.93
Average	5.68	8.46	2.47	4.00	0.07	0.12	8.22	12.58
Difference	2.78%		1.53%		0.05%		4.36%	
P	0.0034		0.044		0.40		<0.001	

[1] Given 9% hydrolyzed casein-basal diet and 0.09% of DL-tryptophan without (C) and with (E) 0.40% of DL-threonine.

the imbalanced rats over that by the controls, was highly significant ($P = < 0.001$).

DISCUSSION

The growth inhibition produced by the addition of 400 mg of threonine to 100 gm of our basal diet is due to an amino acid imbalance as defined by Harper ('58) and is similar to those produced by Henderson et al. ('53) and Koeppe and Henderson ('55). The effect of the imbalance in this study was somewhat variable. For example, in pair 2 (table 1), the difference in total weight gain between the experimental rat and its control was only 2 gm, whereas the other pairs showed a greater weight difference. Whether this is due to individual differences, difference in niacin reserve or any other factor cannot be ascertained. These occasional inconsistencies, however, do not appear to be uncommon in cases in which imbalance is induced by the addition of a single amino acid to a low-protein diet (Morrison and Harper, '60).

The administration of radiotryptophan on the thirteenth day of the experiment made possible the estimation of the extent of excretion of tryptophan and its degradation products at a time when the imbalance was clearly manifest. At the same time, since the radiotryptophan was incorporated into the diet, we avoided any possible metabolic alterations resulting from parenteral or separate oral administration of the amino acid.

The diminished food intake by the imbalanced rats, especially in the pairs that showed marked growth retardation (pairs 1 and 4, table 1), is in agreement with the observations of others (Deshpande et al., '58; Kumta et al., '58; Kumta and Harper, '60). That this reduction in food intake is an integral part of the imbalance phenomenon is supported by the observation that the further addition of tryptophan to a total level of 0.10% or the use of small amounts of DL-threonine in the preliminary experiments (fig. 1) failed to reduce food intake and retard growth. It is possible that this difference in intake may account for at least part of the increased catabolic loss of tryptophan by the imbalanced rats. The food intakes of both groups while in the metabolism cage were somewhat less than they were during the previous two-week period. This may be due in part to the more elaborate precautions provided in the metabolism cage to prevent food scattering.

The average total excretion of products of radiotryptophan by the experimental rats by each of the three modes of excretion tested was approximately the same or slightly more than that excreted by the control rats. This was offset by the differences in dietary intake (and hence radiotryptophan intake) between the control and experimental groups. There was no essential difference in the pattern of excretion between the experimental and control groups; about 69% of the excreted activity appeared as carbon dioxide, about 30% in the urine and 0.8% in the feces in both control and experimental groups. The observation that the fecal content of tryptophan and its metabolites in the two groups were not significantly different infers that reduced absorbability is not a part of the imbalance mechanism.

The lack of growth inhibition in the experimental rat in pair 2 is associated with a rather low urinary and fecal excretion of radioactivity (table 2). That an imbalanced condition was produced, however, is evident from the increase in radioactivity of the expired carbon dioxide (table 3).

When the excretion is expressed as the percentage of radiotryptophan ingested, it becomes apparent that the imbalanced rats excreted a decidedly greater percentage of the administered radiotryptophan (12.6%) than did their controls (8.2%). The difference, 4.4%, represents the portion of the ingested activity excreted by the experimental rats in excess of that excreted by the controls. Assuming that this difference in excretion between the two groups could be expressed in terms of quantity of tryptophan, it may be reasoned that this amount added to the diet of the experimental animals should prevent amino acid imbalance caused by 0.40% of threonine. The difference in excretion of 4.4% is equivalent to 3.96 mg of tryptophan /100 gm of diet, suggesting that growth with a diet containing 0.094 to 0.095% of tryptophan would not be inhibited by 0.40% of threonine. This is in accord

with the results of our preliminary study in which it was found that the use of 0.10% of tryptophan added to the 9% hydrolyzed casein-basal diet plus 0.40% of threonine prevented growth retardation, whereas the use of 0.09% of tryptophan did not.

The observed increase in the excretion of administered C^{14} activity by the experimental rats is in agreement with the observations of Sauberlich and Salmon ('55), who found an increase in the urinary excretion of both "free" and "total" tryptophan among rats fed imbalanced diets. These authors produced the imbalance by the addition of proteins lacking in one or a few amino acids (gelatin or oxidized casein) as contrasted with the addition of a single amino acid. Although Harper ('58) expressed the doubt that adding a relatively small amount of a single amino acid to cause an imbalance could produce an increased loss of the most limiting amino acid, the results of the present study demonstrate such an effect. Sauberlich and Salmon ('55) concluded that the observed increase in the urinary loss of tryptophan among their imbalanced rats was not sufficient to account for the pronounced growth depression of the animals, but Salmon ('58) expressed the opinion that the excretion of unchanged amino acids may only be a part of the imbalance effect and that the rest of the excretion may be in the form of metabolites. The validity of this conclusion is obvious from the results of the present study.

The observation of an increased catabolism of the limiting amino acid in amino acid imbalance supports the hypothesis of Salmon ('54). It is also compatible with the finding of Kumta and Harper ('61) who reported higher urea levels in rats fed an imbalanced diet, and with the data of Deshpande and Harper ('58) who found an immediate decrease in the retention of nitrogen in imbalance. Apparently when the limiting amino acid, already minimal in amount, becomes inadequate for the maximum synthesis of tissue protein as a result of the addition of other amino acids, the surplus amino acids are excreted and in the process there is a wasting of the limiting amino acid. This catabolic loss further diminishes protein synthesis and retards growth. In this way, there is not necessarily a diminished capacity of the animal to utilize the ingested amino acids but rather an accentuated deficiency of the limiting essential amino acid as a result of its increased catabolism or excretion. The effect, therefore, differs little from that produced by the administration of diets deficient in some essential amino acids. In fact, a similar increase in the urinary excretion of amino acids has been found in several studies (Pearce et al., '47; Schweigert, '47; Sauberlich et al., '48) among rats and mice fed incomplete proteins or proteins of low biologic values.

SUMMARY

Amino acid imbalance was produced in rats fed a 9% salt-free acid hydrolyzed casein niacin-free diet with 0.09% of DL-tryptophan and 0.40% of DL-threonine. By inclusion of radiotryptophan in the diet toward the end of the experiment, the extent of excretion of tryptophan and its metabolities through carbon dioxide, urine and feces was estimated. It was shown that the experimental rats excreted a greater percentage of the administered radiotryptophan through carbon dioxide, urine and feces than their controls. These results support the hypothesis that in amino acid imbalance, concomitant with the excretion of the surplus amino acids, there is a wasting of the limiting amino acid through catabolism and excretion, thus increasing the deficiency and retarding growth.

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Effect of Vitamin B_{12} Deficiency on Cholesterol Metabolism

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Earlier studies implicating the anti-pernicious anemia agent in cholesterol metabolism were conducted by Bloor and Macpherson ('17) who demonstrated low blood cholesterol levels in anemia, and by Muller ('30) who found low serum cholesterol levels in pernicious anemia in relapse. The latter demonstrated further a definite increase in the serum cholesterol level concomitant with the response in the reticulo-endothelial system which was proportional to the intensity of the remission.

In contrast to the reports of low serum cholesterol levels in cases of human pernicious anemia, Hsu and Chow[2] reported higher levels of serum and adrenal cholesterol in their vitamin B_{12}-deficient rats and chicks. Later, Ignatova ('61) found that administration of vitamin B_{12} lowered the serum cholesterol levels of 69 out of 75 patients with atherosclerosis.

Further observations pointing to a possible derangement in lipid metabolism in vitamin B_{12} deficiency were made by Ling et al. ('56), Hsu and Chow ('60), and Boxer et al. ('55), who noted higher coenzyme A levels in the liver of deficient animals. These studies suggest a possible involvement of vitamin B_{12} in cholesterol metabolism and prompted us to study the role of this vitamin in cholesterol metabolism. Total cholesterol content was determined in certain tissues in male and female rats fed diets deficient in vitamin B_{12} and supplemented with vitamin B_{12}. The utilization of acetate 1-C^{14} in the liver of these two groups of rats was also examined.

METHODS

Progeny from rats of the McCollum strain, fed a soybean diet deficient of vitamin B_{12} during pregnancy and lactation, were divided into two groups and placed in individual cages with wide mesh and screen bottoms under comparable environmental conditions. During the entire period of the experiment, one group was offered a vitamin B_{12}-deficient soybean diet (table 1), while corresponding littermates of this group were offered the same basal ration supplemented with vitamin B_{12} (50 μg/kg of diet). All rats were fed ad libitum, and weighed periodically to study their growth response. Microbiological assay of vitamin B_{12} in serum was carried out according to the procedure of Skeggs et al. ('50). The samples were processed according to the method of Okuda as described by Gaffney et al. ('57).

TABLE 1
Composition of diet

	%
Soybean meal	60
Salt mixture[1]	4
Sucrose	32
Corn oil[2]	4
Water-soluble vitamins[3]	+
Fat-soluble vitamins[4]	+

[1] Hegsted et al. ('41).
[2] Mazola, Corn Products Company, Argo, Illinois.
[3] Composition of water-soluble vitamins/liter in 50% alcoholic solution (10 ml/100 gm of diet) (in milligrams): thiamine·HCl, 200; riboflavin, 300; pyridoxine·HCl, 250; biotin, 10; folic acid, 20; and (in grams) niacin, 5; inositol, 10; Ca pantothenate, 2; p-aminobenzoic acid, 25; and choline·HCl, 100.
[4] Each kilogram of diet contained the following: α-tocopherol, 23 mg; menadione, 2.1 mg; vitamin A, 1,200 USP units; and vitamin D, 170 USP units.

Animals were killed under light ether anesthesia and blood was drawn by cardiac puncture.

Determination of total cholesterol in serum and liver. Serum and liver choles-

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[2] Hsu, J. M., and B. F. Chow 1957 Vitamin B_{12} deficiency and hypercholesterolemia. Federation Proc., 16: 63.

terol levels were determined colorimetrically according to the Sperry and Webb ('50) modification of the Shoenheimer and Sperry method. Analyses were carried out in duplicate samples and the results checked to within 3% of their means. Portions of total liver were homogenized by means of Potter Elvehjem glass homogenizer and cholesterol was then extracted with a mixture consisting of equal volumes of acetone and absolute alcohol.

Effect of vitamin B_{12} repletion on the incorporation of acetate 1-C^{14} into cholesterol in the liver of deficient rats. Ten 5-month-old vitamin B_{12}-deficient and 5 vitamin B_{12}-treated littermates were selected for this experiment. Five of the vitamin B_{12}-deficient (group A) and 5 of the vitamin B_{12}-treated littermates (group C) were injected with one milliliter of physiological saline solution every other day for three weeks. The remaining 5 vitamin B_{12}-deficient animals (group B) received one milliliter of vitamin B_{12} (5 μg/ml) in physiological saline solution every other day for the same period of time. All animals received intraperitoneal injections of acetate 1-C^{14} and were killed for the determination of total cholesterol in liver.

Oxidation of acetate 1-C^{14}. Ten rats from each dietary group were selected for this experiment. All were given intraperitoneal injections of acetate 1-C^{14}, 5 μc/100 gm of body weight (specific activity of 25 μc/mmole). Carbon dioxide was collected as described previously and radioactivity determined.

Collection of carbon dioxide. Animals were placed in an enclosed glass chamber. Carbon dioxide-free air was drawn through the chamber, and the carbon dioxide evolved was collected in an aqueous NaOH solution and precipitated as $BaCO_3$. Its radioactivity and the weight of the insoluble carbonate were determined.

Incorporation of acetate 1-C^{14} into cholesterol. An equal number of littermates from each dietary group was given intraperitoneal injections of acetate 1-C^{14} (5 μc/100 gm of body weight). The specific activity was 25 μc/mmole. Exactly 30 minutes after injection, the animals were killed. Blood was collected by cardiac puncture and the liver was rapidly excised. The total cholesterol in the specimens was

TABLE 2

Vitamin B_{12} levels in liver and serum of rats fed vitamin B_{12}-deficient and vitamin B_{12}-supplemented diets

Group[1]	Sex	Body wt	Serum vitamin B_{12} levels	Liver vitamin B_{12} levels
		gm	*μμg/ml*	*mμg/gm*
Vitamin B_{12}-deficient	Male	276 ± 10[2]	84.8 ± 5.1	20 ± 1.4
Vitamin B_{12}-treated	Male	397 ± 8	573 ± 46	174 ± 2.8
Vitamin B_{12}-deficient	Female	213 ± 7	73.9 ± 2.1	19 ± 1.6
Vitamin B_{12}-treated	Female	211 ± 6	498 ± 37	172 ± 3.3

[1] Ten rats (4 to 5 months old) were used in each group.
[2] Mean ± SE.

TABLE 3

Serum and liver cholesterol in rats fed vitamin B_{12}-deficient and vitamin B_{12}-supplemented diets

Group	Sex	No. of rats	Body weight	Serum cholesterol	Liver cholesterol
			gm	*mg/100 ml*	*mg/gm*
Vitamin B_{12}-deficient	Male	24	273 ± 9[1]	61 ± 4.8[1]	1.95 ± 0.18[1]
Vitamin B_{12}-treated	Male	24	392 ± 12	83 ± 3.8	2.34 ± 0.13
t value				3.6	1.77
Vitamin B_{12}-deficient	Female	23	201 ± 8	93 ± 3.5	2.45 ± 0.10
Vitamin B_{12}-treated	Female	23	219 ± 4	87 ± 4.6	2.46 ± 0.05
t value				1.1	0.01

[1] All results are expressed as the mean ± SE.

determined colorimetrically, and the radioactivity was also measured.

Radiometric determinations. The C^{14}-labeled cholesterol was precipitated as a digitonide which was dissolved in hot methanol and quantitatively transferred into aluminum planchets and dried by means of an infrared lamp. The radioactivity in the samples was counted for sufficient time to insure a probable counting error of less than 3.5%. The actual counts per minute were always at least three times greater than the background. The Nuclear Chicago gas-flow detector, Model D-47, with a micromil window was used for radiometric measurements. Corrections for self-absorption were made, whenever necessary.

RESULTS

In an attempt to evaluate the vitamin B_{12} nutriture of our animals, the growth response and the microbial activity of vitamin B_{12} in their sera were measured. The average vitamin B_{12} content of the liver and serum of the deficient and treated groups are compared in table 2. The deficient animals of either sex had a markedly lower vitamin B_{12} level in both serum and liver. The results shown in table 2 are typical of the animals used for the studies reported in this paper. The cholesterol levels of the different dietary groups are shown in table 3. Because of the difference in weight response by male and female rats to vitamin B_{12} therapy, the results obtained from both sexes are presented separately. The total cholesterol concentration in the serum of vitamin B_{12}-deficient male rats was significantly less than that of their vitamin B_{12}-treated littermates ($P < 0.001$). The cholesterol in the liver was similarly diminished in deficiency, but the difference did not reach a 5% level of significance. The mean cholesterol levels in both serum and liver of both dietary groups were essentially identical in the female rats.

The incorporation of acetate 1-C^{14} into cholesterol in the liver of both deficient and treated male and female rats is presented in table 4. The deficient animals differed from the controls in having a higher specific activity of cholesterol in the liver. The percentage of incorporation

TABLE 4

Incorporation of acetate 1-C^{14} into cholesterol in the liver of male and female rats fed vitamin B_{12}-deficient and vitamin B_{12}-supplemented diets[1]

Sex	Treatment	Final body wt	Liver[2]	Liver cholesterol	Specific activity[3]	% Conversion[4]	Serum cholesterol
		gm	*count/min/gm*	*mg/gm*	*count/min/mg*		*mg/100 ml*
M (8)[5]	Vitamin B_{12}-deficient	273 ± 17	620 ± 118	1.86 ± 0.09	350 ± 82	0.297 ± 0.04	51 ± 4.5
M (8)	Vitamin B_{12}-treated	397 ± 9	392 ± 40	2.42 ± 0.11	164 ± 23	0.124 ± 0.03	73 ± 5.4
	t value		1.82	4.12	2.16	3.46	3.06
F (10)	Vitamin B_{12}-deficient	210 ± 7.1	1218 ± 226	2.52 ± 0.63	481 ± 76	0.39 ± 0.06	84.6 ± 4.6
F (10)	Vitamin B_{12}-treated	219 ± 7.2	522 ± 51	2.53 ± 0.06	205 ± 16	0.16 ± 0.01	71.0 ± 4.1
	t value		3.00	0.19	3.54	2.25	2.20

[1] All animals received acetate by injection and were killed 30 minutes later.
[2] Expressed as counts/minute/gm of weight wet tissue.
[3] Specific activity expressed as counts/minute/mg cholesterol.
[4] Expressed as total counts retained in liver × 100 divided by total counts injected.
[5] Numbers in parentheses indicate number of animals used.

of acetate 1-C^{14} into cholesterol in the liver was likewise higher in both male and female deficient animals. The activity per gram of liver (column 3, table 4) of the deficient animals was greater than that of the control animals. The serum cholesterol was higher in the treated males, but lower in the treated females than the corresponding controls. For this we have no explanation at this time.

Carbon dioxide was collected from another set of vitamin B_{12}-deficient and treated rats; the deficient and control animals did not differ with respect to (1) percentage of acetate converted to CO_2; (2) total CO_2 output; or (3) in the specific activity of CO_2 (table 5).

In table 6 are presented the data on the repletion experiments. The apparent differences between groups A, B and C were evaluated statistically using analysis of variance. Differences were considered significant only when the P value was < 0.05. Here, the deficiency state resulted again in an increased incorporation of acetate into liver cholesterol and higher specific activity of cholesterol in the liver. Such an increase can be reduced markedly although not completely upon repletion with vitamin B_{12} (cf. group B vs. group C). The serum cholesterol levels of the repleted group are not different from those of the controls. The effect of repletion was more marked in the females in comparison with the males. And the level of serum cholesterol of the male deficient rats was lower than that of the controls. This is in accordance with the observations in table 3. Although the mean value for cholesterol in the female deficient rat was higher than the mean values of either the repleted or control group, differences were not statistically significant.

The specific activities of cholesterol in the repleted animals and control are different, i.e., ($P < 0.05$).

DISCUSSION

In the past two decades there has been an increasing attempt to correlate cholesterol levels with diseased states. As a consequence, this parameter has been used by many investigators as an index of cholesterol metabolism. The level of serum cholesterol in experimental animals is maintained relatively constant by homeostatic mechanisms. The total cholesterol in the body at any given time is the resultant of two processes, that tending to increase it by biosynthesis and intestinal absorption and that to decrease it by degradation and excretion.

Our data on the mean serum cholesterol level are in harmony with the results obtained by Muller ('30) in cases of human pernicious anemia but are not in harmony with the observations of Hsu ('57) who assayed cholesterol content in adrenals and sera of vitamin B_{12}-deficient and treated female rats. The elevation or lowering of cholesterol levels in serum is a composite influence of relatively long duration of many factors. These, besides nutritional ones, may include endocrine and psychogenic factors. For this reason, the observation of abnormal serum cholesterol levels in man or in laboratory animals deficient in vitamin B_{12}, need not reflect the vitamin B_{12} nutriture alone. We, therefore, deemed it worthwhile to compare the synthesis of cholesterol by vitamin B_{12}-deficient and treated rats following the oral

TABLE 5

Conversion of acetate 1-C^{14} into CO_2 by rats fed vitamin B_{12}-deficient and vitamin B_{12}-supplemented diets

Group[1]	Body weight	Total CO_2[2] output	Specific[3] activity	Incorporation[4]
	gm			
Vitamin B_{12}-treated	314 ± 31	3.0 ± 0.07	8.32 ± 1.18	23.5 ± 7.4
Vitamin B_{12}-deficient	249 ± 15	4.2 ± 0.95	5.44 ± 0.77	23.8 ± 6.9
t value		1.25	2.04	0.03

[1] Ten rats were used in each group.
[2] Expressed as total output in moles/100 gm rat/30 minutes.
[3] Specific activity expressed as counts/mmole × 10^{-5}.
[4] Total counts × 100/counts injected.

TABLE 6

Effect of vitamin B_{12} repletion on the incorporation of C^{14} acetate into cholesterol in the liver of male and female rats fed vitamin B_{12}-deficient and vitamin B_{12}-supplemented diets[1]

Sex	Treatment	Liver[2]	Liver cholesterol	Cholesterol[3]	% Conversion[4]	Serum cholesterol	Final body wt
		count/min/gm	*mg/gm*	*count/min/mg*		*mg/100 ml*	*gm*
M	Vitamin B_{12}-deficient + saline injection	480 ± 97	1.79 ± 0.13	286 ± 74	0.24 ± 0.03	54 ± 4.9	279 ± 5.2
M	Vitamin B_{12}-deficient + vitamin B_{12} injection	302 ± 72	2.28 ± 0.48	136 ± 31	0.12 ± 0.03	79 ± 7.9	327 ± 10.9
M	Vitamin B_{12}-treated + saline injection	160 ± 32	2.37 ± 0.14	71 ± 15	0.10 ± 0.01	73 ± 4.2	407 ± 6.9
F	Vitamin B_{12} deficient + saline injection	1601 ± 361	2.52 ± 0.48	634 ± 117	0.44 ± 0.08	92 ± 6.8	209 ± 5
F	Vitamin B_{12} deficient + vitamin B_{12} injection	592 ± 271	2.51 ± 0.08	233 ± 93	0.19 ± 0.06	71 ± 10.4	218 ± 8
F	Vitamin B_{12}-treated + saline injection	608 ± 84	2.63 ± 0.16	230 ± 0.16	230 ± 23	68 ± 3.4	218 ± 11

[1] Five rats were used in each group.
[2] Expressed as counts/minute/gm wet tissue.
[3] Expressed as counts/minute/mg cholesterol.
[4] Expressed as total counts retained in liver × 100 divided by total counts injected.

administration of C^{14}-labeled acetate. Vitamin B_{12}-deficient male and female rats showed an increased incorporation of acetate into liver cholesterol as compared with their treated controls. The greater specific activity of cholesterol in the liver of deficient females when compared with the males is in agreement with the observation of Fillios et al. ('58) who found a relatively higher rate of cholesterol biosynthesis in female rats. Although the specific activities of cholesterol in the liver of the vitamin B_{12}-deficient group was definitely increased, such a result may be brought about by a differential dilution due to either a smaller-size acetate compartment or a smaller-size cholesterol pool. As the counts per minute per gram of liver (table 4, column 3) are markedly greater in the deficient group as compared with its vitamin B_{12}-treated littermates, the possibility that the result obtained was due only to a smaller size cholesterol pool appears unlikely. It should be emphasized that in this study that we explored the incorporation of acetate 30 minutes after injection of the radioactive material. No time curve was included. Such data, therefore, need not bear a direct relationship on the net cholesterol synthesis.

The data on the specific activity of CO_2 failed to reveal any difference between the deficient and control groups. Although the specific activity of CO_2 in the control group appears to be higher than in the vitamin B_{12}-deficient group, differences were not statistically significant. It thus appears that a differential dilution of tracer acetate by a smaller-size acetate compartment is unlikely. In the absence of apparent differences in the size of the acetate pool in both groups of animals the high specific activity of liver cholesterol in the vitamin B_{12}- deficient group can be interpreted as increased cholesterolgenesis. Boxer et al. ('55) found an increase in CoA levels in vitamin B_{12}-deficient animals. It seems reasonable to surmise that the observed increase in cholesterolgenesis is related to this finding.

The data on the CO_2 production further suggests that the oxidation of tracer acetate is not impaired in vitamin B_{12} deficiency.

The repletion experiments (table 6) demonstrate that the increased incorporation

of acetate into cholesterol can be reversed by vitamin B_{12} injection. These results are completely consistent with the concept that vitamin B_{12} plays a role in cholesterol metabolism.

The observations of increased cholesterolgenesis in both male and female rats and low serum cholesterol levels only in male rats, suggests a probable sex difference in catabolic rate between the two groups. This aspect will be considered in our next paper.

SUMMARY

The role of vitamin B_{12} in cholesterol metabolism was investigated. It was demonstrated that vitamin B_{12} deficiency results in (a) low levels of total cholesterol in the serum and the liver of male rats, but not in female rats; (b) an increase in the percentage of incorporation of C^{14} acetate into cholesterol in liver which was reversed by vitamin B_{12} administration; and (c) a higher specific activity of cholesterol in the liver after administration of tracer acetate. It was demonstrated further that the oxidation of acetate is not impaired.

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Effect of Nonessential Nitrogen Supplements on Growth and on the Amino Acid Content in Plasma and Muscle of Weanling Rats Fed a Low-Protein Diet[1]

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There is some evidence that in human subjects the amount of essential amino acids required for nitrogen balance is increased when nonessential nitrogen is added to diets containing minimal quantities of essential amino acids (Tuttle et al., '59). It appears also that different sources of nonessential nitrogen may vary in their effect on nitrogen retention under these dietary conditions (Swendseid et al., '60).

A number of investigators (Sauberlich and Salmon, '55; Harper, '58; Kumta and Harper, '61) have reported that weanling rats ingesting diets low in protein show retarded growth rates when proteins of poor nutritive quality or selected essential amino acids are fed as supplements. This growth retardation is prevented by dietary additions of the limiting essential amino acids. However, the effects of nonessential nitrogen supplements in low-protein diets have not been studied extensively. In a recent report, Sauberlich ('61) investigated the effect of adding 5% levels of nonessential as well as essential amino acids to low-protein diets and found varying degrees of growth depression in weanling rats.

The present report is a study of the growth effects in young rats fed low-protein diets supplemented with various kinds and amounts of nonessential nitrogen. Also, the effect of additions of the limiting essential amino acids to these supplemented diets has been investigated. In some experiments, the free amino acids in plasma and muscle tissue have been determined.

EXPERIMENTAL PROCEDURE

Male weanling rats of the Sprague-Dawley strain weighing between 50 and 60 gm were placed singly in wire-mesh cages and fed the experimental diets ad libitum for periods of two or in some instances, four weeks. Each diet was fed to a group of 6 rats in at least two separate experiments. The animals were weighed at regular intervals and their food consumption was measured.

All animals were fed a basal diet which contained 8% of vitamin-free casein plus 0.3% of DL-methionine as the source of nitrogen. Additional components of the basal diet were as follows (in per cent): cottonseed oil, 4.5; salt mixture (Hegsted, '41), 4.0; choline chloride, 0.15; vitamin mixture, 0.02; dextrose to total 100. The vitamin mixture contained in mg/100 gm of diet: thiamine·HCl, 0.5; riboflavin, 0.5; nicotinic acid, 2.5; Ca pantothenate, 2.0; pyridoxine·HCl, 0.25; menadione, 0.05; biotin, 0.01; folic acid, 0.02; inositol, 10.0; vitamin A and D mixture (units/gm vitamin A = 500,000 USP, vitamin D=50,000 USP),[3] 0.8; α-tocopherol, 0.01; and vitamin B_{12}, 0.002.

For certain groups of rats, a nonessential nitrogen supplement, which varied as

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[2] Part of the data are taken from a thesis submitted by Janice B. Hickson to the Graduate School, U.C.L.A. in partial fulfillment of the requirements for the degree of Master of Science.

[3] Pfizer Crystalites, Chas. Pfizer and Company, Inc., New York.

to source and amount, was added to the basal diet. The supplements fed were glycine, glutamic acid, a combination of glutamic acid and diammonium citrate, or a mixture of the nonessential amino acids present in protein. The amounts used are shown in table 1. In some studies, 0.3% of threonine and 0.2% of tryptophan, the essential amino acids shown to become limiting for growth in low-casein methionine-supplemented diets (Griffith and Naurocki, '48;[4] Harper, '59) were added to the nonessential nitrogen supplement. All amino acids used in this study were C.P. grade and they were included in the diet at the expense of dextrose.

In selected experiments, free amino acids were determined in picric acid extracts of blood plasma and gastrocnemius muscle tissue prepared according to procedures of Tallan et al. ('54). The analyses were made by ion-exchange chromatography using an automatic instrument (Spackman et al., '58).

RESULTS

In table 1, the weight gains of weanling rats can be compared when their basal diets containing 8% of casein and 0.3% of methionine were supplemented with the various nonessential nitrogen sources. When either L-glutamic acid or glycine was added in an amount such that the diet contained 1.75% of nitrogen, growth retardation occurred (groups 2 and 4 vs. 1). This effect on growth was more pronounced when the quantities of the nonessential amino acids were increased so that the diet contained 2.5% of nitrogen (groups 3 and 5 vs. 1). Likewise, a mixture of glutamic acid and diammonium citrate or a mixture of the nonessential amino acids proportioned to the amino acids in casein (with the exception that proline was omitted) caused growth retardation when fed in a 2.5% nitrogen

[4] Griffith, W. H., and M. F. Naurocki 1948 The effect of threonine in choline deficiency. Federation Proc., 7: 288.

TABLE 1

Weight gain of rats fed a low-casein basal diet supplemented with various nonessential nitrogen sources: effect of threonine and tryptophan additions

Group no.	Nonessential nitrogen supplement	0.3% DL-threonine and 0.2% L-tryptophan added	Avg gain in 2 weeks[1]	FER[2]
			gm	
1	None	–	45 ± 2.3	26.9
2	6.9% L-Glutamic acid[3]	–	39 ± 1.7	23.6
3	15% L-Glutamic acid[4]	–	29 ± 1.8	17.7
4	3.5% Glycine[3]	–	31 ± 1.7	18.1
5	7.5% Glycine[4]	–	24 ± 1.1	18.6
6	None	–	52 ± 2.5	25.7
7	None	+	58 ± 3.1	30.2
8	7.5% Glycine[4]	–	24 ± 1.4	17.3
9	7.5% Glycine[4]	+	36 ± 1.7	16.5
10	12% Nonessential amino acid mixture[4,5]	–	33 ± 2.7	14.5
11	12% Nonessential amino acid mixture[4,5]	+	56 ± 1.6	23.6
12	7.3% L-Glutamic acid + 5.6% diammonium citrate[4]	–	36 ± 1.8	20.3
13	7.3% L-Glutamic acid + 5.6% diammonium citrate[4]	+	53 ± 2.0	30.4
14	7.5% Glycine + 0.001% folic acid	+	28 ± 1.6	17.1
15	None	–	51 ± 0.9	29.0
16	4.9% Glycine[6]	–	35 ± 2.3	23.2
17	4.9% Glycine[6]	+	49 ± 2.3	26.6

[1] Mean of 6 rats ± standard error.
[2] Food efficiency ratio (gm gained/100 gm food consumed).
[3] Diet contains 1.75% N with 0.65% N as supplement.
[4] Diet contains 2.5% N with 1.4% N as supplement.
[5] Percentage composition: DL-alanine, 8.0; DL-aspartic acid, 17.6; L-glutamic acid, 54.9; glycine, 4.7; and DL-serine, 15.8.
[6] Diet contains 2.0% N with 0.9% N as supplement.

diet (groups 10 and 12 vs. 6). The growth-retarding effects of glycine were more severe than those of the other nonessential nitrogen sources when comparisons are made on an isonitrogenous basis. Food consumption was not reduced appreciably with any of the nonessential nitrogen supplements and hence lower food efficiency ratios were obtained in all instances.

Also when threonine and tryptophan were added to diets containing 2.5% of nitrogen there was a slight stimulation of growth for animals fed the basal diet (groups 6 vs. 7) (table 1). When nonessential nitrogen supplements were used, the addition of these amino acids prevented growth retardation except when glycine was the source of nonessential nitrogen (groups 9, 11 and 13 vs. 6 or 7). However, with lesser amounts of glycine in the diet, threonine and tryptophan appeared to prevent the depression in the rate of growth (groups 15 vs. 17), although in this experiment threonine and tryptophan were not added to the basal diet. Other investigators (Dinning et al., '49) have shown that when glycine is added to diets containing adequate amounts of protein, the toxic effects can be prevented by supplementing with folic acid. In the present experiments, where protein is limiting for growth folic acid when added either alone (not shown in table) or in combination with threonine and tryptophan had no effect on the growth retardation produced by glycine (groups 14 vs. 8).

The free amino acids in plasma and muscle tissue of nonfasting rats receiving the basal and the nitrogen-supplemented diets were also determined in a limited number of experiments. The amino acids were assayed in pooled samples of plasma

TABLE 2

Free amino acids in plasma and muscle of young rats fed diets containing various nonessential nitrogen supplements

Amino acid	Plasma amino acid content				Muscle amino acid content		
	Group fed no supplement	Group fed 7.5% glycine	Group fed 12% NEAA[1] mixture	Group fed 15% glutamic acid	Group fed no supplement	Group fed 7.5% glycine	Group fed 12% NEAA mixture
	µmoles/100 ml				*µmoles/100 gm wet weight*		
Essential[2]							
Threonine	6.6	5.3	5.1	9.3	23.1	10.7	20.2
Valine	10.4	7.4	9.3	25.0	16.4	8.0	10.4
Cystine	tr	tr	tr	1.7	—	—	—
Methionine	4.3	3.8	3.0	11.8	5.0	5.3	7.0
Isoleucine	5.5	3.9	4.5	12.1	5.9	4.9	4.6
Leucine	8.5	5.9	7.3	19.5	9.6	5.8	7.1
Tyrosine	6.4	2.9	4.1	7.6	11.5	2.2	4.6
Phenylalanine	3.5	2.6	3.0	4.2	2.8	2.2	1.4
Lysine	47.5	28.6	34.6	50.6			
Histidine	3.9	2.6	2.9	6.9			
Arginine	9.8	tr	5.5	6.7			
Total	106.4	63.0	79.3	155.4			
Nonessential							
Aspartic acid	1.5	—	5.8	8.7	22	22	44
Serine	27.6	57.2	56.2	40.3	241	330	461
Glutamine and asparagine	62.4	69.7	70.5	92.4	664	382	602
Proline	16.8	17.5	12.6	29.6	66	14	21
Glutamic acid and citrulline	19.3	16.3	17.4	69.4	165	101	160
Glycine	14.6	237.0	24.1	11.2	233	2450	401
Alanine	49.2	40.7	43.4	110.6	329	157	273
Total	191.4	438.4	230.0	352.2			
E/N ratio[3]	0.56	0.14	0.34	0.44			

[1] Indicates nonessential amino acid mixture.
[2] Includes cystine and tyrosine.
[3] The ratio of essential to nonessential amino acids.

or of gastrocnemius muscle tissue obtained from the group of 5 or 6 rats which had been fed the experimental diets for a 4-week period. The results are shown in table 2. It was found that there was always an increase in the total amount of nonessential amino acids in the plasma of rats fed the nonessential nitrogen supplements (groups 2, 3 and 4 vs. 1). In most instances this was due largely to an increase in the concentration of the supplemented amino acid. There was a definite elevation of glycine in the plasma from animals receiving this supplement and serine was also increased (group 2). The amount of plasma glutamic acid was likewise increased with the glutamic acid supplement and in addition aspartic acid, proline and alanine were elevated (group 4). The accumulation of nonessential amino acids with the feeding of the nonessential amino acid mixture was not as marked although serine and glycine were increased (group 3). The value for proline, which was not included in the supplement fed, appeared to be lowered.

Concomitant with the increase in nonessential amino acids in plasma, there appeared to be a decrease in the total amount of essential amino acids in the groups where the diet was supplemented with either glycine or the nonessential amino acid mixture. The decrease resulted from reduced amounts of most of the individual essential amino acids. With the glutamic acid supplement, there appeared to be an actual increase in the essential amino acids. However, since there was a consistent increase in the nonessential amino acids, all of the supplements fed caused a reduction in the ratio of essential to nonessential amino acids in plasma (E/N ratio, table 2). The greatest reduction in this ratio occurred with the glycine supplement.

The changes in plasma amino acid concentrations are reflected to a degree at least in the free amino acids of muscle tissue. There was an accumulation of glycine and serine in muscle with the group fed the glycine supplement and also with the group fed the nonessential amino acid mixture. For those essential amino acids that were measured, the supplements appeared to cause decreases and these decreases were more pronounced with the glycine supplement.

DISCUSSION

This study showed that nonessential nitrogen supplements retard the growth of young rats fed low-casein methionine-supplemented diets and that this effect can be prevented in most instances by the addition of threonine and tryptophan. Since threonine and tryptophan are the amino acids that become limiting for growth in a casein diet to which a sulfur-containing amino acid has been added, it appears that the nonessential nitrogen supplements act metabolically to increase the requirement for these essential amino acids, when requirements are expressed as percentage of the diet. With high levels of glycine, when threonine and tryptophan do not completely prevent growth depression, it is possible that a deficiency of other essential amino acids may develop. The toxicity of glycine in these experiments with low-protein diets is not mitigated by folic acid.

Reports of several investigators (Grau, '50; Allison, '55; Harper, '58) have shown that requirements for individual amino acids increase with increasing protein intake. The present study provides additional evidence that the amino acid requirements for the growing rat are not constant factors and shows that these amino acid requirements can be influenced by the total nitrogen intake in a dietary situation where the proportionality pattern of the essential amino acids remains unchanged. It appears, therefore, that when the nutritive value of a protein is assessed, the ratio of essential to nonessential nitrogen should receive consideration.

Evidence has been presented indicating that the decrease in the ratio of essential to nonessential nitrogen, which characterizes the supplemented diets, results in a decrease in the ratio of essential to nonessential amino acids in plasma. This is at least partly the result of an increase in the amino acids fed as supplements and their metabolic derivatives. Sauberlich ('61) has also shown that amino acids fed in excess in the diet accumulate in the plasma. It is possible, however, that changes in the concentration of the essen-

tial amino acids might be more directly related to the growth-retardation effect. In the present experiments, there were some instances where the essential amino acids appeared to be lowered. However, it has been found[5] that the plasma level of essential amino acids in animals receiving the basal diet is only 50% of the amount present in animals fed an 18% casein diet and that threonine is reduced by approximately 85%. Hence, under the conditions that were studied here, the effects of nonessential nitrogen supplements may be difficult to demonstrate.

SUMMARY

Various nonessential nitrogen supplements including glycine, glutamic acid, a mixture of glutamic acid and diammonium citrate and a mixture of the nonessential amino acids proportioned as in casein were found to retard the growth of young rats when they were added to diets containing 8.0% of casein and 0.3% of methionine. The effect can be prevented by additions of threonine and tryptophan except in the case of the 7.5% glycine supplement. Increases in the nonessential amino acids fed as supplements and their metabolic derivatives were found in the plasma and muscle tissue. In some instances, the essential amino acids appeared to be decreased. On an isonitrogenous basis, glycine supplementation caused the greatest reduction in growth rate and produced the greatest changes in plasma amino acid concentrations.

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[5] Unpublished results of the authors.

Proceedings of the Twenty-Sixth Annual Meeting of the American Institute of Nutrition

SHELBURNE HOTEL, ATLANTIC CITY, NEW JERSEY
APRIL 14–18, 1962

COUNCIL MEETINGS

The American Institute of Nutrition Council met on Thursday evening, April 12, Friday morning and evening, April 13, and Monday afternoon, April 16. Formal actions of the Council were reported at the Institute business meetings and are included in the following minutes.

SCIENTIFIC SESSIONS

A full 5 days of scientific sessions on nutrition and related sciences were held as part of the annual meetings of the Federation of American Societies for Experimental Biology. A total of 219 abstracts was submitted to the Institute for inclusion on the scientific program. Of these, 27 were transferred to programs of other societies and intersociety sessions. Seventy-two abstracts were received by transfer from other societies. A nutrition program of 21 half-day sessions was arranged and published in the March issue of *Federation Proceedings*, part 2. In addition, 5 half-day intersociety sessions on atherosclerosis were held. Of special interest were two half-day symposia on "Contributions of Intestinal Microflora to the Nutrition of the Host Animal" and "Agriculture's Contribution to the Solution of World Food Supplies," the latter in honor of the celebration of the Centennial of the establishment of the Agricultural Experiment Stations in the United States. The symposia will be published in future issues of *Federation Proceedings*.

BUSINESS MEETINGS

Business meetings were held on Saturday, April 14, and Tuesday, April 17, attended by approximately 280 and 250 members, respectively. Dr. Paul György, president, presided at both meetings.

I. Minutes of 1961

The minutes of the 1961 meeting, as published in *The Journal of Nutrition*, 75: 132, 1961, were approved.

II. Election

The Secretary transmitted the sealed ballots to the Tellers' Committee, Dr. P. Harris, chairman, and Dr. H. Sarrett. At the second business meeting the committee reported the election results from 539 ballots received, as follows:

Effective July 1, 1962:
President: Dr. L. C. Norris
President-Elect: Dr. Grace A. Goldsmith
Councilor (three-year term): Dr. Harold H. Williams
Treasurer (three-year term): Dr. Douglas V. Frost

Effective July 1, 1963:
Secretary (three-year term): Dr. Olaf Mickelsen

Effective May 1, 1962:
Editorial Board, JOURNAL OF NUTRITION
Four-year term: Dr. John G. Bieri
Dr. Harry Broquist
Dr. R. G. Hansen
Dr. R. T. Holman
Three-year term: Dr. E. L. R. Stokstad
One-year term: Dr. George M. Briggs

The names of individuals with the 10 highest number of votes for suggested members of the Nominating Committee were submitted to the President. (See complete list of officers and committees at the end of these proceedings.)

III. Constitutional Amendments

By over two-thirds of all votes cast, the following amendments to the constitution and by-laws were adopted by mail vote:

A. By a vote of 493 for to 17 against, Article VII, Section 3, has been changed to read as follows:

Section 3. The Official Journal(s) of the Institute. A. The editorial responsibility for the

official journal(s) of the Institute shall be vested in an Editorial Board of sixteen members and the Editor-in-Chief. B. The Editor(s) shall be appointed by the Council of the Institute with advice from the Publications Committee and the Editorial Board(s) of the Journal(s), to serve a term of five years beginning July 1 of the year in which he is chosen and shall be eligible for reappointment. The Editor shall have the power to appoint an assistant and such an appointee shall be called Associate Editor. C. Four members of the Institute shall be nominated by the Nominating Committee for membership on the Editorial Board each year to serve a term of four years and to take office May 1 of the year in which they are elected. D. Retiring members of the Editorial Board shall not be eligible for renomination until one year after their retirement.

B. By a vote of 487 for to 19 against, Article IX, Section 3 has been changed to read as follows:

Section 3. Divisions of the Institute may hold separate meetings. They may publish or sponsor publication of official journal(s). The Editor(s) and the Editorial Board(s) of such publication(s) will be selected by each division according to its constitution. Contractual agreements concerning such publication(s) may be entered into by the individual divisions with the approval of the Council of the American Institute of Nutrition.

IV. *Membership Status*

The Secretary reported that as of April 1, 1962, there were 812 members of the Institute — 749 active, 53 retired and 10 honorary members, this being a net increase of 65 members since last year. Four members retired during the year. The Clinical Division reported a total membership of 89.

Members present at the business meeting stood for a moment of silence in memory and in recognition of the following members of the Institute who had passed away since the last meeting:

*Richard J. Block, February 4, 1962
Norman Jolliffe, August 1. 1961
*Elliott P. Joslin, January 29, 1962
E. W. McHenry, December 20, 1961
Milton Silverman, February 3, 1962
Jennie Tilt, January 23, 1960
Jerome A. Uram, February 4, 1962

* Charter members.

Appropriate resolutions which had been received for deceased members were read and approved. Resolutions were read in honor of charter members, Dr. Block and Dr. Joslin and also for Dr. Jerome A. Uram. Resolutions for charter members are included in the minutes as per custom.

RESOLVED. That the American Institute of Nutrition, assembled at Atlantic City, New Jersey, in its Annual Meeting, April 14, 1962, place in its minutes for permanent record this statement of deep regret and sorrow at the passing of its distinguished member, Richard J. Block.

Richard Joseph Block, a charter member of the American Institute of Nutrition and an international authority on nutritional biochemistry, and his wife, Margaret, a pioneer in establishing psychological programs in remedial reading in schools, were among 18 persons killed on February 4, 1962 when a commercial airliner crashed into a mountain near Tingo Maria, Peru.

The accident occurred in the course of a scientific mission in Latin America sponsored by the Department of Health, Education, and Welfare and the National Institutes of Health in connection with the International Program on Nutritional Studies. Dr. Block's primary purpose was to study and advise on the improvement of amino acid content in basic foods. A further objective of the trip was to obtain drug plants from remote areas which had been reported to have shown promise in the alleviation of cancer. These collections were to have been based on observations that had been made by Dr. Theodor Binder of the Hospital Amazonico Albert Schweitzer, Pucallpa, Peru. In this survey Dr. Block was accompanied by another member of AIN, Dr. Jerome A. Uram of the Massachusetts Institute of Technology. Both Dr. and Mrs. Uram perished in the plane crash with Dr. and Mrs. Block.

Dr. Block was born in Macon, Georgia on May 4, 1906. He attended Yale University, completing his undergraduate work in 1928 and receiving the Ph.D. in physiological chemistry in 1931.

From a postdoctoral fellowship at Yale, Block proceeded to Munich, Germany, where he was a consulting chemist in the laboratory of Prof. F. von Muller. He then became a chemist at the New York State Psychiatric Institute and Hospital. In 1943 he was appointed a Professorial Lecturer at New York Medical College and in 1961 he was designated Visiting Professor of Experimental Pathology.

He was associated with the Boyce Thompson Institute for Plant Research as Nutritional Biochemist from 1951 until his death. At the time of his death he was directing four programs of research in nutritional biochemistry and protein chemistry supported by the National Institutes of Health.

He had also served as consultant to many segments of industry, government, and UNICEF. The longest and most continuous of these relations was with The Borden Company, extending over a period of 24 years. He was also Visiting Professor to the Department of Physiology and Biochemistry, and Associated Member of the

Bureau of Biological Research of Rutgers, the State University of New Jersey, as well as chairman of the Sub-committee on Amino Acids of the Committee on Biological Chemistry of the National Academy of Sciences-National Research Council.

Dr. Block's major fields of scientific interest were basic amino acids in proteins; proteins of the brain; serum proteins; intermediary metabolism of cysteine and methionine; cystinuria, vitamin B; hormones and mental disease; amino acids in plant and animal tissue proteins; chromatographic separation of cell constituents; human and animal nutrition; separation and isolation of tumor proteins; sulfur metabolism in plants and insects; and thyroid diseases.

He was the author, or co-author of 142 original articles and had a score of patents in the above fields. Also Dr. Block was the author of four reference books and manuals, and was engaged in the preparation of two more at the time of his death. The best known of his books are: "The Amino Acid Composition of Proteins and Foods" (2nd edition, 1951), written with Diana Bolling, and "A Manual of Paper Chromatography and Paper Electrophoresis" (2nd edition, 1958), with Emmett L. Durrum and Gunther Zweig as junior authors.

Dr. Block was a Fellow of the American Association for the Advancement of Science and of the New York Academy of Sciences. His professional memberships, in addition to the AIN, included the American Society of Biological Chemists, the American Chemical Society, the Institute of Chemists, the Biochemical Society (Great Britain), the Society for Experimental Biology and Medicine, the Harvey Society and Sigma Xi.

RESOLVED. That the American Institute of Nutrition, assembled at Atlantic City, New Jersey, in its Annual Meeting, April 14, 1962, place in its minutes for permanent record this statement of deep regret and sorrow at the passing of its distinguished member, Elliott P. Joslin.

Dr. Elliott P. Joslin, a charter member of the American Institute of Nutrition, dedicated his life unswervingly throughout every year of this Century until his recent death, to all aspects of diabetes. The various eras in the treatment of diabetes came and went during his time, but by keeping the goal of a better life for the diabetic ever before his colleagues, he led the way to progressive but safe applications of advances from the basic sciences to the bedside of the diabetic. The field has now lost its cornerstone and anchor with Dr. Joslin's departure.

V. *New Members*

The Council received 89 nominations for active membership, of which 82 were unanimously approved by members at the business meetings. All of these have accepted membership in the Institute. They are as follows:

NEW MEMBERS — 1962*

†Lynn DeForrest Abbott
†Margaret J. Albrink
J. Craig Alexander
Leonard W. Aurand
Eugene M. Baker
Horace A. Barker
†Adelia M. Beeuwkes
James Karl Bletner
†William P. Boger
Georg Borgstrom
S. A. Broitman
Miriam Kelly Brush
L. B. Carew, Jr.
Bernard Century
Charles Church
Irwin Clark
†William E. Connor
H. R. Conrad
Laurence M. Corwin
James W. Craig
Joaquin Cravioto
†Lewis K. Dahl
R. E. Davies
Carl Lee Davis
H. J. H. de Muelenaere
William E. Drucker
John Fabianek
†Elaine B. Feldman
Joseph P. Fonetnot
Hazel Fox
†Arthur B. French
†Joseph H. Fries
Robert Ball Grainger
†Helene A. (Nathan) Guttman
†George Hamwi
Phyllis Hartroft
†Sami Hashim
Virgil W. Hays
Franklin W. Heggeness
D. E. Hogue
Farris E. Hubbert, Jr.
†Frank Iber
†Doris Johnson
†Martin S. Kleckner, Jr.
Frank Konishi
Roland M. Leach
†Carroll M. Leevy
Kam C. Leong
W. T. Wu Leung
Gilbert A. Leveille
†Charles S. Lieber
Joseph A. Liuzzo
John B. Longenecker
Colin C. Lucas
Gerald Wayne McWard
†Sherman Mellinkoff
Edward C. Miller
W. J. Miller
Mary Alice Morrison
Lura M. Morse
†Robert C. Muehrcke
Bruce R. Poulton
Harold Arch Ramsey
Lillian Recant
Juan Claudio Sanahuja
†Fred Sargent, II
Jacques Sherman
Helen Ryan Skeggs
Melvin D. Small
Frank Houston Smith
H. C. Spruth
†Parker Vanamee
†Edward J. Van Loon
Oliver W. Vaughan
Robert W. Wannemacher
†Louis R. Wasserman
William W. Wells
Ethelwyn B. Wilcox
†John J. Will
Robert G. Yaeger
Shiang-Ping Yang
Z. Z.Ziporin

* For institutional affiliations and addresses of new members see the September issue of *Federation Proceedings*.

† In addition, elected to membership in the Clinical Division.

VI. *Treasurer's Report*

A report by the Treasurer, Dr. James B. Allison, from April 1, 1961, to April 9, 1962, was read and approved. The Auditing Committee, Dr. Harry W. Titus and Dr. Robert L. Squibb, submitted a report that the Treasurer's accounts were correct and complete. The report was approved.

Dues. The President, Dr. György, reported on the recommendation of the Council that the dues would remain the same as for last year, namely, for AIN members, *The Journal of Nutrition* — $10.00, Federation dues — $6.00, AIN dues — $2.00.

TREASURER'S REPORT

April 1, 1961 — April 9, 1962

Balance brought forward		
Cash	$4,761.47	
U. S. Series K Bond	500.00	$ 5,261.47
Receipts		
Interest on U. S. Series K Bond	13.80	
Institute dues from 738 members	1,476.00	
Federation dues from 737 members	1,422.00	
Subscriptions to *Journal of Nutrition* from 691 members	6,910.00	
Institute 1960–61 payments made in 1962	99.00	
Wistar Institute for Editorial Office	9,000.00	
Wistar Institute for Editorial Office, 1960–61 final payment included in '62 statement	2,250.00	
Federation reimbursement of 1961 annual meeting	615.53	
Mailing receipts	1.75	
Bank premiums	.25	
Overpayment from members	38.00	
Deposits of 1960–61 members included in '62 statement	91.85	
American Society for Clinical Nutrition	691.00	25,609.18
Total receipts and balance brought forward		$30,870.65
Expenditures		
Federation Office:		
Dues	3,060.00	
Addressing of dues notices	6.24	
Addressing of dues envelopes	5.23	
Addressing of envelopes with plates of entire membership of Society 5	5.66	
Mail meter charges for fall meeting	97.12	
Wistar Institute, subscriptions to *Journal of Nutrition*	6,910.00	
Wistar Institute, subscriptions to *Journal* by 1960–61 members	36.00	
Wistar addressing of dues cards and window envelopes	67.79	
American Society for Clinical Nutrition	690.50	
Secretary's Office expenses	250.00	
Treasurer's Office expenses:		
Petty cash	5.00	
Directory	5.00	
Speaker expense	40.00	
Binding of *Journals*	280.00	
Cornell University, for Editorial Office	9,000.00	
Cornell University, for Editorial Office final payment of 1960–61	2,250.00	
Bank charges	13.28	
Refund to overpaid members	38.00	
Total expenditures		$22,759.82
Balance on hand, April 9, 1962		
Cash	7,610.83	
U. S. Series K Bond	500.00	
TOTAL BALANCE		$ 8,110.83

JAMES B. ALLISON, *Treasurer*

Editing and Publication Operations

(January 1, 1961 — December 31, 1961)

	1958	1959	1960	1961
Volumes published	64, 65, 66	67, 68, 69	70, 71, 72	73, 74, 75
Pages published	1862 (old format)	(1 vol. in new format)	1486 (new format)	1399 (new format)
(Scientific papers only)				(1357)
Papers published (Inc. 3 biographies)	150	173	208	203
Papers submitted	201		231	287
Papers rejected	34		29	59
Rejection rate	17%		13%	20%
Supplements	—	—	—	—

Biographies: Elmer Martin Nelson, Russell Wilder, and Eugene F. DuBois

Operating Schedule	1960	1961
Average time lapse from receipt of manuscript to publication date:		
Av. no. days with reviewer	17.24	20.6
Av. no. days out for revision	17.3	18.3
Av. no. days in office, in mail or in unavoidable delay	27.13	21.8
	61.67	60.7
Total av. time in Editorial Office (months)	2.1	2.0
Av. no. months with Wistar	3.0	3.2
	5.1	5.2

VII. *Editor's Report*

The Editor of *The Journal of Nutrition*, Dr. Richard H. Barnes, submitted his report. It was approved and is summarized below.

Summary of Finances in the Operation of the Editor's Office — *Journal of Nutrition*

1961–62	
Balance brought forward, actual (estimated 3/15/61 as $689.88)	$ 671.86
Receipts — Wistar	9,000.00
Total receipts and balance brought forward:	9,671.86
Expenditures, partially estimated, per schedule	11,311.35
Estimated balance June 30, 1962 (loss)	$(1,639.49)

The AIN Council approved payment from the AIN Treasury to cover the deficit of the Editor's office for the current year to June 30, 1962.

During the next two years, the additional cost for publishing beyond 1,500 pages per year is estimated to be approximately $4,500. This sum is to be paid by AIN to Wistar.

VIII. *Reports of Committees and Representatives*

A. *Committee on Publication Management:* Dr. W. J. Darby, chairman.

The contract agreements with Wistar were reviewed by Dr. Darby. The AIN Council approved the contract. A copy of the final contract with Wistar, which is effective July 1, 1962, for a two-year term, is on file in the Secretary's office. Conditions of the contract are as follows:

1. Wistar will underwrite the expenses of the Editorial Office at a level not to exceed $9,700 per year. The AIN shall submit annual statements of the expenditures for editorial purposes, and payment shall be made by Wistar to the treasurer of AIN on a quarterly basis. Any unexpended balance will be returned to Wistar at the end of the year or credited to the first quarterly payment of the subsequent year.
2. The subscription price of *The Journal of Nutrition* to members of the American Institute of Nutrition will continue to be $10.00 per year for the next two years.

3. Wistar will print 1,500 pages a year for the Institute and excess pages beyond this number will be paid for by the American Institute of Nutrition at a rate not to exceed $44.50 per page. At the end of the year, if the cost per page to Wistar for those pages in excess of 1,500 is less than this amount, the American Institute of Nutrition will pay only the actual cost of the publication of the additional pages.
4. The American Institute of Nutrition will set and collect a page charge from authors publishing in *The Journal of Nutrition.* These charges will be determined by the American Institute of Nutrition and the funds accruing to the AIN. This procedure is to assure that funds will be available to AIN for the payment of cost of printing in excess of 1,500 pages per year.
5. Subscription income will be divided equally between the Wistar Institute and the American Institute of Nutrition on all new subscriptions by non-members of the American Institute of Nutrition in excess of the subscription list pertaining on January 1, 1962, and in excess of the number of new subscriptions by members of the American Institute of Nutrition beyond those pertaining on that date.

Page Charges for The Journal of Nutrition

In order to meet the future deficit from the publication of *The Journal of Nutrition,* which is estimated to be approximately $6,000 per year, the following was approved:

1. That a page charge of not more than $20.00 per page is authorized with the provision that if an author does not have funds available to defray this cost, he will be excused from such an assessment. The exact page charge is to be determined by the Committee on Publication Management. Funds so obtained would be used to defray the following:
   a. Wistar's assessment of $44.50 per printed page above the agreed 1,500 pages per year
   b. To reimburse the Editor-in-Chief's office for expenses beyond the $9,700 which is paid by Wistar
   c. Unexpended funds to be held in the treasury of the American Institute of Nutrition.

B. *Public Information Committee:* Dr. P. L. White, chairman.

It is the consensus of the Committee that there is need for a more positive identification of members with the AIN, with its goals, opportunities and functions.

Committee Recommendations:

1. To help meet the need for improved communications within the AIN, in order to promote membership solidification and realization of aims and to aid identification of members with this organization, it is recommended that the official organ of the AIN be made available for internal communications.
2. That the AIN significantly increase its efforts in positive public information and in combatting or exposing misinformation. To this end, the following recommendations are directed:
   a. That the size and scope of the AIN Public Information Committee be increased.
   b. That the *ad hoc* Committee to study the establishment of a permanent secretariat in the Federation Office give consideration to the need for active participation in a public information program, possibly in cooperation with other groups active in this area, while planning for the secretariat. Further, that consideration be given to nutrition careers promotion as a function of the secretariat. Should such activities be included among the responsibilities of a secretariat, it is recommended that the Public Information Committee be considered as a counselling and advisory group to the secretariat.
   c. That study groups be formed to examine current needs and then advise on future public information activities. Such groups could be organized on a regional basis in order to be cognizant of specific needs around the country.
   d. The coordination and co-sponsorship of various professional and public information programs with other national nutrition groups would serve to establish the identity of the AIN as a professionally unbiased, multi-discipline organization.
   e. That ultimately the AIN secretariat establish an information center of national significance with appropriate professional information specialists in its employ.

C. *Joint Committee on Biochemical Nomenclature:* Dr. P. L. White.

Steps are now being taken to encourage coordination of all U. S. agencies involved in biochemical and nutritional nomenclature. There are more than 16 agencies with active interest.

The International Union of Pure and Applied Chemistry (IUPAC) has a Commission of Biochemical Nomenclature, with Professor W. Klyne as chairman. The International Union of Biochemistry (IUB) has no commission on nomenclature. However, a Commission on Enzymes recently completed work on the nomenclature of enzymes. It has been the practice of the IUB to refer nomenclature matters to IUPAC. The IUB has given responsibility to its Commission of Biochemical Editors to set up international committees on nomenclature to report to the editors in each country involved. There is no single counterpart in the United States with which Dr. Edsall could work as editor of the Journal of Biological Chemistry.

An *ad hoc* Committee on Problems Connected with Nomenclature and Biochemistry was called together by the NAS-NRC. Drs. Luck, Harte, and White represented the Joint Federation Committee on Biochemical Nomenclature. The proceedings of this meeting were mailed to all the members. The Secretary has on file the report by Dr. J. Murray Luck, Chairman of the Joint Committee on Biochemical Nomenclature.

The *ad hoc* Committee protested the recommended nomenclature on folic acid and derivatives being considered by IUPAC. Recommendations were as follows:

1. That V-13.2 be changed to read: The pteroylglutamic acids may be designated generically as "folic acids (G)," the "G" suffix indicating the number of glutamic acid residues.
2. That V-13.3 be referred to the USP and NAS-NRC for advice.
3. That V-13.4 be revised to correspond with V-13.2, thus pteroyltriglutamic acid would be named "folic acid (III G)."

Chairman Luck recommended that the Societies explore the advisability of disbanding the Joint Committee as soon as action is taken by the NAS-NRC on the formation of the Information Center on Biochemical Nomenclature. Dr. White endorsed the recommendation by Dr. Luck.

Council and membership approved the above report.

D. *U. S. National Committee — International Union of Nutritional Sciences:* Dr. R. W. Engel, secretary.

1. It was pointed out that the provisions specified for investment of funds remaining after the Fifth International Congress on Nutrition, as reported in the Proceedings of the 25th Annual Meeting of the American Institute of Nutrition (Journal of Nutrition, 75: 138, 1961), could not be carried to completion since the National Academy of Sciences Investment Fund found it impossible to follow through on the desired specifications; namely, that such fund would share fully in both capital gains and dividend earnings and that no handling or overhead charges by the Academy would be made against this fund. In view of this, in the fall of 1961, a committee was appointed to investigate the most satisfactory solution to investing this fund, which to date totals $32,779.00, with the possibility of additional income from the sales of the Proceedings of the Fifth International Congress. A new proposal for the investment of this fund and change in the action taken by the membership was presented and approved by the Council and membership:
   a. That $25,000 of this fund, designated "U. S. — IUNS," be placed in the Pine Street Fund, Inc., 30 Wall Street, New York 5, N. Y., and that the remainder of the fund, namely, $7,779.00 plus interest earnings to date, be deposited in the National Savings Bank, Washington, D. C. Information furnished by the Federation business office indicates that this would be a sound investment procedure, based on the experience for similar investments by the American Physiological Society.
   b. Other provisions for use of the fund designated "U. S. — IUNS," as contained in the Proceedings of the 25th Annual Meeting would remain in effect but with the following revision (addition is in italics): "3. Authorization for expendi-

tures of earnings or principal fund requires a majority vote of the *U. S. National Committee on Nutrition and approval of the Council of the AIN.*"

c. Since the major use designated for this fund is that of supporting travel grants of U. S. scientists attending any future International Congresses on Nutrition, the Council recommends to the U. S. National Committee on Nutrition that consideration be given to establishing travel grants from the major portion of the fund proposed for deposit in the National Savings Bank, namely, the major portion of the $7,779.00, plus its interest earnings, for use in supporting travel grant applications from U. S. scientists to the Sixth International Congress on Nutrition, Edinburgh, Scotland, in August, 1963. In emergency, the Chairman of the U. S. National Committee of IUNS and the President of the AIN are authorized to approve expenditures from this fund for special conferences or meetings in which U. S. nutritional scientists should be represented.

2. Other Actions of the U. S. — IUNS.
   a. The U. S. National Committee of IUNS recommended to the Organizing Committee, Sixth International Congress on Nutrition, that each participant receive a copy of a special printing of the publication by Dr. E. V. McCollum entitled "History of Nutrition." This action was taken after the committee was informed by Dr. McCollum that an anonymous donor had graciously offered to furnish the funds to cover costs of the special edition.
   b. The U. S. National Committee, jointly with the Food and Nutrition Board, National Academy of Sciences — National Research Council, has been active in an international program for establishing machine methods for documentation and retrieval of knowledge in nutrition and food science and technology, including industrial technology. An *ad hoc* committee, composed of Dr. Paul György, chairman, Dr. R. W. Engel, secretary, Dr. J. C. Ayres, Dr. R. A. Schilling, Dr. D. B. Hand and Dr. F. N. Peters, has agreed to proceed with an evaluation of the total volume of food science literature with the objective of determining the feasibility of establishing an abstract procedure which would facilitate the use of machine methods. Dr. James Moyer, Food Scientist, New York Agricultural Experiment Station, Geneva, New York, is currently proceeding with this evaluation under a project supported by NIH. Hopefully, an information center for food science can be developed in this country which would serve as a companion to a Nutrition Information Center based on the knowledge contained in the *Nutrition Abstracts and Reviews*, Aberdeen, Scotland. The Aberdeen group is proceeding with the machine documentation of knowledge contained in all the volumes of *Nutrition Abstracts and Reviews*. The joint *ad hoc* committee (U. S. National Committee on Nutrition and the Food and Nutrition Board) is coordinating its current activities with that of the Committee on Food Science Abstracts of the Institute of Food Technologists (IFT). Dr. J. C. Ayres of the *ad hoc* committee is Chairman of the Committee on Food Science Abstracts in the IFT.

E. *Representative to the AAAS Council:* Dr. E. L. Hove.

As your delegate to the Council of the American Association for the Advancement of Science for Section C (Chemistry), the following report on the annual meeting of the Association held in Denver last December 26–31 (1961) is submitted:

At this convention, Section C (Chemistry) sponsored three symposia. The first was entitled "Recent Advances in Carbohydrates — Part 1, Monosaccharides: Part 2, Polysaccharides." The second was titled "Extraterrestrial Biochemistry and Biology" with 9 papers dealing with such matters as "The Biological Profile of Mars," "Interstellar Panspermia," and similar subjects. The third was entitled "Geochemical Evolution" with 8 papers considering the origin of elements, atmosphere and life; the role of trace elements in plants and animals, and the implications of trace element distribution over the surface of the earth.

The Council of the Association held two meetings, at which the following information was given:

Individual membership of the Association now stands at about 73,000 but only 2,700 were registered at the Denver meeting.

The net gain in new members during the past year was a record 6,500 (about a 10 per cent annual growth).

The number of affiliated societies reached 301 with the addition of 5 new affiliates.

The Council of the AAAS is composed of delegates from each of the affiliated societies plus the officers of the Association and its 20 major sections. The number of people on the Council is 519, of these about 160 attended the Council meeting.

The Council heard reports from several of its study committees. Of special interest is the recommendation of the Committee on International Scientific Communication that the Board of Governors explore the feasibility of establishing an International Science Register, to include the world's principal scientists and engineers, with appropriate biographical and bibliographic data.

In another action, the Council went on record as considering it unwise to use any criterion in addition to merit for the selection of N.S.F. fellows ("Loyalty Oath Problem").

Finally, action was deferred for at least another year on the plan to streamline the Council by excluding representation on the basis of affiliated societies.

F. *Representative to the Division of Biology and Agriculture of the National Research Council:* Dr. N. R. Ellis.

Dr. Ellis reported the following:

Your representative to the Division of Biology and Agriculture of the National Research Council attended the several meetings of the organization during the past 12 months. These include the Food and Nutrition Board, the Agricultural Board, the Agricultural Research Institute, and also a recent joint meeting of the Division of Biology and Agriculture with other Divisions of the Council.

The Food and Nutrition Board presently consists of 20 members with Dr. Grace A. Goldsmith as chairman. Attention on worldwide nutrition problems continues to increase. The Committee on International Nutrition Programs, organized in 1959, has prepared a booklet on background principles in the application of nutritional science in programs of various agencies. Further studies on implementation of programs are in progress. Domestic problems continue to dominate, especially those concerned with chemical residues in foods, pesticides, radioactive fallout, fats, decreases in consumption of milk products and others. The Food and Nutrition Board has also taken a hand in development of recommendations on water and food supplies and allowances for fallout shelter stocking. There has been a strong demand for Publication no. 575 on "The Role of Dietary Fat in Human Health." This has been revised to bring it up-to-date and republished. A subcommittee is currently bringing together recommendations on milk as related to public reactions about fats, fallout, and residues and reflected in decreasing per-capita consumption. There has been issued also a statement of general policy in regard to the addition of specific nutrients to foods. The proceedings of a conference held in 1960 have been issued as Publication no. 843 under the title "Meeting the Protein Needs of Infants and Children."

From the work of the Food Protection Committee there has appeared Publication no. 877 based on the proceedings of the symposium in December, 1960, and entitled "Science and Food: Today and Tomorrow," along with Publication no. 887 on "The Use of Chemicals in Food Production, Processing, Storage and Distribution."

The Agricultural Board is continuing review of reports on the nutrient requirements of domestic animals and publication of revisions. A volume of 384 pages on "Basic Principles and Techniques in Range Research," has been published (Publication no. 890).

At the fall 1961 meeting of the Agricultural Research Institute there was an excellent symposium on agricultural research. There has been discussion during the year of a possible reorganization affecting both the Agricultural Board and the Agricultural Research Institute by which

the two would become the Agricultural Research Board.

Dr. T. C. Byerly of the U. S. Department of Agriculture becomes Chairman of the Division of Biology and Agriculture on July 1, succeeding Dr. H. Burr Steinbach.

IX. Report of the Clinical Division:
Dr. Robert E. Hodges,
Secretary-Treasurer of ASCN

Dr. Hodges stated that the Division hopes to continue to serve as a bridge between the parent group and clinicians. The membership of the American Society of Clinical Nutrition is 89, with 31 candidates approved for membership by both AIN and ASCN for 1962. AIN members interested in clinical nutrition are welcome to apply for membership. The Division has a surplus of approximately $1,000 in the treasury. Newly elected officers are listed on the last page of these minutes. ASCN hopes to create an award similar to the Osborne and Mendel Award for meritorious performance. Dues of the ASCN will remain the same, but the cost of the *American Journal of Clinical Nutrition* will be increased from $6.00 to $8.50 per year for members and to $12.00 for nonmembers.

X. *Report of AIN Council Actions:*
Dr. P. György

The Council met on November 11, 1961, in Washington, D. C., and on April 12, 13 and 16 in Atlantic City.

A. The Council approved the nomination of Dr. L. A. Maynard to attend the Nutrition Society of Canada meeting June 8–9, 1962, with Dr. R. W. Engel as alternate.

B. The Council approved the creation of a committee consisting of three former presidents of AIN to receive and submit lists of eligible candidates for nomination to the status of Honorary Membership.

C. The Council recommended that the AIN join the AIBS as an affiliate member and that the annual dues of $100.00 be defrayed by the treasury of the AIN. A motion so directing the Council was unanimously passed by the membership.

D. Dr. György discussed the proposal which the AIN Council had received from the Nutrition Society of Canada for organizing a joint meeting of the two societies. A joint organizing committee will be appointed by the two societies consisting of three U. S. and three Canadian members. Suggested date of the meeting would be the early part of September, 1964. The organizing committee is to report to the membership at the next annual meeting.

E. Dr. György reported that the Council had approved appointment of a committee to investigate the possibility of establishing a secretariat office in the Federation headquarters in the space now allocated to AIN. Such an office may be established as a joint executive secretary with another society or a part-time employee. The function of this office, in addition to serving as secretariat, would be to coordinate public information activities for the Institute and handle a portion of the Treasurer's duties.

F. Announcement was made regarding the Sixth International Congress on Nutrition which will meet in Edinburgh, Scotland, in 1963 from August 9 to August 15. The announcement and program were published in *The Journal of Nutrition*, 76: 223, 1962. According to the report of the Tellers' Committee, 203 members of AIN indicated by mail ballot that they plan to attend this meeting.

ANNUAL DINNER
AND PRESENTATION OF FELLOWS
AND AWARDS

The annual banquet was held on April 18 at the Shelburne Hotel with approximately 310 members and guests attending. Dr. György, as toastmaster, introduced the special guests and awardees.

Dr. R. W. Engel presented Certificates of Fellow to the following persons selected in 1962 for their distinguished careers in Nutrition:

LYDIA J. ROBERTS

"For a distinguished career extending over more than half a century, as first Chairman of the Committee on Dietary Requirements of the National Academy of Sciences, National Research Council, and for 15 years of service in Puerto Rico, following retirement from the University of Chicago, in the spirit of devotion and humility, to serve children and the common man in applied nutrition and preventive medicine."

J. S. HUGHES

"For a distinguished career in experimental nutrition extending over more than half a century in the research areas of major and minor elements for livestock, studies on vitamins A, D, and C, nutritive evaluations of feedstuffs, hormones, and many others."

W. D. SALMON

"A charter member of the American Institute of Nutrition, who has been distinguished as a teacher, in research, and an inspiration to all of his many friends — for pioneer investigations on the vitamin B-complex, amino acid imbalances and for original observations of the induction of hepato-carcinoma in the rat as a result of choline deficiency, and for the original observations of the value of zinc in the prevention and cure of parakeratosis in swine."

J. S. HUGHES

LYDIA J. ROBERTS

W. D. SALMON

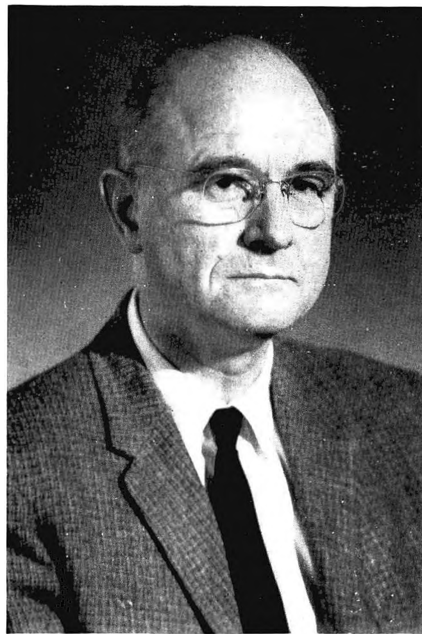
Osborne and Mendel Award

The 1962 Osborne and Mendel Award of $1,000 and a scroll was presented to Dr. William J. Darby, Chairman, Division of Nutrition, Vanderbilt University School of Medicine, for his studies of folic acid metabolism in animals and man, his investigations of maternal and infant nutrition and his contributions to knowledge of the epidemiology of malnutrition. This award is sponsored by the Nutrition Foundation, Inc.

WILLIAM J. DARBY

Borden Award

The 1962 Borden Award of $1,000 and a gold medal were presented to Dr. Horace A. Barker, Department of Biochemistry, University of California, in recognition of his significant contributions to our knowledge of the nutritional value of milk and milk products, important dietary sources of the "Animal Protein Factor," through his isolation of vitamin B_{12} coenzymes and his clarification, in large measure, of their metabolic role and their chemistry.



HORACE A. BARKER

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AMERICAN INSTITUTE OF NUTRITION

July 1, 1962 — June 30, 1963

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National Research Council Boards and Divisions: N. R. Ellis (1963)

American Association for the Advancement of Science: Joseph H. Roe (Section N, Medical) (1963), P. B. Pearson (Section C, Chemical) (1964)

Food and Agriculture Organization: B. S. Schweigert (1964)

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Respectfully submitted,

ARNOLD E. SCHAEFER, *Secretary*