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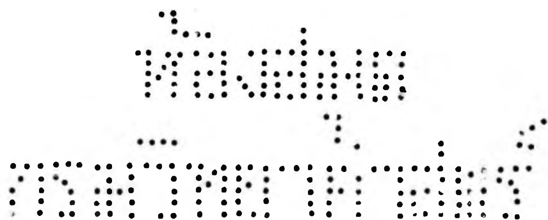

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μg	microgram	mm	millimeter
mμg	millimicrogram	μ	micron
μμg	micromicrogram	mμ	millimicron
		μμ	micromicron
Volume		*Area*	
m^3	cubic meter	m^2	square meter
cm^3	cubic centimeter	cm^2	square centimeter
mm^3	cubic millimeter	mm^2	square millimeter
l	liter		
ml	milliliter		

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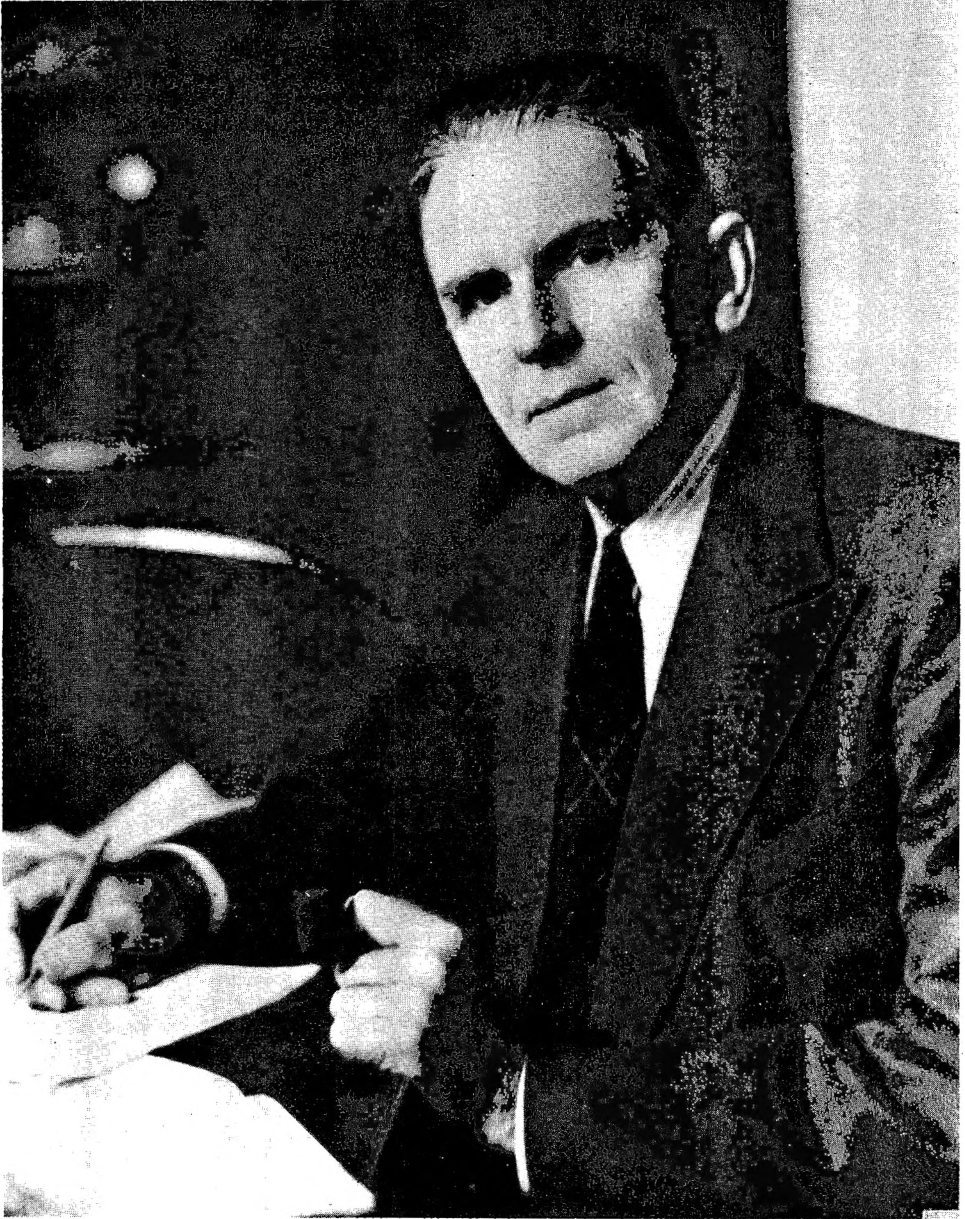
DAVID BREESE JONES

(1879 – 1954)



DAVID BREESE JONES

David Breese Jones
— *A Biographical Sketch*

(October 5, 1879 — September 5, 1954)

A hundred years or so ago, the town of Cambria, Wisconsin, was the center of a growing Welsh community, most of whose people came from agricultural northern Wales to farm the rich prairie land northeast of Madison. Among the arrivals in 1848 was a Jones family and their young son, John. This family was known as Jones, Coety (a contraction of "Coed Ty," meaning "Wooden House"), as it was a custom of the Welsh to add the name of the family farm to the surname in order to distinguish between the many Joneses, Evanses, Williamses, or other names which were so common.

John D. Jones, Coety, later married Mary Breese, whose parents had also come from Wales, and on October 5, 1879, their first son, David Breese Jones, was born. Two more sons were added to the family—John Edward, who was later to go into the Presbyterian ministry and is now retired in Indiana where he served one church for over 30 years; and Llewelyn, who followed his eldest brother's profession, becoming a chemist in the U. S. Food and Drug Administration laboratories in Chicago and Kansas City; he is now retired in Denver, Colorado.

The three brothers—Dave, John, and Lew—grew up in Cambria, working on the Coety farm and, when the family moved into town, engaging in numerous activities of which those in the Welsh Church took an important part. Young Dave had two teachers in school who left a lasting impression on him—an English teacher who instilled in the pupils a deep appreciation of literature and poetry, and a science teacher who sparked their imagination by having them perform simple experiments at home. One of Dave's earliest experiments was the demonstration of "organic matter" in bone by tying a knot in a chicken femur after soaking it in dilute acid, and of "mineral matter" by ashing another bone. It was apparently this teacher who first awakened David Breese Jones's interest in science.

After a three-year course at Ripon College Preparatory School in Ripon, Wisconsin, from which he was graduated in 1899, Dave taught for a winter term in a one-room country school three miles south of Cambria. He walked the three miles from home and built the fire in the schoolhouse each morning before opening school for his 20-odd pupils. Among his later recollections of this period was his surprise one snowy January morning to find a roaring fire in the stove and several other indications that tramps had spent a cozy night in the schoolhouse.

Dave entered Ripon College in 1900 and was graduated in the class of '04. He took all the chemistry courses offered, supplementing these and the other science courses with a sound background in the classics, on which more emphasis was placed then than nowadays. Four years of Latin and three each of Greek and German came easily to one who was linguistically inclined and who had spoken Welsh at home before he spoke English. He earned almost all of his way through college—as janitor in a church, waiting on table, cutting wood, and at a wide variety of other jobs. But the years at Ripon were not all study and work by any means; there were many social events in which he took part, as well as declamatory contests, choir singing, college club activities, and tours as a member of the glee club and the football team.

After receiving the Bachelor of Arts degree from Ripon, David Jones (or D. Breese Jones, as he usually signed his name from about that time) taught high school for two years in Princeton, Minnesota. He was not only the science

teacher—teaching chemistry, physics, and zoology—but he also taught bookkeeping, algebra, geometry, and history, coached the football team, and served for a while as acting principal.

He obtained an appointment as assistant in chemistry at Yale University and, in the fall of 1906, he left the Midwest to take up his new duties in New Haven. He soon arranged with Russell H. Chittenden, then Director of the Sheffield Scientific School, and with Dean Phillips of the Yale Graduate School to take courses toward an advanced degree; his first research was carried out under the direction of Professor Treat B. Johnson in the organic chemistry department during the winter term of 1907–08. The results of this work were published in the American Chemical Journal as two papers in Johnson's extensive series on "Researches on Pyrimidines."

In the spring of 1908 he left the Sheffield campus to take a research fellowship vacated by F. W. Heyl (who later became research director of the Upjohn Company) in the laboratory of Thomas B. Osborne at the Connecticut Agricultural Experiment Station at New Haven. Osborne was already recognized as the outstanding authority in the field of vegetable proteins and, aided by a grant from the Carnegie Institution, he and his associates were intensively engaged in characterizing proteins by their amino acid composition. This was an active period of early development in the chemistry of proteins, as improved methods were being devised to cope with their complexity. Osborne had recently modified Hausmann's method for the differentiation of proteins based on their nitrogen distribution; Sörensen had just published his titration method; and from the laboratories of Fischer, Kossel, Abderhalden, and others results were being published which were to form the basis of much of the later progress in protein chemistry. It is not surprising that the experience in Osborne's laboratory was to be a decisive factor in setting the course of D. Breese Jones's later career.

During the year and a half spent at the Experiment Station before resuming his graduate work under Professor Johnson, he analyzed scallop muscle, ox muscle, egg albumin, and vitellin, by the Fischer ester distillation method. This method, introduced by Emil Fischer in 1901, required large amounts (300 to 500 gm) of protein and involved an extremely laborious and time-consuming procedure whereby each individual amino acid was isolated and weighed in pure form as determined by elementary analysis. The method was being used extensively in Osborne's laboratory because, in spite of its shortcomings, it provided the most elegant means known for characterizing a protein. The last two joint publications by Osborne and Jones, in 1910, described a modification of the Fischer method and a critical examination of the method to ascertain the sources of the errors involved.

Returning to the Sheffield Scientific School, he completed his graduate work and was awarded the doctoral degree in 1910. He received an appointment as head of the chemistry department at Morningside College in Sioux City, Iowa, for the following year, and in August he married Clara Abigail Chase, with whom he had become enamored when they were both on the high school faculty in Princeton. While he had been in New Haven, Miss Chase had continued teaching, having obtained her Master of Arts degree in English at the University of Michigan; of course Breese (as he was often called) had managed to go to Wisconsin each summer to visit his family and to see Clara Chase. One summer he worked for a month or so as a streetcar conductor in New Haven, and otherwise he supplemented his income by tutoring.

The year at Sioux City was followed by 4 years as instructor in organic chemistry at the University of Wisconsin. The teaching schedule in Professor Louis Kahlenberg's department at Madison was a full one; many were the evenings spent correcting papers and abstracting articles for Chemical Abstracts, the latter being an activity Doctor Jones pursued for many years to come. The research for which he found time was on certain organic reactions of alpha-halogen palmitic acids. One summer he taught organic chemistry at the University of Pittsburgh. In 1915 he left Madison and the academic field to enter the government service in the U. S. Department of Agriculture, a service in which he

was to continue even after his retirement in 1949.

Under Harvey W. Wiley, who may be regarded as "the father of pure food and drug legislation in the United States," the Chemical Division of the U. S. Department of Agriculture had become designated by an Act of Congress in 1901 as the Bureau of Chemistry. Wiley resigned as chief of the Bureau in 1912 and was succeeded by Carl L. Alsberg to whom the suggestion was made by T. B. Osborne that, in view of the importance of proteins as essential factors in human nutrition and in the feeding of farm animals, the Bureau of Chemistry should have a laboratory devoted to the study of proteins. Upon Alsberg's recommendation, the Protein Investigation Laboratory was established in 1914; and Carl O. Johns, assistant professor of organic chemistry at the Sheffield Scientific School of Yale, was appointed chemist in charge of the new protein unit. Actual work on proteins began the following year when D. Breese Jones joined the laboratory, having received the second highest rating on the Civil Service examination.

The Joneses arrived in Washington with their three-year-old son, Chase, during an unseasonably hot spell in September. A house was soon located, and in the fall of the following year a daughter, Clara Gwendolyn, was born. The environment at the Bureau of Chemistry was very congenial; Doctor Johns had been an instructor at Yale during the time Doctor Jones was a graduate student there, the two had often sailed together on Long Island Sound, and now a friendship developed not only between their families, but also with the other members of the newly organized laboratory.

On first arriving in Washington, Doctor Jones started work immediately on the isolation and characterization of proteins from the jack bean, *Canavalia ensiformis*. Three proteins were isolated and were characterized by elementary analysis and nitrogen distribution: an albumin, and two globulins to which the names canavalin and concanavalin were assigned. The report of this work was the first of 13 papers published with Johns on the composition of proteins of various seeds, including the peanut, coconut, kafir, corn, several species of beans, and on lactalbumin. The methods used in the investigation of these proteins were essentially those that Osborne was using in New Haven, with new modifications as these were developed. In a paper on the determination of tyrosine in proteins, Johns and Jones called attention to the higher value they had found for this amino acid in kafirin when determined by the colorimetric method of Folin and Dennis than by actual isolation. Later, the use of amino acid isolation methods in the analysis of proteins yielded more and more to the use of such methods as Van Slyke's nitrogen distribution method and the more newly developed colorimetric methods.

As an example of the regard in which the early work of this laboratory was held, we may cite a letter dated October 29, 1919, from Donald D. Van Slyke, editor of the Journal of Biological Chemistry, to Doctor Alsberg, chief of the Bureau of Chemistry. The letter reads in part: "Dear Carl: We just received a paper from Jones and Johns on, 'The hydrolysis of stizolobin, the globulin of the Chinese velvet bean, *Stizolobium niveum*,' which is so well done a piece of work that I want to express my admiration of it to you. I am afraid that they are about the only ones who are continuing the systematic attempt to make the amino-acid analysis of proteins complete, and I hope they will keep it up."

While the chemical work on proteins was proceeding, a rat colony was established in 1917, and nutrition studies were begun following the general line of approach that Osborne and Mendel had been pioneering since 1909. The first feeding experiment D. Breese Jones carried out was with Finks and Johns on the comparative nutritive properties of the proteins of the cow-pea, *Vigna sinensis*, and the field pea, *Pisum sativum*. It was found that the cow-pea proteins are deficient in cystine and are indigestible before cooking, being similar in these respects to beans of the genus *Phaseolus* to which the cow-pea is closely related botanically, whereas the field pea proteins promoted good growth in rats without requiring cystine supplementation or cooking.

After Doctor Johns resigned in 1920 to become director of research in the develop-

ment department of the Standard Oil Company of New Jersey, Doctor Jones was placed in charge of the Protein Investigation Laboratory, which was later named the Protein and Nutrition Research Division, as its scope was extended to include investigations on vitamins and other aspects of nutrition. In 1927, the Bureau of Chemistry was combined with the Bureau of Soils and the Nitrogen Fixation Laboratory to become the Bureau of Chemistry and Soils, and this was later reorganized into the Bureau of Agricultural Chemistry and Engineering. Throughout these changes the Protein and Nutrition Division continued under Doctor Jones's direction as an integral unit of the Bureau. The laboratory was located in the old red brick Bureau of Chemistry building on 13th Street, S.W., until 1935 when the newly constructed South Building of the Department of Agriculture was occupied. In 1943, the Division was consolidated with the Bureau of Home Economics to form the Bureau of Human Nutrition and Home Economics, located in Beltsville, Maryland. Henry C. Sherman of Columbia University headed the new bureau for its first year and was then succeeded by Dr. Hazel K. Stiebeling.

From the time Doctor Jones assumed full responsibility for the Protein Investigation Laboratory, he and his team of co-workers continued the systematic studies on proteins, being the first to isolate the proteins from scores of foods, especially seeds and grains, and to characterize these proteins by amino acid analyses. The studies on proteins of different species of beans were extended, and they revealed that all, except the proteins of the soybean, are deficient in cystine and are characterized by indigestibility which can be corrected by heating. Without cystine supplementation or heating, neither the isolated proteins nor the bean meals supported growth at a satisfactory rate; indeed some of them, particularly the proteins of the navy bean, supported life in rats for only a short time when furnished as the sole source of protein in an otherwise adequate diet.

The proteins of cereals were the subject of many publications. Among those investigated were: wheat, corn, oats, rye, barley, buckwheat, rice, feterita, and milo. A series of three papers described the chemical and nutritive properties of a globulin, an albumin, and a prolamin, isolated from wheat bran. The glutelins of several cereals were given special attention in a series of 6 papers appearing between 1927 and 1930.

In 1931, a U. S. Department of Agriculture Bulletin was published which made available a new set of factors Doctor Jones had worked out for converting the percentages of nitrogen in foods and feeds into percentages of protein equivalents. By the use of these factors, protein contents could be calculated with a much higher degree of accuracy than by the indiscriminate use of the conventional factor 6.25 hitherto generally employed. The new factors were derived from data on the nitrogen contents of more than 100 different proteins obtained from plant and animal sources, more than half of which had been isolated under Doctor Jones's direction.

A series of 7 papers was published on the digestibility of proteins *in vitro*, in which one of the reported findings was that addition of a small amount of gossypol to casein or to the globulin isolated from cottonseed inhibited to a marked degree the digestibility of the protein by pepsin and trypsin, thus explaining the incomplete digestion by animals of the protein content of cottonseed meals and flours, in which gossypol is an ingredient. Also, the effects of peptic and tryptic digestions of casein were studied, with special attention to the comparative amino acid contents of the partial cleavage products, the rate of liberation of cystine, and the lability of cystine toward alkali.

In the vitamin work, attention was given to the study of methods and techniques for vitamin assay, the vitamin contents of certain sea foods, and to the effect upon vitamins of commercial processes used in connection with the production and preparation of foods. Studies were made on the effect of long continued storage at low temperature on the vitamin A content of eggs, the effect upon the vitamin content of tomatoes when the unripe fruit is treated with ethylene gas to develop the color characteristic of the ripe fruit, and the effect upon vitamin C when such fruits as apricots are treated with sulfur dioxide as in the commercial drying of the fruit.

Feeding experiments and chemical studies published in the 1930's on the problem of the toxicity of wheat grown on selenium-containing soils led to the isolation by Horn and Jones of a crystalline substance that appeared to be an isomorphous combination of an unsymmetrical thio-ether diamino acid[1] with its selenium analogue. Pursuing this work further, Horn, Jones and Ringel isolated for the first time a symmetrical thio-ether diamino acid, which they named lanthionine, from the acid hydrolysate of wool that had been treated with hot dilute sodium carbonate solution. They showed this compound to be the thioether analogue of cystine. Subsequently, they isolated lanthionine from other keratins, and from lactalbumin, after treatment with dilute alkali; they suggested the probability that lanthionine may be similarly obtained from most cystine-containing proteins. Both the meso and racemic diastereoisomers were isolated, and feeding experiments showed that DL-lanthionine can replace cystine and methionine for growth, whereas mesolanthionine cannot do so.

The storage of large quantities of grain in the United States raised the question as to the effect of storage on grain proteins. Jones, Gersdorff and Divine investigated the chemical and nutritive changes that occurred under various conditions of storage during periods up to two years in the proteins of soybean meal, wheat, white flour, whole wheat flour, ground corn, and whole shelled corn.

In connection with the characterization of proteins by amino acid analyses, mention should be made of the development of methods published between 1945 and 1950 by Horn, Jones and Blum for the determination of each of the ten so-called essential amino acids. The methods consist of microbiological assays, except for the colorimetric procedures for tryptophan and methionine. These methods were applied to the analyses of over 30 proteins and foods, and the results were published with other reported values for comparison.

It was perhaps fitting that the last paper published by D. Breese Jones, submitted almost a year and a half after his retirement, should have been published in this Journal, and that it should have had as its *raison d'être* a careful survey he had made of a large number of feeding experiments conducted under his supervision over a period of several years. The survey brought to light a correlation between sex and growth behavior of young rats on experimental diets. When either the quality or the quantity of the protein in the diet was inadequate, the females showed greater weight gains than the males; on the other hand, the males showed the greater weight gains in experiments that were similar except that the diets were adequate in all respects. This paper also reported experiments in which it was found that the females were better able to survive than the males on a diet grossly inadequate in protein quality and quantity.

Over the course of 40-odd years, more than 160 publications appeared with D. Breese Jones as author or co-author, of which about 60 were published in the Journal of Biological Chemistry and most of the rest in other journals concerned with some aspect of protein chemistry or nutrition. In government publications, lectures, and semi-popular articles, he was instrumental in disseminating current knowledge of nutrition, emphasizing among other aspects the importance of the nutritive qualities of proteins in the diet. He regretted that with the passing years his executive duties allowed him less time for actual laboratory work; whenever possible he arranged to have a small laboratory near his office, where he could "get his hand in" as opportunity permitted. All the work which he had the responsibility of supervising, as that which he himself conducted, was marked by its thoroughness and meticulous attention to details.

In 1947, the first year that Department of Agriculture Honor Awards were made, he was presented by Clinton P. Anderson, Secretary of Agriculture, with a Superior Service Award. The citation read: "For his contribution to science through research into the chemical nature, digestibility, and biological value of proteins and their constituent amino acids." In the same year he was awarded a Ripon College Alumni Citation, "in recognition of outstanding

[1] This compound was later synthesized by Brown and du Vigneaud; they named it cystathionine.

ability and distinguished accomplishments in the field of Biochemistry." After he reached the compulsory retirement age of 70, in 1949, his desk, books, and files were taken from Beltsville to the South Building in Washington where office space with ready access to the library was made available for his use. He was thus enabled to keep in touch with the Department and was available for consultation in the field of his specialties. This was a volunteer service on his part and a recognition on the part of the Department of the contributions he could continue to make to its program. He enjoyed the freedom of his retirement, taking advantage of pleasant days to get out on the golf course, and making more frequent trips to visit his relatives and close friends. However, he devoted almost half his time to the field in which he had taken such an active part for so many years. He was at his desk in the South Building for the last time only a few days before he suffered a fatal heart attack on August 31, 1954.

I have mentioned my father's thoroughness and attention to detail in his scientific work. These same attributes applied also to his personal life. He practised and often quoted a maxim of Theodore Roosevelt's: "If a job is worth doing, it is worth doing well." Orderliness, precision, and punctuality are characteristics descriptive of the manner in which he conducted his affairs. To these should be added kindness, sterling integrity, imagination, and a quiet sense of humor. Although by nature quite reserved, he had a warm and lively spirit that expressed itself to those who were close to him. He possessed a quiet dignity that was manifested in the erect bearing with which he carried his tall, somewhat slender frame.

Throughout his life he had a great liking of nature and the out-of-doors; this probably stemmed from the experiences of his boyhood days on the farm and in the countryside around Cambria. As a young man, he keenly enjoyed tramping through fields and woods. My mother shared this love of nature and combined with it a good knowledge of botany. During their early married life they went on many fishing trips in the wilds of northern Wisconsin and Michigan and, with photography as a hobby, my father took and processed scores of pictures recording these trips. At a later period this interest in nature centered around the field study of birds. Among my own early memories are those of accompanying my father and mother on early Sunday morning walks with field glasses, bird guide, and notebook through wooded areas around Washington that have long since disappeared with the growth of the suburbs.

My father and mother enjoyed travelling, and they often combined this with camping and with visiting sites of scenic and historic interest. One of the features they liked about living in Washington was the ready accessibility of scenic and historic places in nearby Virginia, Maryland, and Pennsylvania. During the era of the "tin can tourists," our family went on many week-end and vacation trips, and pitching the tent became a routine procedure.

My mother had been a source of inspiration for my father and had shared many common interests with him. After her death in 1925, he did not resume so many activities with his former enthusiasm. Gardening was a recreation he had enjoyed for several years, and he took pride in his vegetables and flowers, some of which he raised on a semi-experimental basis. He continued this activity until he moved from the suburbs into an apartment in Washington when my sister and I went away to school in 1930; for the rest of his life his main outdoor recreation was golf.

My father was always allured by adventure, both the experiencing of it and the hearing or reading of accounts by others. As a boy he had thrilled to the tales told by men who had returned from "Indian Territory" farther west; in later life he enjoyed reading the works of such naturalists and explorers as Burroughs, Muir, Theodore Roosevelt, Amundsen, and Stefansson. Historical biographies also attracted his interest, and "The Diary of John Quincy Adams" was one of his later favorites.

When he attended the 1938 International Physiological Congress in Zurich as a delegate of the U. S. Department of Agriculture, one of the highlights of the trip, on which my sister accompanied him, was a side trip to Wales—especially the region

around Mount Snowdon where his father had lived as a young boy—and the opportunity it afforded him to speak the Welsh language in its native land. He always enjoyed reading and speaking Welsh whenever occasion permitted. He was an active member and one time president of the St. David's Society of Washington, named after the patron saint of Wales and made up of persons of Welsh birth or ancestry. The Welsh are noted for their choral singing, and of course this was one of the favorite activities of the Society where my father also sang bass in the male quartet.

He was active in a number of scientific societies and organizations. In the American Chemical Society he was elected chairman of the Division of Biological Chemistry in 1930 and was president of the Washington Section of the Society in 1934. He was a Fellow of the American Public Health Association, in which he served as chairman of the Committee on Nutritional Problems from 1934 to 1938, and as chairman of the Food and Nutrition Section in 1939. He was elected chairman of the Washington Section of the Society for Experimental Biology and Medicine for the year 1941, and was a member of at least two committees of the National Research Council. His memberships also included the American Institute of Nutrition, the Institute of Food Technologists, the American Society of Biological Chemists, the Washington Academy of Science, and the Cosmos Club.

In the field of protein chemistry and nutrition, the amount of progress my father and his co-workers made may indeed be considered remarkable when the limited methods then available are viewed in contrast to the much more rapid and precise methods of today. The work that has been reviewed in outline here, as well as other contributions which could not be included in a sketch such as this, constituted an essential part in the development of knowledge in this area. As time goes on, those who continue in this field of research will find that their own task has been made easier and their efforts more fruitful as a result of this earlier work.

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Vitamin K Deficiency in Rats Induced by the Feeding of Irradiated Beef[1]

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Evaluation of the nutritive value and appraisal of the toxicity of irradiated foods are imperative before this processing method is commercially applied. In short-term studies, it was shown that irradiation sterilization had little or no deleterious effect on the biological value of animal, legume and cereal proteins (Metta and Johnson, '56; Metta, Norton and Johnson, '57; Metta and Johnson, '59) or on the metabolizable energy of synthetic diets and their macronutrients (Johnson and Metta, '56). The authors then undertook a longevity-reproduction study for appraising the wholesomeness of beef and flour irradiated with gamma radiation and stored for 6 months at 76°F. When irradiated beef and flour, incorporated into balanced diets at 35% dry solids each according to a factorial design, were fed to growing rats ad libitum, a significant number of male rats (but not females) died from hemorrhage in less than 8 weeks of feeding of the irradiated beef diets. After confirming this unexpected observation, a series of experiments was undertaken to elucidate the mechanism involved in this syndrome.

The purpose of this paper is to report on the hemorrhagic syndrome observed in rats consuming irradiated[2] beef and on its prevention by dietary vitamin K supplementation.

METHODS

Ground beef, irradiated at 2.79 and 5.58 million rad,[3] and raw ground beef were supplied to us in sealed cans by the Department of the Army. The raw beef was stored at 0°F. and the irradiated beef at 76°F. for 6 months after processing. Proximate analysis of the beef samples gave average values of 61.1% moisture, 19.8% protein and 17.9% ether extract. The raw and the irradiated beef were cooked in an autoclave for 25 minutes at 17 pounds pressure, and then incorporated wet into two balanced diets to provide 35% dry solids (see table 1 for diet composition). These diets contained all the known vitamins and minerals required in the diet of the rat. No vitamin K was added to any of the basal diets. Sufficient diets were mixed once at the beginning of each experiment to last for the entire period (4 to 8 weeks). After mixing, the diets were preserved in closed jars and stored frozen at 0°F. The food jars were removed from the freezer every third or 4th day and kept in the cold room (40°F.); the rats were fed

TABLE 1
Composition of the experimental diets

Constituents	Per cent
Ground beef (untreated or irradiated)	35[1]
Wheat flour	35
Sucrose	19
Cod liver oil	1.5
Wheat germ oil	0.5
Mineral mixture 446[2]	4
Vitaminized cerelose[3]	5

[1] Dry substance; however, wet beef was used in the diets.
[2] Spector ('48).
[3] The percentage composition of the vitaminized cerelose is as follows: Calcium pantothenate 100 mg; nicotinic acid 40 mg; thiamine hydrochloride and riboflavin 20 mg each; pyridoxine hydrochloride 10 mg; folic acid 2 mg; vitamin B_{12} 0.1 mg; biotin 0.06 mg; choline chloride 2 gm; and cerelose 97.8 gm.

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[1] These studies were supported in part under contract no. DA-49-007-Md-544, with the Office of the Surgeon General, Department of the Army. The opinions expressed in this publication are those of the authors and not necessarily those of the Department of the Army.
[2] Irradiation with gamma rays was carried out at the Atomic Energy Commission's Material Testing Reactor (MTR) at Arco, Idaho, by the Phillips Petroleum Company.
[3] Rad is defined as 100 ergs of radiant energy absorbed per gram of the substance.

from these jars ad libitum. Left-over diets after 4 days of feeding were discarded and freshly withdrawn diets were used for feeding. Proximate analysis of the diets when calculated on a moisture-free basis gave average values of 22.3% for protein (N × 6.25) and 20.1% for fat (ether extract). Prothrombin times were determined on whole plasma according to Quick ('38).

Weanling rats of the Sprague-Dawley strain were used in all experiments. The rats were housed in individual wire cages and had access to drinking water and food at all times.

TABLE 2

Effect of irradiated beef on growth and mortality

No. and sex of rats	Daily growth	Deaths from hemorrhage
	gm[1]	
	Non-irradiated beef	
20 males	6.9 ± 0.13	0
20 females	4.9 ± 0.15	0
	Irradiated beef[2]	
20 males	6.7[3] ± 0.34	14
20 females	4.7 ± 0.07	1

[1] Average daily gains (with standard errors) during the first 20 days of feeding.
[2] Beef irradiated at 5.58 mega rad.
[3] Average of 5 rats.

EXPERIMENTAL RESULTS AND DISCUSSION

Series A experiments. Growth and mortality data on male and female rats eating irradiated and non-irradiated beef are shown in table 2.

A significant number of rats consuming irradiated beef died from internal hemorrhage within 46 days, the first death of a male rat occurring on the 11th day of feeding. This rat became sluggish on the 8th day of the regimen and started refusing food. He continued morbid during the next two days, did not eat any food, lost weight and appeared anemic. He was found dead on the 11th day. Post-mortem examination showed hemothorax, the blood had not clotted; there was bleeding also in the epididymis. Autopsy of the other 13 male rats also revealed epididymal hemorrhage. Hemothorax, subcutaneous hemorrhages and hypertrophy of the thymus gland were also observed in a few rats. In the case of the dead female rat fed irradiated beef, profuse bleeding in the thorax was found.

After 46 days of feeding, the surviving male rats were removed from the experiment but the female rats were continued on their respective regimens for 34 more days. During this period the female rats were apparently healthy and active, and no hemorrhages were observed.

This hemorrhagic syndrome in the rat consuming irradiated beef was confirmed in a second experiment and has since been produced in 12 subsequent experiments. A number of agents may be responsible for the hemorrhagic diathesis. Since the composition of the diets is the same except for the irradiation of the beef, the syndrome must be due to irradiation-induced changes in the beef. Radiation will induce autoxidation (Mead, '52; Polister and Mead, '54)

TABLE 3

Effect of oral supplements on the survival of male rats fed irradiated beef

Diet	Oral supplement	No. of rats	Deaths from hemorrhage
$Beef_O$[1] + CLO[2] + WGO[3]	—	8	1
$Beef_R$[4] + CLO + WGO	—	8	3
$Beef_R$ + CLO	WGO	8	6
$Beef_R$ + CLO	Vitamin E + methyl linoleate	8	1
$Beef_R$ + WGO	CLO	8	4
$Beef_R$ — WGO	Vitamins A and D	8	4
$Beef_R$	Vitamins A, D and E + methyl linoleate	8	4

[1] Non-irradiated beef.
[2] Cod liver oil.
[3] Wheat germ oil.
[4] Beef gamma irradiated at 5.58 million rad.

and might conceivably produce compounds causing this syndrome.

Series B experiments. In order to test the possibility that free radicals, oxidation products, etc., produced in beef by irradiation might react with the unsaturated fats of wheat germ oil and cod liver oil of the diet to produce a hemorrhagic agent such as α-tocopherylquinone (Woolley, '45) for example, wheat germ oil and cod liver oil were withdrawn from the diet. In one experiment these oils were orally administered to the rats separately or in combination and in another experiment they were replaced by vitamins A and D, α-tocopherol and methyl linoleate. It was found that when the oils were withdrawn from the diet, hemorrhage still occurred although somewhat less extensively (see table 3).

Series C experiments. The original longevity-reproduction study was designed to appraise the wholesomeness of irradiated stored beef. Therefore, the irradiated beef used in these experiments had been stored for over 6 months at room tmperature after irradiation. The question arose as to whether hemorrhage was induced as a result of changes which occurred during irradiation of the beef, storage of the irradiated beef, or storage of the diets. This series was designed to study this problem.

Four experimental diets similar in composition to that reported in table 1 were used. Beef was irradiated at 5.58 mega rad. Cod liver oil (1.5%) and 0.5% of wheat germ oil were incorporated into each of these diets during mixing. No vitamin K was added to any of the diets. In diet 1, irradiated beef, which was stored for over 6 months at 76°F., was used. In diet 2 freshly irradiated beef was used. Diet 3 contained control beef. These three diets were mixed and stored frozen for 4 weeks before the start of the experiment. Diet 4 was similar to diet 1, except that it was mixed every other day, and thus there was no appreciable storage of this diet after mixing.

These diets were fed ad libitum to 4 groups of male rats for 40 days. From the data in table 4 it is obvious that the harmful effects caused by ingesting irradiated beef are not due solely to storage of the beef or of the diets.

Series D experiments. It has been reported many times (e.g., Nightingale et al., '47) that the rat does not require vitamin K in the diet and that the requirement of this vitamin is normally supplied by in-

TABLE 4

Effect of the storage of irradiated[1] beef and of irradiated beef diet on survival of male rats

Type of beef in diet	No. of rats	Deaths from hemorrhage
Stored irradiated beef[2]	9	9
Fresh irradiated beef[2]	9	2
Non-irradiated beef[2]	6	0
Stored irradiated beef[3]	9	9

[1] Ground beef was gamma ray irradiated at 5.58 mega rad.

[2] Diet was mixed and stored frozen for the entire feeding period of 40 days.

[3] Diet was mixed every other day; no storage of diet.

TABLE 5

Effect of vitamin K oral supplementation on survival of rats fed irradiated beef

Type of beef in diet	Supplementation	No. of rats	Deaths from hemorrhage
	Experiment 1		
$Beef_O$[1]	None	10	0
$Beef_O$	1–3 μg vitamin K_3/gm of diet	4	0
$Beef_R$[2]	None	14	9
$Beef_R$	1–3 μg vitamin K_3/gm of diet	14	0
	Experiment 2		
$Beef_R$	3 μg vitamin K_3/day	8	0
$Beef_R$	10 μg vitamin K_3	8	0
$Beef_R$	30 μg vitamin K_3	8	0

[1] Non-irradiated beef.

[2] Beef gamma irradiated at 5.58 mega rad.

TABLE 6

Relative effectiveness of water-versus fat-soluble vitamin K in rats fed irradiated beef (a three-week trial)

Oral supplement per rat per day	No. of rats	Plasma prothrombin time	Deaths from hemorrhage
		Seconds	
None	8	24	2
1 μg vitamin K_3[1] in water	6	27	0
3 μg vitamin K_3 in water	6	16	0
6 μg vitamin K_3 in water	6	16	0
One drop corn oil	6	23	0
One drop corn oil + 1 μg vitamin K_3[2]	6	20	0
One drop corn oil + 3 μg vitamin K_3	6	15	0
One drop corn oil + 6 μg vitamin K_3	6	14	0

[1] Sodium salt of 2-methyl-1,4-napththoquinone diphosphate ester.
[2] 2-Methyl-1,4-naphthoquinone.

testinal bacterial synthesis. Thus, our experimental diets contained no added vitamin K even though ionizing radiation has been shown to destroy vitamin K in beef (Richardson et al., '56). However, the irradiated beef in the diet might in some way alter this normal pattern, and so experiments involving vitamin K supplementation were carried out.

The results are given in table 5. These data show that while no hemorrhages occurred in growing rats receiving non-irradiated beef with or without oral supplementation of vitamin K, 9 of 14 rats receiving irradiated beef without added vitamin K died in 4 to 8 weeks, and that oral administration of vitamin K_3 (as the sodium salt of the phosphate ester of menadione) completely prevented deaths due to hemorrhage in rats receiving irradiated beef. One rat on the irradiated beef diet plus vitamin K did develop anemia after 35 days possibly due to hemorrhage and was cured by giving 50 μg of vitamin K-sodium-phosphate salt intraperitonially. These results indicated that a dietary requirement for vitamin K became necessary when rats were fed irradiated beef.

A second experiment was undertaken to determine the lowest level of vitamin K that would prevent hemorrhage in rats receiving irradiated beef. The data (table 5) clearly show that as low as 4 μg of vitamin K_3 per day administered orally can effectively protect the rat.

Another experiment (table 6) has demonstrated that 3 μg of water-soluble or 3 μg of fat-soluble vitamin K_3 were equally able to completely prevent hemorrhage and restore the plasma prothrombin times to normal in rats consuming irradiated beef. These data indicate that there is nothing in the irradiated beef which severely inhibits the absorption of dietary vitamin K.

SUMMARY AND CONCLUSIONS

Consumption of diets containing gamma-ray irradiated (2.79 or 5.58 × 10^6 rad) beef resulted in internal hemorrhages and prolonged prothrombin times in growing male rats. Generally the female rat did not show this syndrome. The lesion was induced by freshly irradiated beef as well as irradiated beef which has been stored for over 6 months at room temperature.

Supplementation with vitamin K prevented the hemorrhagic diathesis in rats consuming irradiated beef.

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The Site of Action of Lactose in the Enhancement of Calcium Utilization

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Although the inclusion of lactose in a diet has long been recognized as a means of improving the utilization of calcium, the mechanism by which this is accomplished has remained obscure. Fournier ('55a) and Dupuis and Fournier ('58), in attacking this problem, have presented evidence that the action is more than just fermentation of lactose with the subsequent beneficial acidification of the intestinal contents. Some of the sugars that had the greatest effects on calcium metabolism were those which were attacked with difficulty by intestinal bacteria. The sugars which improved calcium utilization included lactose, D-galactose, D- and L-xylose, D- and L-arabinose, mannose, melibiose, and raffinose. These were designated by Fournier ('54, '55b) as "structural carbohydrates" and considered to act by participating in some manner with the bone cell.

In investigations by Lengemann, Wasserman and Comar ('59) lactose was shown to increase calcium absorption from the intestine within 30 minutes after administration; this leads one to doubt that the primary action of lactose is on the bone cell and suggests that it acts in the intestine. The experiments presented in this paper were initiated to test the suggestion that lactose acts primarily on the bone cell.

METHODS

The male and female albino rats used in these studies were of Yale-Wistar origin and had been raised and maintained on a commercial pelleted dog food.[2] For most of the experiments the animals were fasted for 24 hours and then the test solutions were administered while the animals were under light ether anesthesia. After an additional 24-hour fast, the rats were sacrificed and their femurs removed. The radioisotope content of the femurs, as employed by Wasserman, Comar and Nold ('56) was used as an index for the extent of absorption.

When the radioisotope administered was a gamma emitter (Ba^{133}, Sr^{85}, Mg^{28}, and Ra^{226}) the fresh bones were counted in a deep well scintillation counter. When Ca^{45} was the nuclide the bones were ashed, dissolved in 2 N HCl and brought to 50 ml volume. Suitable aliquots were used for precipitation of the calcium as the oxalate; the precipitates were then transferred to counting cups, as described by Comar ('55), and assayed under a Geiger-Mueller tube. The results were expressed as the percentage of the administered dose contained in two femurs.

Further details will be provided in the body of the results.

RESULTS

It was of interest to first determine if lactose in the body proper could influence the passage of calcium from the intestinal tract. For this purpose female rats received oral doses of 0.13 mmole of Ca^*Cl_2 and oral or intraperitoneal administration of 1 mmole of lactose. In addition, a group received the Ca^*Cl_2 intra-abdominally and was contrasted with rats receiving both lactose and Ca^*Cl_2 in this manner. The data of this experiment (table 1) demonstrated that lactose placed in the intestinal tract had a remarkable ability to stimulate calcium absorption ($P \angle 0.01$) but when placed in the abdominal cavity had no effect. The animals that received the Ca^*Cl_2 intraperitoneally showed that lac-

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[2] Purina.

tose had no significant ability to influence the utilization of calcium once the calcium had gained access to the body.

Since it was possible that a lactose effect upon bone might require a period of time before becoming noticeable, an experiment was devised in which rats were given daily injections of lactose for two weeks before receiving the test dose of Ca*. The male rats used for this purpose weighed from 160 to 200 gm at the start and were divided into three groups of 5 rats each. Each rat received a daily intraperitoneal dose of 5 ml of either isotonic saline, glucose or lactose. The sugar solutions were made so that each rat received 2 mmole of sugar per day. Two weeks later the animals were given the test dose of Ca* after having been fasted for 24 hours; however, the daily injection routine was continued.

The "Uptake" experiment of table 2 shows that long term predosing with lactose had no statistically significant effect upon the absorption of calcium from the intestine. The "Removal" experiment was carried out in the same manner as that for uptake except that the rats received the Ca^*Cl_2 24 hours prior to the start of the daily injections. Again, no statistically significant differences existed between groups and so it can be concluded that the lactose within the body is without marked effect upon the absorption of calcium or its removal from the skeleton.

TABLE 1

The effect of the site of administration upon the lactose enhancement of calcium absorption[1]

Solutions administered		Femur uptake	Relative index
Oral	Intraperitoneal		
		% of dose	
Ca^*Cl_2	—	3.34 ± 0.41	100
Ca^*Cl_2 + lactose	—	5.80 ± 0.23	174
Ca^*Cl_2	Lactose	3.39 ± 0.45	100
—	Ca^*Cl_2	7.24 ± 0.24	216
—	Ca^*Cl_2 + lactose	7.34 ± 0.14	229

[1] Values represent mean ± standard error of the mean. Six female rats, weighing 110 to 150 gm, composed a group. Dosing solutions contained 0.13 mmole Ca and 1 mmole lactose, respectively.

TABLE 2

The effect of long-time intraperitoneal administration of saline, glucose and lactose solutions upon the uptake of calcium from the intestinal tract and upon its removal from bone[1]

Experiment	Per cent of dose in femur		
	Saline	Glucose	Lactose
Uptake	2.69 ± 0.14	2.69 ± 0.09	2.70 ± 0.48
Removal	7.22 ± 0.15	6.85 ± 0.06	7.08 ± 0.13

[1] Five male rats per group; weight at start: from 160 to 200 gm. Each rat received daily intraperitoneal injections of isotonic saline, or 2 mmole of a glucose solution or 2 mmole of a lactose solution for a two-week period. The "Uptake" rats received an oral test dose of 0.13 mmole of Ca^*Cl_2 at the end of this period while the "Removal" rats received this dose intraperitoneally 24 hours before the start of the daily injections.

TABLE 3

The absorption of Sr^{85} when lactose was present in the same or adjacent ileal segments[1]

Group	Site of administration	Femur Sr^{85} uptake	Relative index
$CaCl_2$ only	Single segment	1.63 ± 0.27	100
$CaCl_2$; lactose	Adjacent segments	1.65 ± 0.41	101
$CaCl_2$ + lactose	Same segment	3.76 ± 0.73	230

[1] Mean ± standard error of the mean. Six male rats, weighing 120 to 150 gm, composed a group. One half milliliter of dosing solution, containing 0.065 mmole of Ca or 0.25 mmole lactose or both, was injected into the ileal segments as indicated above. The animals were killed 4 hours after dosing.

In support of this, embryonic chick bone, cultured in a medium containing 0, 10^{-4}, 10^{-3}, or 10^{-2} M of lactose, showed no differences in Ca* uptake after 24 hours and 7 days of culture.

The injection of lactose into the abdominal cavity did not eliminate the possibility that metabolic products of lactose, possibly produced in the lumen or the walls of the intestine, could affect the metabolism of the bone cell. Therefore, an experiment was performed (table 3) in which 0.065 mmole of labeled calcium was placed in a ligated segment of the ileum of the rat and 0.25 mmole of lactose was placed in an adjacent portion. Control animals received only labeled calcium or a mixture of lactose and the radioactive calcium chloride. To facilitate the experiment Sr^{85} was used to label the calcium; however, the results could be expected to be quite similar to those for Ca^{45} (Lengemann, Wasserman and Comar, '59). As seen from table 3, lactose was effective ($P < 0.01$) only when it and the calcium were present in the same segment, tending to rule out the possibility that a metabolite was involved.

Since a theoretical metabolite might require time to condition the bone cell, male rats were fed for two weeks on the stock ration to which had been added either 10% (by weight) of glucose or lactose. The animals were then fasted and given oral doses of the test solutions indicated in table 4. As can be seen, prolonged feeding of lactose had no effect upon the absorption of calcium from the intestine when glucose was included in the test solution. This, in conjunction with the data of table 3, made it unlikely that a metabolite of lactose was affecting the metabolism of the bone cell. It is of interest that the response to lactose by rats prefed on glucose was equivalent to that of those receiving lactose in their ration. This indicated that the development of a flora capable of attacking lactose was not essential for the effect and that the enhancement of calcium absorption by lactose was capable of

TABLE 4

Prefeeding of glucose and lactose and their effects upon calcium absorption when glucose or lactose was contained in the test dose[1]

Carbohydrate prefed	Carbohydrate in test dose	Femur Ca^{45}	Relative index
		% of dose	
Glucose	Glucose	3.19 ± 0.09	100
Glucose	Lactose	6.07 ± 0.29	190
Lactose	Glucose	3.12 ± 0.20	97
Lactose	Lactose	6.81 ± 0.39	215

[1] Six male rats per group; average weight; 165 gm. Prior to dosing the rats were fed the stock ration to which had been added either 10% glucose or lactose. The dosing solutions contained 0.13 mmole $Ca^{*}Cl_2$ and 1 mmole of glucose or lactose.

TABLE 5

The effect of lactose and lysine upon the absorption of some alkaline earth metals

Alkaline earth metal	Alkaline earth metal in dose	Control	Lactose	Lysine
	mg	*% of dose in femurs*	*% of dose in femurs*	*% of dose in femurs*
Mg^{28}	2.6	3.35 ± 0.16	4.51 ± 0.14	3.78 ± 0.18
Ca^{45}	4.0	3.87 ± 0.24	6.87 ± 0.34	5.74 ± 0.15
Sr^{85}	2.0	3.29 ± 0.14	6.82 ± 0.19	6.54 ± 0.16
Ba^{133}	0.9	2.66 ± 0.29	8.52 ± 0.75	10.34 ± 0.52
Ra^{226}	0.08	1.67 ± 0.09	10.49 ± 0.29	10.62 ± 0.63

[1] Six female rats per group; weight ranged from 100 to 150 gm. The dosing solutions contained the indicated milligrams of the alkaline earth as the chloride, except that radium was in the form of the bromide. One millimole of lactose or lysine was included in the test dose when indicated.

being sustained over at least a two-week period.

The enhancing effect of lactose was not limited solely to calcium but was general for the members of group IIa of the periodic table (table 5). The increase was significant for all ($P < 0.05$). Lysine also enhances calcium absorption (Wasserman, Comar and Nold, '56), and so it was of interest to contrast the effect of lysine with that of lactose. Lysine acted in a similar manner to lactose except that it did not enhance the absorption of magnesium. This may indicate that though both lactose and lysine can increase the absorption of calcium their modes of action may differ.

DISCUSSION

In attempting to explain the mechanism of action of lactose and other sugars upon calcium absorption, Fournier ('54) suggested the existence of a group of sugars that exert a beneficial effect upon ossification and named these "structural carbohydrates." It was implied that these sugars or some metabolite act at some point within the body and result in an increasing utilization of calcium. The purpose of the experiments reported here was to test this concept. The results have demonstrated that lactose per se in the tissues of the body had no effect upon the absorption of calcium from the intestine or the distribution of absorbed calcium. Allowing lactose a period of two weeks to prime a possible mechanism also failed to produce favorable evidence.

The possibility that an intermediate of lactose metabolism was the functional material to act on the bone cell had previously been challenged by finding that glucose and galactose could not reproduce the effect of lactose, and that a favorable influence on calcium absorption could be detected as early as 30 minutes after the administration of lactose (Lengemann, Wasserman and Comar, '59). The data of table 3 reinforced this argument against a metabolite by showing that the lactose had to be in the same ileal segment as the calcium to be effective. In addition, glycine, an intermediate mentioned by Fournier ('55b), and calcium salts of acetate, lactate, and glucuronate have been shown to be ineffective in promoting calcium absorption (Wasserman, Comar and Nold, '56; Lengemann, Wasserman and Comar, '59).

Fournier ('55a) and Dupuis and Fournier ('58) dispelled the possibility that the intestinal bacteria might be involved in the production of intermediates by noting that some of the "structural carbohydrates" are attacked with difficulty, and that feeding of aureomycin failed to stop the lactose effect. Lengemann, Wasserman and Comar ('59) found that neither feeding of sulfadiazine or injection of neomycin into the intestine could stop the response to lactose. This also removed acidification of the digestive tract as a factor, as did the fact that lactose did not reduce the pH of the digestive tract of rats fed a meat diet (Robinson and Duncan, '31) but still improved calcium absorption.

In all, the data of this paper suggest that lactose acts in the digestive tract and not at some site within the body. The mechanism is still unknown.

SUMMARY

1. Single or multiple intraperitoneal injections of lactose failed to stimulate the absorption of calcium from the intestine of the rat.

2. Daily intraperitoneal doses of lactose, given for a period of two weeks, failed to influence the mobilization of labeled calcium from the skeleton of the male rats.

3. Calcium absorption from the ileum could be stimulated only if calcium and lactose were together in the same segment; if in adjacent segments no effect was observed.

4. Rats fed for two weeks on a diet containing 10% of lactose did not absorb a greater proportion of a test dose of Ca^*Cl_2 than did rats fed a diet containing 10% of glucose.

5. The enhancing effect of lactose was not limited to calcium but included magnesium, strontium, barium and radium. Lysine resembled lactose but failed to stimulate the absorption of magnesium.

6. It was concluded that the site of action of lactose is in the intestine and not within the body.

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Effect of a Physiological Cation-Anion Imbalance on the Growth and Mineral Nutrition of Rabbits

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Keener and Thacker ('58) have reported that calves and rabbits suffered poor growth, anemia, inappetence and enlarged thyroids when fed a timothy hay grown on heavily fertilized Paxton loam soils. The land producing this forage was limed and seeded in 1950 and fertilized each subsequent year with a fertilizer mixture containing 7% nitrogen, 7% phosphorus and 7% potassium at a rate of 600 lbs. per acre in April and 400 lbs. per acre after the first crop of the season was removed. Their first assumption was that the timothy hay was deficient in iodine and one or more organic factors. However, in the continuation of these investigations it became evident that the deficiency in the hay was related to its mineral composition. The studies to be reported demonstrate that the rabbit responds to mineral supplements of a particular nature that suggests that when a diet is fed containing timothy hay from an area in New Hampshire a physiological cation-anion imbalance is created that has implications in the metabolism of calcium, potassium, sodium and magnesium by the rabbit.

EXPERIMENTAL

Dutch belted rabbits of mixed sexes, 4 to 5 weeks of age, were used. When the rabbits were two weeks of age, the stock colony ration was replaced by a basic experimental ration. The dams were removed daily for supplemental feeding of the stock ration (alfalfa hay and wheat and oat grain). At three weeks of age, the young were weaned, caged individually and fed, preexperimentally, for one to two weeks on the basic experimental ration. The experimental rabbits were selected on the basis of an adequate food consumption in the preliminary period and divided into groups according to weight and sex. The size and number of groups was determined by the number of diets to be examined and the replications desired. The individuals of a weight-sex group were randomly assigned to diets and to cage space. Water and feed were available to the rabbit at all times. Food consumption was recorded daily and body weights weekly.

After preliminary studies had suggested that the growth response obtained with a salt mixture supplement might not be related to a specific mineral deficiency, the effect of supplementing a basal diet with calcium, potassium, magnesium and phosphorus salts in all possible combinations was studied in the first experiment (table 1). The basal and preexperimental diet was composed of timothy 50%, soybean protein[1] 20%, dextrose[2] 21.5%, sodium chloride (iodized) 0.5%, hydrogenated vegetable oil[3] 8.0%, plus the fat soluble and water-soluble vitamins.[4] By analysis, this diet contained 0.65% K, 0.25% Ca and 0.1% Mg. All supplements were added at the expense of the dextrose. The dietary levels and salts selected for the mineral supplements were: calcium, 0.45% as calcium citrate, potassium, 0.7% as

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[1] ADM Assay Protein, Archer-Daniels-Midland Co., Cincinnati 41, Ohio.

[2] Cerelose, Corn Products Refining Co.

[3] Primex, Procter and Gamble, Cincinnati, Ohio.

[4] The vitamins were supplied in milligrams per 100 grams of diet: thiamine·HCl 0.7, riboflavin 1, calcium pantothenate 2.5, pyridoxine 0.7, niacin 20, inositol 10, p-aminobenzoic acid 10, folic acid 0.1, biotin 0.05, choline 100, orotic acid 1, thioctic acid 0.1, vitamin A 666, calciferol 0.01, α-tocopherol 7, menadione 0.075 and cobalamin 5 μg.

potassium acetate, magnesium, 0.06% as magnesium oxide, phosphorus, 0.27% as sodium dihydrogen phosphate. The concentration of the mineral elements in this supplement are comparable to their concentration in the Hawk-Oser salt mixture ('31). A treatment was included for comparative purposes in which the basal diet was supplemented with 4% of the Hawk-Oser salt mixture. The 17 treatments were examined in a randomized block designed in 8 replications.

All surviving rabbits were autopsied and the left tibia and fibula removed, dried at 100°C, extracted with di-ethyl ether and dried for bone ash determinations. Cardiac and skeletal muscle, and kidney tissues from moribund animals sacrificed in the course of the experiment and from selected animals autopsied at the completion of the first experiment were examined histologically.

The second experiment was designed to study the supplementary value of comparable milliequivalent levels (22.5 meq./100 gm diet at the expense of dextrose) of the acetate, bicarbonate (or carbonate), chloride or sulfate salts of sodium, potassium, calcium or magnesium. The basal diet was similar to that used in the first experiment except for the addition, at the expense of dextrose, of the following mineral supplement (mg/100 gm diet): $MgSO_4$, 148; MgO, 50; $Ca(H_2PO_4)_2 \cdot H_2O$, 755; $CaSO_4 \cdot H_2O$, 755; K_2SO_4, 900; and a trace mineral mixture. This supplement raised the mineral composition of the basal diet to approximately: potassium 1.0%, calcium 0.6%, magnesium 0.1% and phosphorus 0.48%. The trace mineral supplement furnished in p.p.m.: copper 10, iron 100, manganese 30, zinc 50, molybdenum 2, cobalt 1 and selenium 1. The 16 salts were examined in a randomized block design with 6 replications. A basal and a 4% Hawk-Oser salt mixture supplemented diet with 12 rabbits on each treatment were included in the randomization.

RESULTS

The results of the first experiment are shown in table 1. Potassium and magnesium salts substantially improved growth and survival of the rabbits although the

TABLE 1

Response of the rabbit to the factorial supplementation of a timothy diet with salts of phosphorus, magnesium, potassium and calcium

Experiment 1

Additions to basal diet	Initial weight	Gain		Gain/Feed	Bone ash[1]
		3 Weeks	6 Weeks		
	gm	*gm/day*			%
None[2]	272	2.6(7)	2.0(2)	—	50.8(1)
0.27% P[3]	282	3.0(7)	3.0(2)	—	57.0(2)
0.06% Mg	278	13.8	10.6	0.27	57.4
Mg + P	282	12.1	10.9	0.27	62.0
0.7% K	277	14.1	12.7	0.27	58.5
K + P	279	12.3	12.9	0.30	61.4
K + Mg	271	15.8	13.2	0.28	60.8
K + Mg + P	272	10.6	11.4	0.28	60.5(7)
0.45% Ca	274	17.7	17.1	0.32	64.5
Ca + P	281	20.6	18.7	0.31	67.7
Ca + Mg	277	18.2	18.0	0.32	66.4
Ca + K	264	18.0	18.2	0.31	66.5
Ca + Mg + P	279	18.1	17.4	0.30	68.4
Ca + K + P	270	21.2	19.2	0.32	68.1
Ca + Mg + K	279	18.9	18.0	0.31	66.6
Ca + Mg + K + P	278	18.6	17.9	0.32	67.3
4% Hawk-Oser minerals	271	19.9	18.3	0.31	66.9
Standard error[4]		±1.14	±0.98	±0.010	±0.64

[1] Dry fat-free bone.

[2] Eight animals on each treatment, survivors indicated within parentheses.

[3] Fed as $NaH_2PO_4 \cdot H_2O$, Mg as MgO, K as $K\,C_2H_3O_2$, Ca as $Ca_3(C_6H_5O_7)_2 \cdot 4H_2O$.

[4] Standard errors were derived from analysis of variance.

growth response was not as great as that observed in the presence of the calcium salt. Supplements of the potassium salt generally resulted in a greater gain than did those of magnesium but the differences were not of sufficient magnitude to be statistically significant. The growth and survival of the rabbits receiving the supplement of sodium dihydrogen phosphate were not different from those of the animals fed the basal diet. In terms of body gain, additive effects or interactions among the mineral supplements were absent. However, the second order interactions were significant when the bone ash data were considered. Each of the mineral supplements alone increased bone ash with calcium effecting the greatest increase over that of the basal diet. Any combination of minerals increased the bone ash level further and if calcium was present in the combination, the level was comparable to that found when the diet was supplemented with the Hawk-Oser salt mixture.

The rabbits fed the basal and phosphorus-supplemented diets exhibited definite hindquarter paralysis and extremely thin bones as well as a decided growth failure. The presence of normal reflexes and repeated recovery and relapse of the paralytic condition suggested that it was of muscular origin. Histologically, the skeletal muscle appeared to be edematous, showed areas of muscle nuclei proliferation and cellular infiltration. This syndrome is reminiscent of potassium deficiency in the rabbit, although not as severe as that described by Hove and Herndon ('55). These authors also mentioned the fragile bones in their potassium-deficient animals. They concluded that the rabbit has a high requirement for potassium. Their diets contained salts with only inorganic anions.

In the absence of additive effects, in relation to growth, of the several minerals in combination, a simple deficiency of calcium, potassium and magnesium did not appear to be an adequate explanation of the observed results. The response sug-

TABLE 2

Response of the rabbit to supplementation of a timothy diet with organic or inorganic salts of sodium, potassium, calcium or magnesium

Experiment 2

Additions to basal diet	Initial body weight	Daily gain	Gain / feed	Hemoglobin/ 100 ml blood	Urinary pH	Bone ash[1]
	gm	*gm*		*gm*		*%*
None	321	2.6(9)[2]	0.12	7.8	5.1	49.1
$NaC_2H_3O_2$[3]	322	17.9	0.32	11.4	6.2	59.3
$NaHCO_3$	299	18.7	0.31	11.3	6.5	59.6
NaCl	322	2.7(5)	0.12	8.3	5.2	51.0
Na_2SO_4	339	4.0(4)	0.17	8.2	5.1	47.1
$KC_2H_3O_2$	330	18.6	0.32	11.3	6.4	59.0
$KHCO_3$	304	18.3	0.32	10.6	6.7	59.1
KCl	317	3.0(5)	0.12	7.5	5.0	51.3
K_2SO_4	338	2.3(4)	0.10	7.5	5.3	49.0
$Ca(C_2H_3O_2)_2$	329	20.1	0.32	10.9	5.4	59.5
$CaCO_3$	312	20.9	0.33	11.0	5.1	59.8
$CaCl_2$	300	— (0)	—	—	—	—
$CaSO_4$	324	— (0)	—	—	—	—
$Mg(C_2H_3O_2)_2$	323	16.3	0.32	11.3	6.2	57.4
$MgCO_3$	323	16.8	0.32	11.4	5.9	58.6
$MgCl_2$	322	1.1(3)	0.05	7.8	5.2	45.9
$MgSO_4$	310	1.3	0.07	7.8	5.0	50.4
4% Hawk-Oser salts	315	16.2	0.29	10.8	5.1	59.6
Standard error		±0.75[4]	±0.013[4]	±0.35	±0.12	±0.37[4]

[1] Dry, fat-free bone.

[2] Six rabbits on each treatment with 12 animals on the two control rations. Number of survivors shown within parentheses. Five-week growth period.

[3] All salts added at a level to furnish 22.5 meq. per 100 gm diet.

[4] Standard error associated with response of rabbits fed $C_2H_3O_2^-$, HCO_3 or $CO_3^=$ salts.

gested that a non-specific or sparing action of the salts used was involved with the possibility that an absolute calcium deficiency existed to account for the greatter response in the presence of calcium supplements. The mineral supplements appeared to be effective only when the salts carried an anion capable of being oxidized to CO_2 and H_2O by the animal body.

In the second experiment the mean daily gain and ratio of gain to feed consumed (table 2) were calculated from a 5-week growth period. The blood hemoglobin concentration was determined in the 4th week and the urinary pH values were measured on a 24-hour sample collected in the third or 4th experimental week. The acetate, bicarbonate or carbonate salts of sodium, potassium or calcium or the Hawk-Oser salt mixture when added to the basal diet at comparable milliequivalent levels gave a remarkably similar response in body gain, efficiency of feed utilization, blood hemoglobin levels and bone ash levels. When these cations were fed as the chloride or sulfate salts, no improvement was noted in performance over that shown by the rabbits fed the basal diet. The chloride and sulfate salts of calcium were the only ones that increased the toxicity of the basal diet.

The rabbits fed the diets supplemented by calcium acetate or carbonate or the Hawk-Oser salt mixture excreted urine at pH values as low as did those animals fed the basal or the diets supplemented with the chloride and sulfate salts.

The bone ash values obtained in the second experiment were consistently lower than those found in the first experiment. An explanation for this change in level of bone mineralization is not readily apparent. The basal diet of the second experiment was supplemented with trace and macro minerals and the animals were two to three weeks younger at the time of sacrifice than they were in the first experiment. The bone ash levels of the rabbits fed the basal diet and those diets supplemented with the chloride and sulfate salts were consistently lower than those fed the acetate or bicarbonate or carbonate salts. No statistically significant differences were evident within the two classes of salts, those carrying chloride or sulfate anions and those with anions metabolizable to CO_2 and H_2O by the animal body.

DISCUSSION

These data demonstrate that a timothy hay type of ration which does not support growth, normal hemoglobin or bone ash levels was rendered nutritionally adequate in terms of these criteria when supplemented with a salt of sodium, potassium, calcium or magnesium carrying an anion capable of being oxidized to CO_2 and H_2O by the animal body. These results support the hypothesis that in an effort to satisfy the chemical and physiological requirement that an ion of one charge be concomitantly metabolized with an ion of the opposite charge, the rabbit, under some dietary conditions, is forced to draw on its body stores of certain mineral cations to excrete the physiological excess of anions. Morgen and Beger ('15) found that sodium carbonate increased the mineral content of rabbit bones whereas sodium chloride was ineffective. These authors suggested that the carbonate salt acted to increase the alkaline reserve. By inference, this hypothesis suggests that calcium, potassium, magnesium and sodium deficiencies could be induced in the presence of what otherwise would be adequate dietary levels of these elements by the manipulation of their level and cation-anion balance of the ration.

Wooley ('54) and Wooley and Mickelsen ('54) reported that potassium and sodium bicarbonate, potassium acetate or calcium carbonate were satisfactory replacements for supplements to kale in purified diets for the rabbit. These authors also indicated that these salts overcame an apparent toxicity associated with the level of casein in their purified diets. We have noted with the timothy type of basal diet that mortality increased as the source of the protein component was changed from wheat gluten to soybean protein or to casein. A high-phosphorus protein such as soybean protein or casein in the diet (the phosphate of the protein mainly balanced by an organic cation) would increase the stress on the physiological cation-anion balance of the ani-

mal. This increased stress would explain the marked deterioration in the condition of the rabbit with the above changes in level or source of dietary protein.

SUMMARY

The failure of rabbits to grow, maintain normal blood hemoglobin and bone ash levels when fed a basal diet containing a timothy hay grown at a particular location in New Hampshire was corrected when this diet was supplemented with salts of sodium, potassium, calcium or magnesium carrying an anion metabolized to CO_2 and H_2O by the animal body. Salts of these elements carrying a chloride or sulfate anion fed at the same milliequivalent level were ineffective. It is suggested that under the dietary conditions of this experiment the rabbit suffers a physiological cation-anion imbalance or an acidosis, and that this condition is interrelated with the mineral metabolism of the animal. It was shown that this mineral imbalance induces calcium and potassium deficiencies in the presence of apparent adequate dietary levels of these elements, and it is further suggested that this interrelationship might involve the metabolism of other cations.

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The Effect of Ascorbic Acid in the Diet of Adult Chickens on Calcium Utilization by the Progeny[1]

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The use of ascorbic acid in the ration of the domestic chicken has been studied only occasionally since it is universally known and accepted that this species can synthesize this vitamin. In the current study it was noted, however, that the presence of vitamin C in the parents' diet had a rather striking effect on the skeletal retention and blood levels of Ca^{45} in progeny given a rachitogenic diet.

The exact mode of action or influence of vitamin D on the prevention of rickets has been a controversial subject for many years. It has been established experimentally by many workers as reviewed by Nicolaysen and Eeg-Larsen ('53), that vitamin D is necessary for intestinal absorption of calcium or phosphorus or both, thereby preventing the development of rickets. It was also brought out in this review that others have demonstrated the necessity of vitamin D for normal bone deposition. It follows, then, that vitamin D is necessary for normal bone development and apparently performs this function by either increasing calcium and phosphorus absorption or by promoting the metabolism of these minerals after absorption. It is also probable that vitamin D may be functional at both sites.

The role of ascorbic acid in calcium metabolism has also been studied somewhat in animal species other than the chicken and appears to have some influence. For example, Lanford ('39) found that the addition of orange juice to the diet of rats increased calcium retention. Henry and Kon ('39) conversely, reported that the addition of 2 mg daily of ascorbic acid had no influence on the retention of calcium in rats. Ruskin ('38) and Ruskin and Jonnard ('38 a and b) as reported by Bourne ('56) held that there was an intimate association between ascorbic acid and calcium, believing that calcium was absorbed from the intestine as calcium ascorbate. Leichsenring et al. ('57) found that both orange juice and crystalline ascorbic acid increased calcium retention in women.

The relationship of these two vitamins as regards calcium absorption and metabolism has not been studied to the authors' knowledge. Since both of these vitamins have been shown to influence calcium utilization when studied separately it seems that such studies should be made.

EXPERIMENTAL

A New Hampshire-Delaware cross chick was used in the experiments described here. One-day-old chicks were placed in electrically heated batteries where the temperature was maintained at recommended levels. These batteries were located in a room where the temperature was kept at 74°F. with a uniform light-day of 14 hours. During the first three days the chicks were placed on ground milo and subsequently fed the rations (table 1) for the remaining 25 days. General management conditions recommended for growing chicks were maintained throughout the experiment.

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[2] The data reported herein were taken from a thesis submitted by C. W. Weber to the school of Graduate Studies, Colorado State University, in partial fulfillment of the requirements for the degree, Master of Science in Poultry Nutrition.

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TABLE 1
Composition of the deficient ration

Ingredient	Levels used
Ground milo	70.00
Soybean oil meal (50% protein)	25.00
Ground alfalfa meal (17% protein)	1.50
Ground limestone	0.50
Steamed bone meal	2.50
Salt, iodized	0.50
Manganese sulfate supplement (70% $MnSO_4$)	0.022
Vitamin mix[1,2]	0.113

[1] The vitamin mixture supplied 110,000 I.U. vitamin A, 75 mg riboflavin, 100 mg niacin, 200 mg calcium pantothenate, 40 gm aqueous choline chloride (70%) and 400 μg vitamin B_{12} per 100 pounds of ration.

[2] The control chicks were fed this same diet, but with the addition of 13,500 I.C.U. of vitamin D_3, per 100 pounds of ration.

At three days of age the chicks were divided in a random manner into two groups. The first group was given the vitamin D_3-deficient ration (table 1) and the second was given this same ration supplemented with a recommended level of vitamin D_3, (National Research Council, '54). The vitamin D_3-deficient ration was shown to produce rachitic symptoms readily, in approximately three weeks. Rachitic symptoms were determined visually by an ungainly walk, softness of the beak, unthrifty appearance and slow growth. At 4 weeks of age, the chicks were injected with 0.5 ml of a solution containing approximately 10 μc of Ca^{45} plus 15 mg of Evans Blue dye. The Ca^{45} was in the form of $CaCl_2$ in hydrochloric acid solution.

The injection procedure was carried out by making a small incision just over the crop, thereby exposing this organ, and then injecting the solution directly into the lumen. The birds were sacrificed by asphyxiation in an ether container at specific time intervals following injection. These time intervals were 15, 30, 45, and 60 minutes. Just prior to sacrifice a 1.8-ml blood sample was collected by cardiac puncture in a tube containing a 0.2% solution of sodium oxalate. After sacrifice, the esophagus, crop, proventriculus and ventriculus "upper bowel" were removed as a unit and prepared for Ca^{45} analysis by a method described later. For conciseness and clarity the above intestinal tract sections will be referred to as the "upper bowel." The division of the intestinal tract was made at this particular point since it is commonly accepted that intestinal absorption of most materials does not occur until such substances reach the duodenal loop area. It follows then, that the "upper bowel" in this case can be considered as the non-absorptive segment in relation to Ca^{45} while the "lower bowel" will be thought of as the absorptive area.

The tibiae were next removed and measurements of the epiphyseal-cartilaginous plates were made. The values were then averaged to give an estimate of the degree of vitamin D_3 deficiency. Each of these component parts was then ashed completely at 700°C. After ashing, the ash was taken up in a 25% nitric acid solution and transferred quantitatively to a volumetric flask and made to 100 ml volume. After allowing time for equilibration, a 0.5 ml sample was removed and placed in a small glass planchet. The solution was then dried under a heat lamp. The Ca^{45} activity of these samples was then determined in a gas flow, windowless type scaler.[4] Suitable calculations for self-absorption were made on all samples.

Ten chicks were used for each control and experimental group with an equal distribution between males and females in each group whenever possible. Statistical differences were determined by calculating the standard error and applying the "t" test to such data.

RESULTS AND DISCUSSION

In studying the absorption of materials from the intestinal tract, it seems important that the rate of feed movement to the absorptive area should also be determined. In this study it was observed that the disappearance of Ca^{45} from the "upper bowel" in the control chicks occurred at an exponential rate (fig. 1). The vitamin D_3-deficient group showed this same rate to 45 minutes after Ca^{45} injection. The 60-minute group showed a very low disappearance value. Since the latter value was approximately equal to the 30-minute control and deficient groups, however, it does

[4] Nuclear Measurements Corporation, Indianapolis, Indiana.

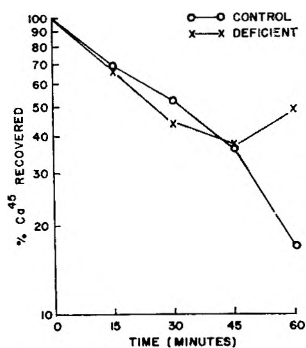

Fig. 1 The recovery of calcium[45] from the upper digestive tract. The term upper digestive tract, as used here, applies to the crop, esophagus, proventriculus and ventriculus.

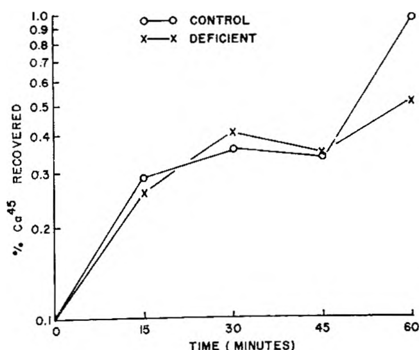

Fig. 2 The recovery of calcium[45] from the blood.

not seem probable that the vitamin D_3 deficiency was instrumental in producing this result.

An estimate of intestinal absorption of Ca^{45} is indicated by the amount of isotope recovered from the blood as well as that deposited in the skeleton. It will be noted (fig. 2) that 15 minutes after injection, Ca^{45} blood levels had gone up sharply in both deficient and control chicks. This may be explained by the rapid movement of the isotope from the upper to the lower bowel during this time. Following this initial uptake of Ca^{45}, the level of this isotope in the blood remained in a rather uniform state for the next 30 minutes.

Sixty minutes after injection, blood Ca^{45} levels of both groups again increased. These blood curves are quite different from those appearing in the literature. For example, Migicovsky and Jamieson ('55) and Keane et al. ('56) showed that specific Ca^{45} blood activity reached a maximum in control chicks approximately 10 minutes after injection, followed by a progressive decline. In the two cases cited, deficient chicks required a much longer period of time to attain a maximal blood Ca^{45} activity (30 to 90 minutes) which also declined thereafter and in each instance the specific activity of the control was much greater than that of the deficient chicks.

The amount of Ca^{45} deposited in the tibiae (fig. 3) showed variable differences in the control and deficient groups to 45 minutes post injection. The wide difference exhibited at 60 minutes may be explained by the failure of the deficient group to send sufficient Ca^{45} from the upper to the lower bowel (fig. 1). This statement is supported by the result of a correlation analysis made between the amount of Ca^{45} leaving the upper bowel and that recovered from the tibiae. The values obtained for the control and deficient groups were 0.979 and 0.941 respectively, which were significant to the one per cent level of probability. It appeared, from these results, that the degree of Ca^{45} accumulation in the tibiae was dependent upon the amount presented to the intestinal absorptive area with the vitamin D_3 deficiency apparently exerting no influence.

Since the above results are quite different from those found in the literature cited, it appeared that some factor was present which was producing these unusual results. Evidence that the deficient

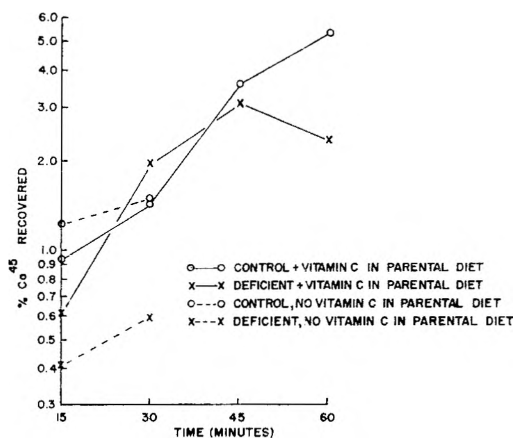

Fig. 3 The recovery of calcium[45] from the tibiae.

chicks were rachitic is shown by the data in table 2. In all cases the control chicks were heavier to a highly significant degree. Furthermore, measurements of the epiphyseal-cartilaginous plates of the tibiae differed markedly between the control and deficient groups. Visual observations of the chicks also indicated strongly that the deficient chicks showed rachitic symptoms whereas the controls showed none.

In reviewing some data from a previous experiment it was noted that the deposition of Ca^{45} in the tibiae of chicks (fig. 3) was quite similar to the values of Migicovsky and Jamieson ('55) and Keane et al. ('56). The chicks used in both studies in this laboratory were progeny of a similar strain. The diet used for the two experiments was also identical, except that ascorbic acid had been added to the parental diet in the last experiment. The vitamin C additions were made at 10, 20, and 40 mg per pound of ration. In view of these differences it appeared that the presence of ascorbic acid in the parents' diet could be influencing the Ca^{45} uptake and retention in progeny given the rachitogenic diet.

The apparent difference in the data of the two experiments was further illustrated by determining the correlation coefficient between the amount of Ca^{45} leaving the upper bowel and that recovered from the tibiae. The values obtained, when the parents were not given dietary vitamin C, were −0.196 and 0.685 for the vitamin D_3-deficient and control chicks respectively. The value for the control chicks was of a highly significant nature. It was shown previously in this study that this relationship was highly correlated in both control and deficient progeny from ascorbic acid supplemented parents.

In view of the above results, a small pilot study was initiated to test further the influence of parental dietary ascorbic acid on rachitic progeny. In this study, eggs were hatched from the control hens and from hens receiving a supplement of 10, 20, or 40 mg of ascorbic acid. All other procedures were similar to those previously described.

The uptake of Ca^{45} in the tibiae was increased statistically, to a highly significant degree in vitamin D_3-deficient females when the parents were given supplementary ascorbic acid (table 3). It was also observed that the width of the epiphyseal plate was increased slightly in the female

TABLE 2

The effect of vitamin D_3 deficiency on the body weight and tibiae epiphyseal plate width of the chick

Treatment[1]	Mean body weight	Epiphyseal plate width
	gm	*mm*
15 minute control	244 ± 8.0[2]	
15 minute deficient	148 ± 8.9[5]	4.29 ± 0.13
15 minute control[3,4]	365 ± 17.6	1.04 ± 0.01
15 minute deficient[3,4]	233 ± 14.0[5]	3.31 ± 0.32[5]
30 minute control	257 ± 4.5	0.96 ± 0.03
30 minute deficient	128 ± 9.8[5]	3.32 ± 0.38[5]
30 minute control[3,4]	338 ± 15.6	0.99 ± 0.01
30 minute deficient[3,4]	209 ± 4.4[5]	3.39 ± 0.52[5]
45 minute control	265 ± 11.7	0.86 ± 0.03
45 minute deficient	139 ± 9.9[5]	3.50 ± 0.56[5]
60 minute control	251 ± 14.4	1.00 ± 0.01
60 minute deficient	121 ± 9.3[5]	4.29 ± 0.35[5]

[1] Values refer to time of sacrifice after Ca^{45} injection.

[2] Standard error.

[3] No ascorbic acid present in the parents' diet, all other progeny were produced by parents given supplementary ascorbic acid.

[4] The heavier average body weights of these groups were due to the chicks being older at the time of weight determination.

[5] Significantly different at the 1.0% level of probability.

TABLE 3

The effect of ascorbic acid in the diet of the parents on calcium metabolism by vitamin D_3-deficient progeny

Parental diet	No. of chicks	Tibia epiphyseal plate width	Tibia Ca^{45} uptake
		mm	*cpm/100 gm body wt.*
Female:			
Basal	7	1.96 ± 0.67[1]	55.3 ± 4.8
Basal + 10 mg[2]	7	2.10 ± 0.87	76.4 ± 2.8[3]
Basal + 20 mg	7	2.10 ± 0.81	85.6 ± 3.4[3]
Basal + 40 mg	7	2.20 ± 0.86	83.0 ± 2.3[3]
Male:			
Basal	7	2.19 ± 0.25	
Basal + 10 mg	7	3.03 ± 0.54	
Basal + 20 mg	6	3.65 ± 0.42[3]	
Basal + 40 mg	6	3.28 ± 0.92	

[1] Mean ± standard error.
[2] Refers to level of ascorbic acid per pound of ration in the parental diet.
[3] Significant at 1.0% level of probability when compared to the basal group.

groups by this treatment. In the males, however, Ca^{45} determinations were not made but the epiphyseal plate width was increased noticeably at all three levels of parental ascorbic acid supplementation (table 3).

To show further the effects of ascorbic acid, certain correlation calculations were made concerning the relationship of bone development and the chick's ability to utilize Ca^{45} as measured by Ca^{45} content in the tibiae (table 4). In the case of the progeny from the vitamin C-supplemented adults, there was no relationship between epiphyseal plate width, tibiae ash weight and the amount of Ca^{45} utilized whereas these factors were highly related in progeny of parents not given vitamin C.

These results further indicated that the presence of vitamin C in the parents' diet has an influence on calcium uptake in rachitic progeny. In addition, the results suggested that this uptake effect had little influence in preventing the appearance of rachitic symptoms. In fact, the last study indicated that the presence of vitamin C in the parents' diet may have increased the severity of rickets despite the birds' ability to deposit more Ca^{45} in the bone.

In light of the data observed in this study, several suggestions may be made. First, it appears that the birds' ability to absorb calcium will not necessarily prevent the appearance of rachitic symptoms. Secondly, these results indicated that vitamin D_3 does not function primarily for promoting intestinal absorption of calcium. Thirdly, it was shown that ascorbic acid or some agent produced by the presence of this vitamin in the parental diet acts on rachitic progeny to help maintain calcium absorption. Fourthly, it appeared that the presence of this vitamin in the parents' diet may increase the degree of rickets when the progeny are given vitamin D_3-deficient diets.

TABLE 4

The influence of parental ascorbic acid on bone development and Ca^{45} utilization by rachitic progeny

Correlation calculation	r Value
Tibiae epiphyseal plate width and Ca^{45} uptake:[1]	
non-supplemented parents[2]	−0.941[4]
supplemented parents[3]	−0.190
Tibiae ash weight and Ca^{45} uptake:	
non-supplemented parents	0.797[4]
supplemented parents	0.258
Tibiae ash weight and epiphyseal plate width:	
non-supplemented parents	0.939[4]
supplemented parents	0.482

[1] Ca^{45} uptake refers to that amount found in the combined tibiae.
[2] Parental diet contained no vitamin C.
[3] Parental diet contained vitamin C.
[4] Significant at the 1.0% level of probability.

SUMMARY

The influence of ascorbic acid in the parental diet on the subsequent utilization of Ca^{45} by rachitic and non-rachitic progeny has been studied using the chick.

The presence of vitamin C in the ration of the parents apparently changed the early Ca^{45} uptake by the progeny. The absence of vitamin D_3 in the diet of the progeny appeared to have no effect on these results. In control and vitamin D_3-deficient chicks produced from parents given dietary ascorbic acid, it was observed that the amount of Ca^{45} retained by the tibiae to 60 minutes post-injection was dependent on the amount of isotope presented to the absorptive area of the intestinal tract. This same relationship was observed in control progeny from non-ascorbic acid-supplemented parents; however, vitamin D_3-deficient progeny failed to show this relationship. It was also observed that both control and deficient chicks from the ascorbic acid-supplemented parents showed increasing blood levels of Ca^{45} as late as 60 minutes after injection. These results were widely different from those normally expected, suggesting that the presence of vitamin C in the maternal diet had an influence on the intestinal absorption of both control and vitamin D_3-deficient chicks.

The above results suggested that vitamin D_3 was not involved primarily with intestinal absorption. Furthermore, it was shown that even though parental dietary ascorbic acid promoted normal intestinal absorption of Ca^{45} during vitamin D_3 deficiency, appearance of rachitic symptoms was not alleviated. In short, it appeared that rachitic symptoms may be even more severe under such conditions.

In light of these observations, it seemed that calcium uptake alone, in this case, was not sufficient to prevent rickets. This leads to the suggestion that vitamin D_3 is necessary for normal bone development and that factors other than this vitamin can sustain normal calcium intestinal absorption.

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The Effects of Dietary Nitrate on Rabbits and Rats[1]

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The growing trend toward the use of fertilizers of high nitrogen content in the production of vegetables leads to the question of possible injurious effects upon those who consume the food thus grown; for under some conditions nitrogen in abundant supply in the soil may accumulate in the form of nitrate in the plant up to levels as high as 6.4% in Swiss chard and 11.1% (as KNO_3) in turnips (Gilbert et al., '46). Nitrate as such is a diuretic, but otherwise has no untoward effects in non-ruminant mammals. Varying amounts of it, however, are probably reduced to nitrite in the system, and this ion is known to exert a number of undesirable effects. Of these the conversion of some of the hemoglobin to methemoglobin is the most easily measured, and is probably potentially the most deleterious. In ruminants, toxic effects from this cause have sometimes proved fatal (Bradley et al., '40; Campbell et al., '54; Davidson et al., '41; Lindner et al., '48; Olson and Moxon, '42; Savage, '49). Effects of this kind in humans are much rarer, but methemoglobinemia and occasional deaths have been reported in infants ingesting well water high in nitrates (Walton, '51). No injury of any degree has been reported for non-ruminants attributable to nitrate in plant food. But as far as we have been able to discover no investigation of marginal toxicity has been published.

The work here reported is concerned primarily with levels of methemoglobin compared with levels of nitrate added to or naturally present in the food of laboratory rabbits and rats. Evidence for the compound responsible for the changes in the blood has been sought by means of nitrate balance studies.

Levels of methemoglobin compared with levels of sodium nitrate added to food

Rabbits and rats were chosen as test animals. There were 10 animals of each species in each test group. Rabbits were of mixed breed, half males, half females, and ranged in age from 11 to 15 months during this experiment. Their weight range was from 3150 to 3750 gm. Male rats were used and they ranged in age from 11 to 13 weeks, and in weight from 170 to 217 gm. They were housed in individual cages and all animals remained healthy throughout the experiments.

A double 5 by 5 interlaced Latin square design was used to minimize the effects due to animals and day sequences. In this design, day sequences were rows, animals were columns, nitrate levels were the dietary treatments, and in the rabbit experiments the time interval of bleeding would be represented by three sub-units in each square (see table 1). The rest period between the day sequences averaged three days since preliminary studies had indicated that a time interval of this duration was adequate in removing carry-over effects of previous treatment.

Trial determinations of methemoglobin were undertaken with rats and rabbits to determine the interval after feeding

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TABLE 1

Double, interlaced latin square design

	Animals									
	1	2	3	4	5	6	7	8	9	10
Sequence period	C	C	D	E	B	D	A	B	A	E
	E	A	A	B	D	C	E	D	C	B
	D	B	E	A	A	E	D	C	B	C
	B	D	C	D	E	B	C	A	E	A
	A	E	B	C	C	A	B	E	D	D

		Ration for rabbits consuming increasing levels of nitrate as sodium nitrate	$NaNO_3$ equivalent in grams intake per animal
I	A	90 gm oats	0.004
	B	90 gm Purina Rabbit Chow	0.074
	C	90 gm rabbit chow + 0.2 gm $NaNO_3$/kg body wt.	0.672 (av.)
	D	90 gm rabbit chow + 0.4 gm $NaNO_3$/kg body wt.	1.344 (av.)
	E	90 gm rabbit chow + 1 gm $NaNO_3$/kg body wt.	3.380 (av.)
		Rations for rats consuming increasing levels of nitrate as sodium nitrate	$NaNO_3$ equivalent in grams intake per animal
II	A	5 gm oats	0.0002
	B	5 gm Purina Laboratory Chow + 3.4 mg $NaNO_3$	0.004
	C	5 gm laboratory chow + 0.2 gm $NaNO_3$/kg body wt.	0.040 (av.)
	D	5 gm laboratory chow + 0.4 gm $NaNO_3$/kg body wt.	0.080 (av.)
	E	5 gm laboratory chow + 1 gm $NaNO_3$/kg body wt.	0.200 (av.)
		Rations for rabbits consuming increasing levels of nitrate as it occurs naturally in plants	$NaNO_3$ equivalent in grams intake per animal
III	A	90 gm oats	0.004
	B	90 gm Purina Rabbit Chow	0.074
	C	10 gm collards + 45 gm Purina Rabbit Chow	0.120 (av.)
	D	15 gm collards	0.124 (av.)
	E	25 gm collards	0.207 (av.)
		Rations for rats consuming increasing levels of nitrate as it occurs naturally in plants	$NaNO_3$ equivalent in grams intake per animal
IV	A	5 gm oats	0.0002
	B	0.06 gm greens + 0.01 gm egg	0.007
	C	0.5 gm greens + 0.08 gm egg	0.061
	D	1.1 gm greens + 0.18 gm egg	0.133
	E	2.7 gm greens + 0.45 gm egg	0.328

when highest levels would be found. Minimum methemoglobin levels were obtained in fasting animals while maximum methemoglobin readings were obtained in the rabbits between 4 and 7 hours after ingestion of the nitrate and the intervals of 4, 5.5, and 7 hours were therefore used. Only one bleeding time (5.5 hours after feeding) was used for the rats since closely spaced bleedings are not feasible in so small an animal. The method of Evelyn and Malloy ('38) was used for methemoglobin determinations.

Oats were used as one dietary treatment because they were lower in nitrate than the complete ration otherwise fed. The other rations are shown in table 1, sections I and II. The xylenol method of the AOAC as modified by U. S. Plant, Soil, and Nutrition Laboratory[3] ('53) was used for all nitrate determinations.

The data were analyzed for variance and the treatment means tested by Duncan's new Multiple Range Test ('55). The results are shown in table 2, lines 1 and 2. Of primary interest are the highly significant differences in treatment means. Although statistical significance did not accompany the rise in methemoglobin values at every step, the overall indication is

[3] Personal communication from C. J. Morris.

TABLE 2

Methemoglobin, in grams per 100 ml of blood, in animals consuming increasing levels of nitrate. Means of 10 animals. See table 1 for description of rations

	Rations in gm NO_3/kg				
	A 0.007	B 0.15	C 0.15	D 0.29	E[1] 0.73
1. Rabbits—$NaNO_3$ CV[2]—48	0.07	0.11	0.12	0.14	0.16
2. Rats—$NaNO_3$ CV—55	0.11	0.19	0.24	0.26	0.38
3. Rabbits—plant NO_3 CV—46	0.12	0.16	0.18	0.19	0.18
4. Rats—plant NO_3 CV—60	0.04	0.09	0.12	0.19	0.23

Values not underscored by the same line differ significantly from each other at the 1% level.

[1] Possibly due to the taste the rabbits would not always consume all of the ration containing 1 gm $NaNO_3$/kg body weight in a short period of time and the intake was slightly less than 1 gm $NaNO_3$/kg body weight. Also the C, D and E ration for rabbits on plant nitrate were not the same nitrate/body weight ratio as other C, D and E rations. They were limited by amount of collards the rabbit would eat, see table 1, section III.

[2] Coefficient of variability.

of a significant effect on methemoglobin levels of increase in nitrate in the diet.

To determine whether levels of methemoglobin of this order, or associated effects, could be harmful would require more intensive physiological measurements. Gross discomfort as evidenced by abnormal behavior was not apparent. None of the values in table 2 approached the level where cyanosis would appear in humans (Finch, '48) and no cyanosis was seen in the animals. The transitory nature of methemoglobin in the living animal in the absence of a continuing stimulus (Gelinsky, '40; Neill, '25; Spicer, '50) mitigates against injury.

Levels of methemoglobin compared with amount of nitrate fed in plant food

In order to see whether nitrates as they occur in plants would cause a rise in methemoglobin levels of the same order as those accompanying the $NaNO_3$ additions just reported, a similar experiment was performed using collards for rabbits and turnip greens for rats. The collards were grown with no special intent of producing a high nitrate content and contained 0.61% of nitrate (dry basis). The turnip greens were grown using cultural conditions known to cause nitrate accumulation and contained 8.85% of nitrate (dry basis).

The methods and design used were the same as those described in the previous experiment. The same rabbits were used after a rest period. The ages ranged from 14 to 18 months and weights from 3150 to 3800 gm. The rats ranged in age from 39 to 43 weeks and in weight from 300 to 340 gm.

The results given in table 2, lines 3 and 4, are similar to those reported in lines 1 and 2 in that highly significant rises in methemoglobin levels accompanied certain ranges of rise in nitrate consumed. The generally higher methemoglobin levels of the rabbits in this study as compared with the previous one, including the higher level on the oat ration, may have been an effect of temperature. We obtained higher methemoglobin levels regularly during hot weather. Differences in temperature between the time of the two rat experiments were not marked and the levels of methemoglobin in the second experiment were lower than in the first one.

Mindful of the relation of methemoglobin to total hemoglobin and the fact that nitrates are known to have a diuretic ef-

fect on humans and animals, hematocrits and methemoglobin were determined simultaneously on rats being fed varied rations. The correlation coefficient was not significant [$r = 0.16$, $r^2 = 0.025$]. Less than 3% of the change in methemoglobin can be accounted for by the change in hematocrit.

Highest levels of methemoglobin found

Since the levels of the methemoglobin reported in the foregoing experiments were not manifestly injurious, an effort was made to discover how high the methemoglobin levels could be caused to go, for this is the crux of our interest in this problem. Some individual animals would eat nitrate more readily than others, and for the purposes of this determination these animals were fed as much sodium nitrate as they would consume. The highest level of methemoglobin recorded under these circumstances was 2.49 gm per 100 ml of blood. This occurred in a rat which was eating nitrate at the rate of 10 gm per kilogram of body weight. A high level of methemoglobin was also obtained in one rabbit owing to a peculiarity which caused its levels to rise above the levels found in other rabbits on the same regimen. This occurred during the preliminary studies and by the time the experiment proper was conducted this animal did not show this difference. During the time when the methemoglobin levels were higher than those of the other rabbits a methemoglobin value of 3.2 gm per 100 ml was obtained with the animal on a ration containing $NaNO_3$ at a level of 3 gm per kilogram of body weight. Some cyanosis was evident in the albino rat at the time of the high methemoglobin. No such observation could be made on the rabbit because of its natural pigment. Methemoglobin levels of the order just cited represent reduction in oxygen carrying hemoglobin of approximately 20%. In non-anemic animals not subject to undue exertion this is probably not serious but in animals already anemic the effects could well be critical.

Balance studies

The foregoing results showing rise in methemoglobin with rise in ingestion of nitrates pose the problem of the mechanism of the reaction. Since methemoglobin formation is known to result from the presence of nitrites in the circulation, the assumption has been made throughout the literature that the toxic effects of nitrate manifested by cyanosis are due to the reduction of nitrate to nitrite ions. Tracer studies involving some analyses on blood and tissue would be the best means of obtaining evidence bearing on this problem. Lacking facilities for such studies, we turned to balance studies to supply some evidence.

Only rabbits were used in the balance studies. The urinary excretion of nitrate at various intake levels was measured. Percentages recovered are given in table 3 and agree in general with the findings of Greene and Hiatt ('54).

TABLE 3

Urinary nitrate recovery in rabbits in 24 hours (10 rabbits, 4 24-hour periods)

Supplement to 90 gm of Purina Chow	Recovery in 24 hours
	%
None (Av. equivalent 22 mg $NaNO_3$/kg body wt.)	49.2
22 mg $NaNO_3$/kg body wt.	35.3
44 mg $NaNO_3$/kg body wt.	35.4
66 mg $NaNO_3$/kg body wt.	41.1
132 mg $NaNO_3$/kg body wt.	48.4
198 mg $NaNO_3$/kg body wt.	56.1

Total nitrogen in the feces was determined at two levels of nitrate intake (protein intake being constant), in order to rule out the possibility of nitrate being excreted in the feces.

In an effort to answer the question, "Is any nitrite excreted, and if so how much?," a modification of the alpha-naphthylamine method of the AOAC was used (Dawson, '56) for the determination of nitrite in the urine of 5 rabbits collected for 4 24-hour periods, while the daily ingestion of each rabbit was 90 gm of commercial chow[4] with 200 mg of $NaNO_3$. Nitrates were determined also. Then after a rest period the same 5 rabbits were fed 90 gm of oats for 9 days and nitrites and nitrates were determined in the urine for the last 7 days of this 9 day period. The results of these analyses are shown in table 4.

[4] Purina Rabbit Chow.

TABLE 4

Urinary nitrate and nitrite recovery in rabbits. Mean values of several consecutive 24-hour collections (5 animals per group)

Ration	Mean total nitrate fed per rabbit	Mean total urinary nitrate per rabbit	Mean total urinary nitrite per rabbit	Recovery[1]
	mg	*mg*	*mg*	%
90 gm oats	21	9	0.10	44
90 gm Purina Rabbit Chow + 200 mg $NaNO_3$/kg body wt.	2023	774	7.61	39

[1] The percentage recovery was calculated by adding the total urinary nitrate excreted to the total nitrate equivalent of the urinary nitrite and dividing by the total nitrate fed. In each case the nitrate equivalent of the nitrite in the urine represents 0.5% of the amount recovered.

The chow and nitrate solution were analyzed for nitrite and this small amount of nitrite in the ration was subtracted from the amount of nitrite excreted in urine to obtain the urinary nitrite figures in the table. These results agree with those in table 3 and with those reported by Kameoka and Morimoto ('53), who found that when nitrate was fed to rabbits as 2% of their diet it was completely absorbed but only about 50% excreted. These workers also found an increase in the amount of nitrite in the urine where nitrate in the food was increased. This increase in nitrite excretion following increased nitrate ingestion supports the hypothesis of some conversion of nitrate to nitrite.

SUMMARY

Methemoglobin rises significantly in the circulation of rabbits and rats with increases in the levels of nitrate ingested either as sodium nitrate or as it occurs naturally in plant food.

The levels to which methemoglobin rises are not high enough to cause abnormal behavior or appearance except in occasional animals temporarily aberrant due to unexplained causes, or in a larger number of animals ingesting amounts of nitrate greater than would likely occur under natural conditions.

The fact that large amounts of nitrate ingested cannot be recovered in that form in the excreta permits the assumption that some of it is reduced in the body. The recovery of small amounts of nitrite in the urine substantiates this assumption and provides evidence for its existence in the blood where it could act on the hemoglobin. Some nitrate remains unaccounted for and this raises the question of its fate and the reaction in the system of any other products which might be formed.

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The Effect of Radiation Sterilization on the Nutritive Value of Foods

V. ON THE AMINO ACID COMPOSITION OF MILK AND BEEF

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Earlier work on the effect of irradiation on the destruction of amino acids in proteins has been reviewed previously (Johnson, '57).

The purpose of the present paper is to report the effect of irradiation on the amino acid composition of milk and beef proteins.

EXPERIMENTAL

Protein hydrolysates of non-irradiated beef and milk, 2.8 million rad irradiated beef and milk, 5.6 million rad irradiated beef, and 9.3 million rad irradiated milk have been analyzed by the column chromatographic method of Moore and Stein ('48, '51).

Preparation of samples

Evaporated milk was bought locally, frozen, and stored at −20°C. Three cans of this milk were irradiated with 2.8 million rad γ-ray irradiation;[1] three, with 9.3 million rad; and three were not irradiated. The milk was held frozen before and after irradiation.

Beef round steak was purchased from the University Meats Division. After the visible fat was trimmed off, the steak was ground, canned under vacuum, and frozen. Some cans were irradiated at 2.8 million rad, some at 5.6 million rad, and some were not irradiated.

The milk, after irradiation, was coagulated, making it difficult to obtain representative samples for assay. Therefore, both non-irradiated and irradiated milk proteins were precipitated with 10% trichloracetic acid; the precipitate was filtered, washed with ethanol, acetone, and ether, dried at 40°C under vacuum and stored in a vacuum desiccator at 4°C. The filtrates showed no protein by biuret or ninhydrin tests. Nitrogen recovery was from 98 to 99%.

Both non-irradiated and irradiated beef were extracted with hot ether for 48 hours and dried to constant weight in a drying oven at 50°C under 29 inches of vacuum. The samples were then ground to a fine powder and stored in a vacuum desiccator at 4°C.

Hydrolysis of samples

Both milk and beef samples were placed in sealed tubes and hydrolyzed in 500 volumes of 6 N HCl for 24 hours in a 110°C oven. The hydrolysates were concentrated at 45°C under reduced pressure until nearly dry. Ten milliliters of water were added and the samples reconcentrated to near dryness. They were then made up to volume with pH 3.42 buffer.

The determination of cystine and tryptophan

Although the cystine peak appeared in each determination, it was low, spread-out, and irregular, with at least one dent on the top, and greatly variable. Consequently cystine was determined as cysteic acid. Cystine and cysteine were converted to cysteic acid by performic acid oxidation by the method of Schram et al. ('54). According to previous determinations, the hydrolysates contained no other strongly acidic ninhydrin-positive constituents, and cysteic acid was determined on the Dowex-50, 100-

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[1] 1 rad = 100 ergs energy dissipated per gram of tissue. Gamma irradiation was used to "sterilize" the foods.

TABLE 1
The effect of irradiation on amino acids of milk
(Calculated as grams per 100 gm of crude protein, N × 6.25)

Amino acid	Irradiation dosage in million rad[4]		
	0	2.8	9.3
Aspartic acid	7.05 ± 0.09[1]	6.46 ± 0.07[1]	5.24 ± 0.06[1]
Threonine	4.04 ± 0.11	3.54 ± 0.13	3.33 ± 0.08
Serine	5.14 ± 0.12	3.71 ± 0.16	3.71 ± 0.07
Glutamic acid	21.01 ± 0.24	21.00 ± 0.23	10.67 ± 0.16
Proline	9.01 ± 0.15	9.02 ± 0.08	9.00 ± 0.07
Glycine	2.15 ± 0.06	2.14 ± 0.05	1.63 ± 0.03
Alanine	3.51 ± 0.04	3.72 ± 0.07	3.61 ± 0.06
Cystine—cysteine[3]	0.93 ± 0.02	0.92 ± 0.04	0.89 ± 0.05
Valine	5.21 ± 0.02	5.30 ± 0.03	5.26 ± 0.03
Methionine	2.66 ± 0.06	2.06 ± 0.05	1.90 ± 0.06
Isoleucine	5.20 ± 0.03	4.71 ± 0.04	3.59 ± 0.02
Leucine	9.44 ± 0.04	9.30 ± 0.05	8.22 ± 0.03
Tyrosine	4.17 ± 0.03	4.20 ± 0.02	4.21 ± 0.03
Phenylalanine	4.48 ± 0.04	4.54 ± 0.06	3.98 ± 0.05
Tryptophan[3]	1.02 ± 0.03	1.06 ± 0.02	0.98 ± 0.05
Histidine	2.64 ± 0.06	2.64 ± 0.05	1.85 ± 0.05
Lysine	7.30 ± 0.09	7.21 ± 0.06	6.94 ± 0.07
Arginine	3.23 ± 0.08	3.22 ± 0.12	3.03 ± 0.10
Ammonia	1.76 ± 0.14	1.75 ± 0.16	1.94 ± 0.20
Total	99.95	96.54	80.11

[1] Standard error.
[2] Cystine and cysteine were determined as cysteic acid.
[3] Tryptophan was determined microbiologically.
[4] Four determinations.

cm column. It emerged as a sharp peak from fraction 22 to 25 at 37.5°C and pH 3.42. Standardization indicated that 0.5 mg of cystine yielded 0.4 mg of cysteic acid from the column. Therefore, the quantities of cysteic acid measured in the hydrolysates have routinely been divided by 0.8 to give the final percentage of cystine in the hydrolysate.

Tryptophan was determined microbiologically by the Miller and Ruttinger method ('50).

RESULTS

The effect of irradiation on amino acid composition of milk

Statistical analysis of the data tabulated in table 1 indicated the following: (a) at the 1% level, aspartic acid, serine, methionine and isoleucine in the 2.8 million rad-irradiated milk were significantly lower than in non-irradiated milk; (b) at the 1% level, aspartic acid, glutamic acid, glycine, methionine, isoleucine, leucine, phenylalanine and histidine in the 9.3 million rad irradiated milk were significantly lower than in the 2.8 million rad irradiated milk.

The effect of irradiation on amino acid composition of beef

Statistical analysis of the data in table 2 indicated the following (a) at the 1% level, aspartic acid, threonine, serine, glutamic acid, glycine, methionine and arginine in the 2.8 million rad irradiated beef were significantly lower than in the non-irradiated beef; (b) at the 1% level, aspartic acid, threonine, serine, glutamic acid, glycine, methionine, histidine and lysine in the 5.6 million rad irradiated beef were significantly lower than in 2.8 million rad irradiated beef; (c) the order of destruction of the amino acids in beef was glutamic acid → serine → aspartic acid → threonine → glycine → lysine → methionine → arginine → histidine → proline; (d) although the average effect of irradiation on proline content was not significant, the linear functional relationship (Snedecor, '50) between the level of irradiation and proline content of beef was significant at the 1% level.

TABLE 2

The effect of irradiation on amino acids of beef

(Calculated as grams per 100 grams of crude protein, $N \times 6.25$)

Amino acid	Irradiation dosage in million rad[4]		
	0	2.8	5.6
Aspartic acid	8.23 ± 0.08[1]	6.94 ± 0.06[1]	4.08 ± 0.06[1]
Threonine	4.56 ± 0.10	3.61 ± 0.09	2.84 ± 0.05
Serine	4.42 ± 0.07	2.75 ± 0.06	1.10 ± 0.05
Glutamic acid	15.07 ± 0.13	8.15 ± 0.12	3.38 ± 0.06
Proline	4.79 ± 0.11	4.67 ± 0.07	4.40 ± 0.08
Glycine	6.49 ± 0.09	5.80 ± 0.07	5.09 ± 0.07
Alanine	6.22 ± 0.08	5.90 ± 0.09	6.24 ± 0.06
Cystine—cysteine[2]	1.25 ± 0.04	1.20 ± 0.03	1.18 ± 0.03
Valine	4.51 ± 0.05	4.43 ± 0.06	4.44 ± 0.03
Methionine	2.93 ± 0.06	2.45 ± 0.02	2.16 ± 0.04
Isoleucine	4.58 ± 0.07	4.44 ± 0.04	4.57 ± 0.02
Leucine	7.98 ± 0.10	7.77 ± 0.03	7.53 ± 0.09
Tyrosine	3.37 ± 0.09	3.31 ± 0.08	3.26 ± 0.02
Phenylalanine	4.50 ± 0.08	4.57 ± 0.01	4.14 ± 0.03
Tryptophan[3]	1.32 ± 0.01	1.29 ± 0.03	1.30 ± 0.02
Histidine	2.46 ± 0.03	2.37 ± 0.06	1.98 ± 0.05
Lysine	8.73 ± 0.11	8.46 ± 0.08	6.88 ± 0.10
Arginine	6.90 ± 0.09	5.59 ± 0.08	5.36 ± 0.12
Ammonia	1.40 ± 0.21	1.32 ± 0.19	1.71 ± 0.18
Total	99.71	85.02	71.64

[1] Standard error.

[2] Cystine and cysteine were determined as cysteic acid.

[3] Tryptophan was determined microbiologically.

[4] Three determinations.

DISCUSSION

Irradiation effects on sulfur amino acids

The determination of cystine in terms of cysteic acid gives no information as to how much cystine-cysteine in the protein was rendered useless for nutritional purposes by oxidation to cysteic acid or some intermediate oxidation products. Metta and Johnson ('56) previously shown that the biological value of milk proteins was reduced by 8% upon irradiation at 2.8 million rad. Later the same authors demonstrated that additional cystine restored the biological value of irradiated milk proteins. Thus, some such destruction of cystine must have occurred during irradiation. In addition, some lowering of methionine was found in this study which might also account for the effect of added cystine.

Proctor and Bhatia ('53) found that the order of deamination of the amino acids in aqueous solution, upon irradiation with a cathode ray dose of 250,000 rep was histidine → cystine → phenylalanine → tyrosine → tryptophan. In this study no significant changes of histidine, tyrosine and phenylalanine were found upon irradiation of 2.8 million rad in milk or beef proteins. The susceptibility to damage may depend on the "exposed position" of the amino acid (Bellamy and Lawton, '54). Glutamic acid, aspartic acid, serine and glycine were most seriously damaged in both beef and milk upon irradiation. Fortunately they are non-essential amino acids for animals and man.

SUMMARY

The effect of irradiation at 2.8 million, 5.6 million, and 9.3 million rad levels on the amino acid composition of milk and beef was studied.

It was found that glutamic acid, aspartic acid, serine and glycine were most seriously reduced by irradiation in both milk and beef.

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Nitrogen Balance of Young Adults Consuming a Deficient Diet Supplemented with Torula Yeast and Other Nitrogenous Products[1]

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The nutritive value of torula yeast protein has been extensively studied in this laboratory using different biological procedures (Goyco and Asenjo, '46, '47, '48a, '49, '54; Goyco, '56). The main limitation of this protein appears to be its low content of physiologically available methionine. This finding is in agreement with observations made by Klose and Fevold, ('44); Carter and Phillips, ('44) and Harris, Hajny and Johnson, ('51).

Our studies, so far, had been performed using the albino rat as the experimental subject. Considering that findings made in the rat are not necessarily transferable to other species, particularly man, studies using human subjects became necessary in view of the fact that our ultimate objective is to determine the value of torula yeast protein in human nutrition. One of the main research interests of this department has been the search for a readily available, nutritionally acceptable, low-price protein source to improve the Puerto Rican dietary.

This paper reports the effects of supplementary torula yeast and torula yeast plus methionine on the nitrogen balance of normal young male Puerto Ricans consuming a diet typical in low-income groups. Also the effect of various other supplements was investigated.

EXPERIMENTAL

Subjects. In this investigation healthy young adult male volunteers served as experimental subjects. These were carefully selected from a population of about 400 delinquents in a governmental jail.[3] They ranged from 18 to 24 years of age, from 163 to 168 cm in height, and from 56.8 to 65.9 kg in weight. A thorough medical examination[4] was made upon a group of about 30 selected subjects and only those found to be physically fitted for the experiment were used. In each trial, 4 subjects were used. They were maintained in a metabolic ward under close supervision day and night. Normal physical activity was permitted. Radio, television and several table games were furnished for their entertainment when restricted to the metabolic ward. Excellent cooperation was obtained from these individuals.

Composition of diets. The basal diet used was representative of the food consumed by the low income group of the Puerto Rican population. It was compounded according to the recommendations made by the Bureau of Nutrition and Dietetics of the Department of Health of the Government of Puerto Rico. The items included in this diet were determined from

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[4] The author wishes to thank Dr. Ramón M. Suárez, Director of the Fundación de Investigaciones Clínicas, FIC, Santurce, Puerto Rico, for his invaluable help in determining the physical fitness of all the subjects. Dr. Suárez also did hematological and chemical determinations on blood and urine samples collected at the beginning and at the end of several of the trials. All values were found to be within the normal range.

survey studies carried out previously by different governmental agencies (Descartes, Díaz Pacheco and Nogueras, '41; Roberts and Stefani, '49). The composition of the basal diet was as follows: (grams per day): rice (polished, enriched), 181; red kidney beans, 47; dry codfish, 30; white sweet potato, 100; plantain (green), 50; banana (green), 50; corn meal, 24; tanier, 50; sugar, 100; lard, 48; salad oil, 35; tomato sauce, 25; sweet pepper, 10; onion, 10. Garlic vinegar and table salt were used as condiments. A cup of hot black coffee was included with each meal; besides, 50 ml of strained orange juice was also given, this serving as the vehicle for the supplements.

The meals were prepared by an experienced graduate dietitian. Three different menus virtually identical in composition were developed and used throughout. They were alternated successively to provide a constant intake in proteins and calories and at the same time variation in taste and appearance. Five individual portions were prepared daily from carefully weighed ingredients; 4 of these to be consumed by the subjects and one for chemical analyses.

The calculated average caloric requirement for the type of subject used was from 2400 to 2700 or from 40 to 42 Cal. per kilogram of body weight. The basal diet as compounded will not supply this amount, therefore, additional calories were administered in the form of sugar-cane molasses and a flavored cornstarch dessert. The calculated daily total caloric intake for all the subjects was 2800 Cal. No difficulties were encountered for the complete consumption of every meal. Body weights were recorded daily before breakfast, and all subjects either maintained or gained weight slightly during the experimental periods suggesting that the caloric content of the diet was adequate.

The reduced protein diet used during the depletion periods contained the same ingredients as the basal diet, but the amount of rice, red kidney beans and codfish was reduced to half. Adequate adjustment for calories was made by increasing items such as sugar, lard, molasses, etc.

To reduce, as much as possible, variation in composition all food items that were not perishable were purchased in bulk lots and after mixing were stored in a cooled environment. Perishable food items were purchased in amounts sufficient for each trial.

Balance periods. The nitrogen balance experiment consisted of three different successive periods:

1. A two-day preliminary period during which the subjects continued to consume the institutional diet, but 24-hour urine collections were made. Also, a representative sample of the three meals served and consumed during these two days was collected for analysis. The purpose of this preliminary period was to obtain information as to the amount of nitrogen intake and urinary nitrogen excretion immediately before the subjects were started on the nitrogen metabolism experiment.
2. A 4-day metabolic depletion period during which the reduced protein diet was consumed and complete 24-hour urine and stool collections were made.
3. An 8-day metabolic experimental period during which the basal diet, with or without supplement was consumed. A daily portion of the supplement was equally distributed during the three meals. At mealtime, the dietitian carefully transferred the portion into the orange juice and required the subjects to consume it completely. During several trials, as indicated in table 2, one multi-vitamin capsule[5] was given daily. Complete 24-hour urine and stool collections were made.

PREPARATION OF SAMPLES AND METHODS OF ANALYSIS

Diets. The sample representing the total daily intake by the subjects was quantitatively transferred to a large, weighed

[5] Perfolin capsules was a gift of the American Cyanamid Co., Lederle Laboratory Division, Pearl River, N. Y., through Dr. Fernando Otati, Director of Clinical Research. The inclusion of this multi-vitamin capsule in some trials only, was done on purpose. We wanted to see the over-all effect, on the nitrogen balance, of torula alone and also the effect, if any, of this additional vitamin supplementation. In those trials where the nitrogenous supplement did not supply vitamins a capsule was given to compensate for the vitamins in torula.

In trials (nos. 2–7; 3–8 and 6–11) where only half of the subjects received the vitamin capsule, the difference in nitrogen balance between the two groups was found not significant statistically.

blendor cup. After homogenization aliquot samples were placed in covered glass jars and kept frozen until analyzed. Total solids and total nitrogen determinations were made.

Stools. All samples of stools collected were kept frozen until combined into a composite for a given period. With few exceptions, the proper separation of stools from the different periods by the use of 0.6 gm of carmine was very satisfactory. The frozen pooled stools were allowed to thaw and then quantitatively transferred to a weighed blandor cup for homogenization. Aliquot samples were taken in covered glass jars and kept until analyzed. Total solids and total nitrogen determinations were made.

Urine. Daily 24-hour urine samples were measured and diluted to appropriate volumes with distilled water, and analyzed for their total nitrogen and creatinine content.

Analyses. Total nitrogen was determined in diets, stools and urine, according to the Kjeldahl procedure (A.O.A.C., '55). Duplicate homogenized samples for nitrogen determinations in stools and in food were weighed in specially prepared covered weighing bottles. The weighing bottle together with the accurately weighed samples was put into the digestion flask. This procedure proved to be very accurate and convenient for working with homogenized samples.

Creatinine was determined by the method of Clark and Thompson ('49).

Total solid determinations in diets and stool samples were made by standard official methods. Samples were dried at 110°C until constant weight was recorded.

RESULTS AND DISCUSSION

Preliminary period

The nitrogen balance for each group of subjects during the two-day preliminary period was calculated. The value for nitrogen output in the stool was estimated from the average nitrogen excretion in the stool, found by analysis for each group during the various experimental periods in which they participated. All subjects were in positive nitrogen balance, the average value was +2.73 ± 0.45 gm[6] of nitrogen per day. The average nitrogen intake during these preliminary periods was 12.75 ± 0.44 gm nitrogen per day. These individuals were excellent experimental subjects for this type of balance studies, considering that they had been in confinement, at this jail, for at least one year prior to our studies and, therefore, had been subject to a uniform and rather constant nutritional regime for a substantial period of time. The influence of previous nutritional status of subjects used in balance studies upon the results obtained has been indicated by several authors (Allison, Anderson and Seeley, '46; Hegsted, Trulson, White and White, '55).

Depletion period

In table 1 are summarized the average nitrogen balances for each metabolic depletion period. During these 4 days the subjects were partially depleted of protein, a condition obviously essential for studies upon biological value determinations using the refeeding technique. The degree of negative nitrogen balance achieved in each individual subject did not vary greatly from the average value of all the depletion trials, −2.79 ± 0.14 gm. The fluctuations observed are similar to those reported by Rose, ('49), and by Swendseid, Williams and Dunn, ('56).

Experimental periods

Table 2 summarizes data on the average nitrogen balances for each experimental metabolic period. The average daily output of nitrogen in the urine for the last 4 days of the experimental period was used in computing the nitrogen balance. A constant daily fecal nitrogen output by each subject during each period was assumed. Daily nitrogen balance was calculated for each individual using the daily urinary nitrogen values and the average values for nitrogen intake and fecal nitrogen, but for brevity, only average values for each experimental metabolic period are presented.

The criterion used in evaluating the shifts in nitrogen balance produced by the different supplements given after each depletion period was the one proposed by

[6] Standard error of the mean.

TABLE 1
Nitrogen metabolism of subjects on depletion diet

Trial no.	Number of subjects	Total nitrogen (Gm in 24 hours)					Balance
		Intake	Output				
			Urine	Stool	Total		
1	4	2.90	4.88	1.38	6.26		−3.36
2–7	8	3.02	4.58	1.27	5.85		−2.83
3–8	8	3.09	4.69	1.16	5.85		−2.76
4	4	3.25	4.74	1.05	5.79		−2.54
6–11	8	3.21	4.79	1.12	5.91		−2.70
13	5	3.19	5.48	1.17	6.65		−3.46
10	3	3.29	4.99	1.31	6.30		−3.01
12	4	3.38	4.44	0.81	5.25		−1.87
5	4	3.00	4.89	1.01	5.90		−2.90
9	4	3.29	4.77	0.94	5.71		−2.42
Average:		3.14 ± 0.05[1]	4.80 ± 0.08	1.13 ± 0.04	5.93 ± 0.14		−2.79 ± 0.14

[1] Standard error of the mean.

Leverton et al. ('56): "Nitrogen equilibrium is the zone in which the difference between the intake and the excretion does not exceed ±5% , i.e., the excretion is within 95 to 105% of the intake." Therefore, a negative balance, or nitrogen loss from the body, was considered to exist whenever the excretion was more than 105% of the intake. Conversely, a positive balance or nitrogen retention existed whenever the excretion was less than 95% of the intake.

The statistical significance of changes in N balances was obtained from the daily N balance data for each subject during the last 4 days of the experimental periods. Standard errors for all periods were calculated using the conventional method. The results of the "t" test and the p values obtained using Fisher's table are given in table 3.

Nitrogen balance on basal diet

In trial 1, the basal diet without any supplement was investigated. The amount of nitrogen consumed was low, 4.61 gm of nitrogen per day. With the exception of the nitrogen supplied by the small amount of dry codfish, all the rest was of vegetable origin. Three of the 4 subjects showed a slightly negative nitrogen balance and one a slightly positive balance, the average value being on the negative side but falling within the "zone" of equilibrium.

The performance of these subjects upon the basal diet is of interest. It would appear that the protein in the diet supplied all the essential amino acids in adequate amounts for the maintenance of nitrogen equilibrium and in one case, even more than the required amount, allowing for a slight storage of nitrogen.

Effect on nitrogen balance of a supplement of torula yeast[7]

During trials 2 and 7 a supplement of 15 gm of torula yeast was given. This amount of yeast added approximately 1 gm of nitrogen to the basal diet. The effect of this supplement on the nitrogen balance is of considerable interest. Five subjects showed a positive nitrogen balance that represents definite nitrogen storage. The other three subjects, although in positive nitrogen balance, fell within the "zone" of equilibrium. The average nitrogen balance of this group represented a slight but significant nitrogen storage, +0.58 ± 0.08 gm (table 3).

The effect of increasing the torula yeast supplement to 30 gm per day was investigated during trial 10. Four subjects were started in this trial but one had to withdraw because of sickness. This subject, after being in the experimental period for two days, had a mild diarrhea and some vomiting. It appeared that he could not tolerate the supplement. Therefore,

[7] The torula yeast used was supplied by the laboratories of the Puerto Rico Economic Development Co. at Hato Rey, Puerto Rico, under the direction of Mr. Carlos Vincenty.

TABLE 2

Nitrogen metabolism of subjects on experimental diets

Trial	Supplement	Number of Subjects	Total nitrogen (Gm in 24 hours)					$\frac{\text{N-output}}{\text{N-intake}} \times 100$
			Intake	Output			Balance	
				Urine	Stool	Total		
1	None	4	4.61 ± 0.14[1]	3.37 ± 0.14	1.40 ± 0.12	4.77 ± 0.10	−0.16 ± 0.10	103 ± 2.17
2–7[2]	Torula yeast (15 gm)	8	6.06 ± 0.09	3.85 ± 0.09	1.63 ± 0.11	5.48 ± 0.08	+0.58 ± 0.08	90 ± 1.43
3–8[2]	Skimmed milk	8	6.06 ± 0.10	3.45 ± 0.11	1.47 ± 0.10	4.92 ± 0.10	+1.14 ± 0.10	81 ± 1.77
4	Glycine	4	5.88 ± 0.09	4.66 ± 0.14	1.37 ± 0.08	6.03 ± 0.14	−0.15 ± 0.14	103 ± 2.47
10[2]	Torula yeast (30 gm)	3	7.55 ± 0.12	4.49 ± 0.24	1.99 ± 0.08	6.48 ± 0.24	+1.07 ± 0.24	85 ± 3.22
12[2]	Brewers' yeast	4	6.31 ± 0.09	4.10 ± 0.19	1.97 ± 0.17	6.07 ± 0.20	+0.24 ± 0.20	96 ± 3.17
5	Rice and beans	4	5.61 ± 0.08	3.75 ± 0.11	1.24 ± 0.12	4.99 ± 0.14	+0.62 ± 0.14	88 ± 2.92
9[2]	Rice and beans + DL-methionine	4	6.32 ± 0.09	3.71 ± 0.17	1.62 ± 0.09	5.33 ± 0.16	+0.99 ± 0.16	84 ± 2.68
6–11[2]	Torula yeast + DL-methionine	8	6.43 ± 0.06	3.98 ± 0.11	1.62 ± 0.07	5.60 ± 0.11	+0.83 ± 0.11	87 ± 1.72
13[2]	Torula yeast (30 gm) with 1 gm rice-beans nitrogen removed	5	6.33 ± 0.11	4.40 ± 0.13	1.55 ± 0.10	5.95 ± 0.14	+0.38 ± 0.14	94 ± 2.34

[1] Standard error of the mean. These standard errors were calculated using daily individual values in every case except for the stool N where only one value for each subject was available.

[2] Subjects in these trials received one multivitamin capsule daily.

TABLE 3

Test of significance of differences between nitrogen balances

Trial	Supplement	Mean difference[1]	N	Degrees of freedom	t	P
		gm N/day				%
2–7	Torula yeast (15 gm)	0.74	48	46	4.93	0.1
3–8	Skimmed milk	1.31	48	46	7.89	0.1
4	Glycine	0.02	32	30	0.11	90
6–11	Torula yeast plus DL-methionine	1.00	48	46	6.94	0.1
13	Torula yeast (30 gm) minus rice-beans	0.55	36	34	3.10	1.0–0.1
10	Torula yeast (30 gm)	1.24	28	26	6.66	0.1
12	Brewers' yeast	0.41	32	30	1.82	10–5
5	Rice and beans	0.79	32	30	4.59	0.1
9	Rice and beans plus DL-methionine	1.16	32	30	5.94	0.1

[1] Basal diet vs. supplement.

the values for only three subjects are reported.

The nitrogen intake during this trial was higher because of the extra 15 gm of torula yeast consumed. All three subjects showed a positive nitrogen balance with an average value of +1.07 ± 0.24 gm. This value is considerably higher than that obtained when 15 gm of torula were fed.

Although no true diarrhea was observed among the subjects that tolerated the higher level of torula yeast, all stool samples were of a semisolid consistency and several times stomach discomfort was reported. Besides, on questioning the subjects they all agreed that this higher level of torula yeast was disagreeable organoleptically. Whether the undesirable side effects observed when the torula supplement was increased from 15 to 30 gm are outweighed by the concomitant improvement recorded in nitrogen balance and storage, respectively, is difficult to evaluate at the present time and will require further investigation.

Effect on nitrogen balance of a supplement of torula yeast with the simultaneous removal of an equivalent amount (nitrogen) of rice and beans

In trial 13, 30 gm of torula yeast were given again to 5 new subjects. In this case, however, 1 gm of nitrogen in the form of rice and beans was removed from the basal diet and thus the nitrogen intake was maintained at the same level as that in the standard trials. The conditions established in this trial were, therefore, similar to those in trials 2 and 7, except for the substitution of 1 gm of nitrogen of rice and beans by the same amount of nitrogen in the form of torula yeast.

The results obtained appear to show that this substitution caused untoward effects on the nutritive value of the protein in the diet. The average value for the nitrogen balance was reduced from +0.58 ± 0.08 to +0.38 ± 0.14 gm per day and a corresponding reduction of the nitrogen stored was also produced.

If we compare the results obtained in this trial with those of trial 10, when 30 gm of torula yeast were fed as supplement, the difference is still greater. In the present case the untoward effects are more evident, thus indicating that although torula yeast could be a fairly good supplement to the basal diet protein, it is not a good substitute for the same.

Combined effect on nitrogen balance of a supplement of torula yeast and methionine

The effect of the simultaneous supplementation of the basal diet with 15 gm of torula yeast and 1.1 gm of DL-methionine was evaluated during trials 6 and 11. Of the 8 subjects utilized, 6 showed a strong

positive nitrogen balance that represents a very significant nitrogen storage (table 3). The value for the other two subjects falls within the zone of equilibrium.

The subjects in these two trials, except one, were the same previously used in trials 2 and 7, where 15 gm of torula yeast was the only supplement fed. The over-all response of the group under these two different experimental conditions appears to indicate a better utilization of the protein of the diet when DL-methionine is given together with torula yeast. This confirms observations previously made in this laboratory using the rat as experimental animal (Goyco and Asenjo, '54).

Effect on nitrogen balance of a supplement of brewers' yeast[8]

Goyco and Asenjo ('48c, '49) have reported, on the basis of rat experiments, that brewers' yeast is superior to torula yeast in terms of protein quality. These observations have been confirmed by others. (Harris, Hajny and Johnson, '51).

In order to investigate whether this difference could also be demonstrated in human subjects, the effect of a supplement of brewers' yeast, equivalent in nitrogen to 15 gm of torula yeast, was used in trial 12.

The 4 subjects used in this trial were the same ones utilized in trial 2, when 15 gm of torula yeast was used as a supplement. Although our observations were not extensive, the results obtained with these 4 subjects indicated that under the conditions of this experiment brewers' yeast protein seems to be inferior to torula protein. The average nitrogen balance for the 4 subjects that consumed 15 gm of torula was +0.49 gm with a value of 91% storage. The corresponding values for these same subjects on an equivalent amount of brewers' yeast supplement were $+0.24 \pm 0.20$ gm and $96 \pm 3.17\%$, respectively. This group appeared to retain more of the dietary nitrogen when the supplement was torula than when it was brewers' yeast.

Effect on nitrogen balance of supplements of skimmed milk and glycine

To investigate whether the conditions established for these series of nitrogen metabolism studies could demonstrate the effect between a high quality protein supplement and one supplying only non-essential nitrogen, three additional experiments were performed. Dry skimmed milk and the non-essential amino acid glycine were the supplements used. They were supplied in amounts equivalent to approximately 1 gm of nitrogen, that is, similar to the level used with other supplements.

Skimmed milk was consumed during trials 3 and 8, and all 8 subjects participated. It is apparent from the results presented in table 2, that 1 gm of nitrogen in the form of skimmed milk produced the strongest nitrogen balance ($+1.14 \pm 0.10$ gm) of all trials performed. This represents a marked and highly significant nitrogen storage (table 3).

These results indicated that the experimental conditions established could demonstrate the effect, on the nitrogen metabolism of the subject, of a supplement of approximately 1 gm of nitrogen and also, that when this amount of nitrogen was supplied by a biologically complete protein the nutritive value of our basal diet was significantly improved.

On the other hand, when glycine was fed (trial 4), the over-all effect was found statistically non-significant (table 3). Neither the nitrogen balance nor the percentage of nitrogen stored were modified when compared with the values obtained with the unsupplemented basal diet. These results show that the improvement in nitrogen balance and nitrogen storage observed with the different supplements studied was not due just to an increased nitrogen intake, but to the quality of the protein added as a supplement to the basal diet; in other words, to the essential amino acids present in these proteins and to their availability.

Effect on nitrogen balance of a supplement of rice and beans

The effect of adding as a supplement to the standard basal diet, an amount of rice and beans mixture equivalent to 1 gm of nitrogen, in order to raise the intake of this element to a comparable level with the other trials, was investigated in trial 5. The rice and beans mixture used contained

[8] Fleischmann type 2019.

both ingredients in the same proportion as they are present in the basal diet.

The improvement in nitrogen retention is evident from the results presented in table 2. The subjects used in this trial and in trial 1, where the unsupplemented diet was used, were the same. The average nitrogen balance obtained in trial 1, −0.16 ± 0.10 gm per day was shifted to +0.62 ± 0.14 gm, indicating a significant improvement in nitrogen assimilation (table 3).

The effect of this relatively small amount of extra rice and beans on the nitrogen metabolism of the subjects was very similar to that of a supplement of 15 gm of torula yeast. In part, this strengthens the results of previous experiments with the rat, indicating a limitation in the content of the amino acid methionine in both these supplements (Axtmayer, '47; Goyco and Asenjo, '48b; Asenjo and Goyco, '54).

Combined effect on nitrogen balance of a supplement of rice-beans and methionine

As a final corroboration, in humans, of this last observation the effect of the combined addition of rice and beans with 1.1 gm per day of DL-methionine, on the same group of subjects, was studied in trial 9.

The average nitrogen balance value obtained, +0.99 ± 0.16, was considerably higher than that in trial 5, +0.62 ± 0.14; similar effects were observed in the percentage of nitrogen lost (table 2).

The effect of the addition of this amount of DL-methionine together with the rice and beans mixture was very similar to that obtained when this amino acid was added together with the supplement of torula yeast in trials 6 and 11. In both cases the improvement in the nutritive value of the basal diet was evidenced by a significantly higher nitrogen retention (table 3). These results strongly point to the fact that our basal diet as compounded for these studies is very limited in methionine. The correction of this low level of methionine in the basal diet produced a marked improvement in its protein value. When this was done jointly with the addition of torula yeast as a supplement, the lack of some B complex vitamins in the basal diet (Goyco and Asenjo, '48c) was also corrected.

SUMMARY

The results of nitrogen metabolism studies performed on young male adult Puerto Ricans consuming a diet typical of low income groups are presented. This diet was supplemented with different nitrogenous products.

1. The subjects on the unsupplemented basal diet (4.61 gm of nitrogen per day) maintained nitrogen equilibrium.

2. Supplementation with torula yeast improved nitrogen retention to a small but significant degree. Torula yeast plus DL-methionine induced a greater retention. Brewers' yeast in an equivalent amount produced a lower nitrogen storage.

3. Skimmed milk as a supplement produced the strongest nitrogen retention, while the non-essential amino acid, glycine, was without effect on the nitrogen balance.

4. Supplementation with an extra amount of rice and beans mixture, alone or together with DL-methionine, induced a significant improvement in nitrogen retention very similar to that obtained with torula yeast.

The significant improvement in nitrogen balance whenever DL-methionine was included as a supplement indicates that the protein of the basal diet used has as its principal limiting factor, a low content of available methionine.

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Amino Acid Balance and Imbalance

II. DIETARY LEVEL OF PROTEIN AND LYSINE REQUIREMENT[1]

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There is evidence that the requirement for the most limiting amino acid in a diet rises as the dietary level of protein is increased. Thus the requirement of the growing chick for arginine (Almquist and Merritt, '50), tryptophan (Griminger et al., '56), lysine (Grau, '48) and methionine plus cystine (Grau and Kamei, '50); of the weanling pig for isoleucine (Becker et al., '57), and lysine (Brinegar et al., '50); and of the growing rat for tryptophan (Salmon, '54) have been reported to rise as the level of a protein deficient in these amino acids is increased in the diet. It should be noted, however, that in very few of the studies on this subject has a true increase in amino acid requirement been observed. In most experiments there were two limiting factors, protein generally and one amino acid in particular, so that responses to increasing increments of the amino acid were observed until protein generally became limiting. Then, when the protein level was increased and protein generally was no longer limiting, further responses to supplements of the limiting amino acid were obtained. Such studies, which are merely demonstrations of the law of limiting factors, do not provide evidence that amino acid *requirements* increase if the dietary level of protein is raised but only that the extent of *utilization* of one amino acid is limited if the diet is deficient in another. Elimination of such results from consideration leaves relatively few instances in which amino acid requirements have been shown to increase with increasing levels of protein in the diet and one case (Bressani and Mertz, '58) in which no such effect was observed.

It has been suggested (Allison, '55) that the increased requirement for the most limiting amino acid is a reflection of an increased rate of anabolism, an explanation that would apply to experiments involving two limiting factors; that higher protein intakes might alter the proportions of protein, fat and moisture in the tissues (Mitchell, '44), and that the higher requirement may result from an increased rate of amino acid catabolism (Salmon, '54).

The similarity of the conditions used in studying the influence of the dietary level of protein on amino acid requirements to the conditions under which amino acid imbalances are observed, and observations that the requirement for the most limiting amino acid (expressed as a percentage of the diet) may be increased in both cases, suggest that the two conditions have a common basis (Harper, '58). The work reported in this paper was undetraken to examine this aspect of the problem using wheat gluten, which is deficient in lysine, as a dietary protein for the rat. The influence of the dietary level of wheat gluten on the deposition of fat in the tissues and on protein digestibility have also been examined.

EXPERIMENTAL

Three-week old male weanling rats of the Holtzman strain weighing between 40 and 50 gm were kept for three days in individual raised-bottom cages, and fed on

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a diet containing wheat gluten,[3] 8% ; mineral mixture, 5% ; corn oil 5% ; adequate quantities of vitamins and dextrin to make 100%. They were weighed daily, and those rats which showed satisfactory weight gain during the first three days were separated into similar groups of 5 rats each and were fed on the experimental diets ad libitum. They were weighed on alternate days. All experiments were terminated after two weeks and food consumption for this period was recorded. The temperature of the room was maintained at about 24°C. Food containers were stored in the refrigerator.

Composition of the diets. The diet containing 10% of wheat gluten had the following percentage composition: wheat gluten 10% ; mineral mixture, (Harper, '59) 5% ; corn oil, 4.5% ; fat-soluble vitamin mixture in corn oil, 0.5% ; water-soluble vitamin mixture in sucrose, 0.25% ; choline chloride, 0.15% ; and dextrin to make up 100%. Higher levels of wheat gluten and supplements of L-lysine monohydrochloride were added at the expense of dextrin. The fat-soluble vitamin solution provided per 100 gm of diet: α-tocopherol, 10.0 mg; vitamin A, 400 I.U. and vitamin D, 200 I.U. The water-soluble vitamin mixture provided in milligrams per 100 gm of diet: thiamine·HCl, 0.5; riboflavin, 0.5; nicotinic acid, 2.5; calcium pantothenate, 2.0; pyridoxine·HCl, 0.25; vitamin K (menadione), 0.05; biotin, 0.01; folic acid, 0.02; vitamin B_{12}, 0.002; inositol 10.0; and ascorbic acid, 5.0. Ascorbic acid was included in the diet to minimize the destruction of thiamine (Kandutsch and Baumann, '53).

With higher levels of wheat gluten in the diet, the levels of water-soluble vitamin mixture and choline chloride were increased at the expense of dextrin to provide double the usual level of vitamins with 30% wheat gluten; 4-fold with 40 to 47% ; 6-fold with 50 to 60% and 8-fold in diets containing more than 60% of wheat gluten. For the diet containing 88% of wheat gluten a water-soluble vitamin mixture containing 4 times the usual quantities per 0.25 gm was prepared and 0.5 gm of this mixture was added per 100 gm of diet.

Digestibility determinations. Nitrogen in the diet was determined by the micro-Kjeldahl method using mercuric oxide as the catalyst, and total nitrogen consumed during the two-week experimental period was calculated. Feces were collected on papers kept underneath the cages, and the 14-day collection was pooled, dried and powdered. An aliquot was used for micro-Kjeldahl nitrogen estimation, and the total amount of nitrogen excreted in the feces was calculated. From these values the apparent digestibility was calculated using the relationship

$$\text{Digestibility} = \frac{\text{Nitrogen consumed} - \text{fecal nitrogen excreted}}{\text{Nitrogen consumed}} \times 100$$

This was not corrected for endogenous nitrogen.

Carcass analyses. The rats were stunned by a blow on the head and bled to death by cutting the jugular vein. The gastrointestinal tract was removed and the carcasses were stored at −4°C until the time of analysis. Each frozen carcass was ground to a homogeneous paste in a mincing machine, and 10 gm of the paste was dried at 100°C for 24 hours in a tared aluminum dish. The dry residue was extracted with ether in a Goldfisch continuous extractor for 24 hours. The fat-extracted residue was dried and ground in a small Wiley mill and an aliquot was analyzed for nitrogen by the micro-Kjeldahl method.

RESULTS

The initial experiments consisted of feeding trials using diets containing various levels of wheat gluten, unsupplemented and supplemented to contain 1% of L-lysine. A diet containing 30% of wheat gluten provides the accepted minimum requirements of the rat (Rose, '37) for all of the indispensable amino acids except lysine (Block and Weiss, '56) but, to meet the lysine requirement, the diet must contain 60% of wheat gluten. It was therefore possible, using suitable additions of L-lysine monohydrochloride, to prepare diets containing from 30 to 60% of wheat gluten but only 1% of lysine.

Weight gains of rats fed diets containing different levels of wheat gluten, with-

[3] Nutritional Biochemicals Corp., Cleveland, Ohio. Nitrogen content, 13.7%.

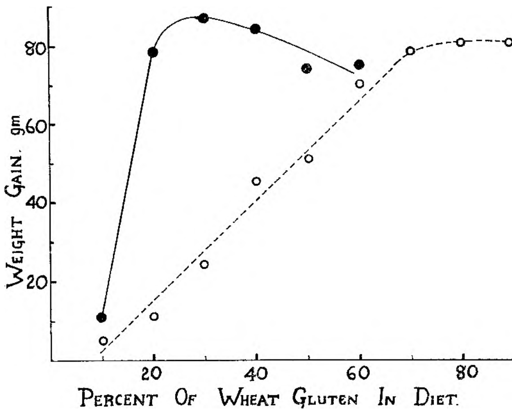

Fig. 1 Weight gains of rats fed on diets containing various levels of wheat gluten. ○ – – – ○, unsupplemented wheat gluten; ● ——— ●, supplemented to contain 1% of L-lysine.

out a supplement of lysine, are represented by the broken line in figure 1. The rate of gain increased stepwise with each increase in the dietary level of wheat gluten from 10 to 70% and, even with 88% of wheat gluten in the diet, there was no depression of the growth rate. The solid line in figure 1 represents the weight gains of rats fed diets containing different levels of wheat gluten supplemented in each case to contain 1% of lysine. The weight gained in two weeks reached a maximum when the diet contained 30% of wheat gluten but, as the level of wheat gluten was increased above this, the growth rate fell off. The weight gain of the group fed the diet containing 50% of wheat gluten was significantly lower ($P < 0.05$) than that of the group fed the diet containing 30 % of wheat gluten.

This suggested that the level of lysine needed in the diet to support maximum growth increased as the level of wheat gluten was raised, so the effect of lysine supplementation was studied in greater detail. Diets with levels of wheat gluten of 30, 47, 53 and 60%, which contained 0.5, 0.8, 0.9 and 1% of lysine, respectively, were selected and the effects of supplementing each of these with three levels of lysine were determined. The results are summarized in table 1. From this table it is seen that for maximum weight gain, 83 gm in two weeks, the lysine requirement was between 0.8 and 0.9% of the diet when the wheat gluten level was 30%. Diets containing 0.9% of lysine did not support this rate of gain when the wheat gluten level was increased to 53%. The dietary level of lysine required for maximum growth increased as the wheat gluten level was raised and was about 1.2% when the diet contained 60% of wheat gluten.

The crude digestibility of the protein in diets containing from 30 to 70% of wheat gluten was determined and the results are presented in table 2. It is clear that the digestibility was uninfluenced by the dietary level of protein.

Analyses for the moisture, fat and protein content of the carcasses of the rats fed on diets containing different levels of

TABLE 1

Weight gains of rats fed on diets containing different levels of wheat gluten and graded levels of lysine

Lysine in diet	Average weight gain in two weeks ± S. E.[1]			
	30% Wheat gluten	47% Wheat gluten	53% Wheat gluten	60% Wheat gluten
%	*gm*	*gm*	*gm*	*gm*
0.50	30 ± 1.4			
0.75	69 ± 6.2			
0.80	78 ± 1.4	52 ± 1.2		
0.90	83 ± 3.7	73 ± 5.9	56 ± 1.5	
0.95		72 ± 4.1		
1.00			66 ± 3.7	65 ± 3.6
1.05		81 ± 2.8	76 ± 3.7	
1.10				70 ± 1.4
1.15			87 ± 1.6	80 ± 1.6
1.20				
1.25				83 ± 4.8

[1] Standard error of the mean for 5 animals.

TABLE 2

Crude digestibility of protein with different levels of wheat gluten in the diet

Wheat gluten in diet	Crude digestibility ± S.E.[1]
%	%
30	93.5 ± 0.4 (4)
47	93.9 ± 0.3 (5)
53	94.9 ± 0.5 (4)
60	95.8 ± 1.0 (4)
70	94.6 ± 0.7 (4)

[1] S.E. = standard error of the mean for the number of animals indicated within parentheses.

wheat gluten were done and the results are given in table 3. With the exception of the group fed the diet containing 47% of wheat gluten, which supported a weight gain of only 52 gm in two weeks, the dietary level of protein did not influence the moisture or the fat content appreciably. The protein content of the carcass tended to increase and the fat content to decrease slightly as the dietary level of protein was raised.

While this work was in progress, it became apparent from other observations in the laboratory (Nath, Harper and Elvehjem, '59) that, although only traces (less than 1%) of fat could be extracted from wheat gluten with ether, an appreciable amount of material could be extracted with *n*-butanol. A maximum yield of 12.7% of lipid-like material was obtained by extracting wheat gluten with *n*-butanol for 48 hours in a continuous extractor, and the major portion (about 65%) of this extracted material was ether soluble.

Lipid, present to this extent in wheat gluten, would cause an increase of 6.4 Cal./100 gm of diet for every increase of 10% in the level of wheat gluten. This is a relatively small increase but as the lysine requirement of the growing rat, expressed as a percentage of the diet, increases with an increase in the caloric value of the diet (Rosenberg and Culick, '55), and as a large increase in the dietary level of protein could increase the caloric content of the diet significantly, the experiment summarized in table 1 was repeated using diets made isocaloric, by the addition of corn oil, with that containing 60% of wheat gluten. The results are presented in table 4, from which it can be seen that, even when the diets were isocaloric, the rates of gain of rats fed diets containing a particular level of lysine fell off as the dietary level of protein was increased. It is evident, however, that the amount of lysine required to support maximal growth with the lower levels of wheat gluten was somewhat higher than was observed previously, indicating that the effect of wheat gluten level on the lysine requirement observed in the previous experiment (table 1) was partly a result of the higher calorie content of diets containing high levels of wheat gluten, and only partly a result of the increase in the dietary level of protein.

The similarity of the effect of higher levels of protein on the lysine requirement to the effect of amino acid imbalance is brought out by the results of the last experiment in which diets containing a mixture of 30% of wheat gluten and 30% of zein supplemented with graded levels of L-lysine monohydrochloride were used. The results are summarized in table 5. It is apparent from this table that, while a level of 0.9% of L-lysine in the diet containing only 30% of wheat gluten supported a weight gain of 80 gm in two

TABLE 3

Carcass analyses of rats fed on diets containing different levels of wheat gluten

Wheat gluten	Moisture	Fat	Protein
%	% ± S.E.[1]	% ± S.E.[1]	% (N × 6.25) ± S.E.[1]
30 + 0.5 L-lysine·HCl	68.7 ± 0.4	11.0 ± 0.8	15.64 ± 0.3
47	66.7 ± 0.5	13.6 ± 0.6	15.21 ± 0.3
60	68.4 ± 0.1	10.3 ± 0.5	16.50 ± 0.5
70	68.6 ± 0.2	10.7 ± 0.5	16.21 ± 0.1
80	68.8 ± 0.5	10.1 ± 0.6	17.65 ± 0.3
88	68.8 ± 0.4	9.7 ± 0.7	18.21 ± 0.6

[1] Standard error of the mean for 5 animals.

weeks, replacing 30% of the dextrin in the diet with zein resulted in a significantly ($P < 0.01$) lower weight gain of only 66 gm in two weeks. Additional L-lysine was needed in the high protein diet to give maximal weight gain.

The effect of the level of wheat gluten in the diet on the amount of lysine required for maximum growth is shown in table 6. These values were obtained using isocaloric diets containing about 12.5% of fat. As the wheat gluten content of the diets was increased from 30 to 53%, the lysine requirement for maximum gain increased from 1.0 to about 1.2% of the diet. The food consumption fell slightly as

TABLE 4

Weight gain of rats fed on diets containing different levels of wheat gluten and graded levels of lysine

(All diets made isocaloric by the addition of corn oil)

Lysine in diet	Average weight gain in two weeks ± S.E.[1]			
	30% Wheat gluten + 3.8% corn oil	47% Wheat gluten + 1.6% corn oil	53% Wheat gluten + 0.9% corn oil	60% Wheat gluten
%	*gm*	*gm*	*gm*	*gm*
0.50	25 ± 1.5			
0.80	62 ± 3.8	40 ± 2.6		
0.90	73 ± 3.5		50 ± 4.6	
0.95		64 ± 3.0		
1.00	83 ± 3.1			65 ± 3.6
1.05		71 ± 4.5	70 ± 3.3	
1.10		79 ± 3.0		70 ± 1.4
1.15			76 ± 3.4	80 ± 1.6
1.20			79 ± 3.5	
1.25				83 ± 4.8

[1] Standard error of the mean for 5 animals.

TABLE 5

Weight gain of rats fed on diets containing a mixture of wheat gluten and zein and supplemented with L-lysine

Diet composition				Weight gain ± S.E.[1]
Wheat gluten	Zein	L-Lysine·HCl	Total lysine	
%	%	%	%	*gm/2 weeks*
30	—	0.5	0.90	80 ± 3
30	30	0.5	0.90	66 ± 4
30	30	0.8	1.15	78 ± 2

[1] Standard error of the man for 10 animals.

Differences between groups 1 and 2 and between groups 2 and 3 are highly significant ($P < 0.01$).

TABLE 6

Effect of level of wheat gluten on amount of lysine required for maximum growth in two weeks

Diet composition		Food intake	Weight gain	Lysine intake
Wheat gluten	Total lysine			
%	%	*gm/2 wk.*	*gm/2 wk.* ± *S.E.*[1]	*gm/2 wk.*
30	0.9	140	73 ± 3	1.3
30	1.0	140	83 ± 3	1.4
47	1.05	134	71 ± 4	1.4
47	1.1	136	79 ± 2	1.5
53	1.05	134	70 ± 3	1.4
53	1.15	138	76 ± 3	1.6
53	1.20	133	79 ± 2	1.6

[1] Standard error of the mean.

the protein content of the diet was raised but the amount of lysine required during two weeks for maximum growth increased from 1.4 to 1.6 gm.

DISCUSSION

From table 1 it is seen that the lysine requirement of the rat for maximum growth increased from 0.9% of a diet containing 30% of wheat gluten to about 1.2% of a diet containing 60% of wheat gluten. The increase in the caloric content of the diet containing 60% of wheat gluten, due to the lipid material present in the gluten, was responsible for an increase of 0.1% in the lysine requirement (compare tables 1 and 4). Nevertheless, an additional 0.15 to 0.2% of lysine beyond this had to be provided in the diet containing 60% of wheat gluten to ensure maximum growth. That this additional requirement is not attributable to a decrease in the digestibility of the wheat gluten is apparent from table 2. Also, since the percentages of moisture and fat in the carcass were but slightly affected by the dietary level of protein (table 3), it is improbable that, with higher levels of wheat gluten in the diet, the amino acids are diverted toward fat synthesis, thus making the limiting amino acid still more limiting.

The need for additional lysine to prevent a fall in the growth rate when the level of wheat gluten is increased in these diets resembles the effect of an amino acid imbalance. When the level of wheat gluten in a diet is increased to 60% in order to provide 1% of lysine in the diet, the accepted minimum requirement of the growing rat for this amino acid (Rose, '37), the levels of all of the other indispensable amino acids in the diet are much above the requirements. This is the type of condition which results in amino acid imbalance and growth retardation (Harper, '58; Salmon, '58). The experiment summarized in table 5 indicates this relationship more clearly. When 30% of zein is substituted for dextrin in a diet containing 30% of wheat gluten supplemented with 0.5% of L-lysine monohydrochloride, the levels of most of the essential amino acids are well above the accepted minimum requirements for the growing rat, and, although lysine remains at the near optimum level of 0.9%, growth is retarded.

The growth-retarding effect of the excess of amino acids from either wheat gluten or zein is prevented by the addition of lysine. The prevention of growth retardation by a supplement of the most limiting amino acid is characteristic of amino acid imbalances (Harper, '58).

Bressani and Mertz ('58) reported that the lysine requirement of the rat did not increase when the corn gluten content of their diets was raised from 16 to 40%. They determined the significance of the differences statistically, a procedure which may not detect true differences in responses that follow the law of diminishing returns (Almquist, '54). The results they present show that as the protein content of a diet containing 0.8% of lysine was increased from 16 to 40%, the gain in 28 days fell from 126 to 106 gm and that, although maximum gain was observed with between 0.8 and 0.9% of lysine in a diet containing 16% of protein, between 1.0 and 1.2% of lysine was required for maximum gain with 40% of protein in the diet. As an increment of 0.2% of lysine may cause a growth response of from 40 to 50 gm in the lower range of lysine levels whereas a similar increment may cause a growth response of only 10 to 15 gm or even less as the requirement for lysine is approached, it is apparent that small but important differences may not be detected by direct statistical treatment of such results.

On the basis of the observations reported in this paper, it is concluded that wheat gluten, due to its very low level of lysine (Block and Weiss, '56), is so severely unbalanced that the lysine it contains is not completely utilized. Certainly it is not possible to ensure maximum growth in a growing rat by merely increasing the level of wheat gluten sufficiently to provide 0.9% of lysine in the diet. However, as was observed in a previous study (Harper, '59), if the level of the limiting amino acid is increased sufficiently to exceed the requirement slightly, either through increasing the level of protein (e.g., 80% of wheat gluten) or by supplementation with the most limiting amino acid, a consider-

able excess of amino acids in the diet can be tolerated. In fact, this protein, with a biological value of only 40, will support maximum growth if the level in the diet is increased sufficiently to provide about 1.2% of lysine.

The presence of an appreciable amount (12.7%) of lipid material in wheat gluten is an important factor which has not received much attention in nutrition studies. The presence of lipid which is released by *n*-butanol may be a feature common to many purified vegetable proteins. Drackett protein extracted with *n*-butanol for 24 hours yielded 6.7% of lipid material. The lipid content of wheat gluten is apparently sufficiently great to cause an increase in the requirement for lysine, when this protein is fed at high levels. The effect of the lipid in wheat gluten on the lysine requirement observed in this study is comparable to that reported by Rosenberg and Culick ('55) when they increased the calorie content of the diet of rats by adding fat.

SUMMARY

Evidence has been obtained suggesting that wheat gluten is so poorly balanced in amino acids that the lysine requirement of the rat for maximum growth is increased when this protein is the sole source of lysine in the diet. An analogy has been drawn between this effect and those observed as a result of amino acid imbalances.

Little change in the moisture and fat content but increased deposition of protein occurred in the carcasses of rats fed on diets containing higher levels of wheat gluten.

The digestibility of the proteins in wheat gluten was about 95% and this was unaffected by the level of wheat gluten in the diet over the range of 30 to 70%.

An effect of the appreciable amount of lipid in wheat gluten on the requirement of the growing rat for lysine has been demonstrated.

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Biological Availability of Amino Acids in Fish Meals and Other Protein Sources[1]

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Considerable attention has been devoted to the nutritional value of proteins and to the amino acid deficiencies which limit their use in foods, but comparatively little is known about the variation in the availability of each of the amino acids in these proteins, or how processing may affect their availability.

From comparisons of the amino acid contents of the food eaten and the feces produced, Kuiken and Lyman ('48) and Kuiken ('52) have estimated the availability of amino acids in some protein sources. Two disadvantages of this method are: (1) that the action of the intestinal microflora on the food, while not specifically known, may be significant (Schweigert and Guthneck, '54; Nasset et al., '55), and (2) that the diet itself may influence the secretion of protein-rich materials into the intestinal tract, thus increasing the apparent undigested residue (Lyman, '57).

In another method, widely used, the growth rate obtained on a diet low in an essential amino acid is compared with the growth rate obtained on a similar diet to which a known amount of the test protein is added. By supplementing the basal diet with several levels of the limiting amino acid, a response curve can be established, and the amount of amino acid in the test protein estimated. Grau and Almquist ('45), using a diet based on raw soybeans to determine the biological availability of methionine, concluded that most of the methionine in feedstuffs was available. However, Schweigert and Guthneck ('54), using a rat diet based on oxidized casein, found the availability of methionine to vary from 44 to 79% of the total, when the total amount was estimated by microbiological assay. Using an oxidized casein diet, Schweigert ('48) reported that the percentage of available tryptophan varied from 20 to 65% for chicks. For weanling rats, Lushbough et al. ('57) and Gupta and Elvehjem ('57) found variations from 73 to 132% for a number of foodstuffs. Their diets were based on mixtures of casein and oxidized casein, or casein, gelatin, and zein.

Diets in which the amino acids were furnished by sesame seed oil meal or by purified amino acids have both been used to investigate the availability of lysine (Schweigert and Guthneck, '53; Guthneck et al., '53). Sesame seed oil meal is a protein source of high quality except for its low lysine content. They found that 49 to 98% of the total lysine in various foodstuffs was utilized for growth by the protein-depleted rat. Kratzer and Green ('57) used a diet based on sesame seed protein to investigate the availability of lysine in several blood meals. They found percentages of availability varying from 64 to 85% for chicks and from 49 to 76% for turkey poults. Gupta and co-workers ('58) used a diet based on wheat gluten to investigate the availability of lysine to the rat. They found variations in availability ranging from 75 to 100%.

The availability of isoleucine in a number of proteins fed to weanling rats has been reported by Deshpande et al. ('57) to range from 30% for zein to almost 100% for several other proteins. Additions to a diet based on blood meal were used in this study.

In the chick assay method we have developed, the amount of an essential

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amino acid in a protein source of unknown value is estimated by using a diet containing crystalline forms of all the essential amino acids (except the one being assayed) at levels higher than the requirements of the chick, and adding the unknown protein as the only source of this one amino acid. The performance of this diet is then compared with that of two other diets: one, the negative control, is a diet containing all essential amino acids except for the one being estimated; the other, the positive control, contains all the essential amino acids at levels higher than the requirements of the chick, and in addition contains a protein source. This source is usually the test protein, but it may be one of similar quality, fed at the same protein level as is used in the test diet. The value of the method depends in part on obtaining essentially normal growth when the test protein plus a complete amino acid mixture is fed. The method also depends on a knowledge of the approximate requirement for each essential amino acid. By using this information, the amount of available amino acid in the different test diets can be evaluated by plotting, on the growth-response curve for the amino acid, the experimentally determined growth rates. An example of such a plot is given in figure 1, where lysine is the amino acid being assayed. Thus a straight line between the negative control (zero available amino acid) and positive control (full requirement of available amino acid) serves as the basis for calculation of the amount of available amino acid in the test material. This method assumes a linear growth response to added increments of amino acid through the major part of the scale. It has been found that most of the essential amino acids do give an approximately linear response from the zero level to levels slightly lower than the requirement.

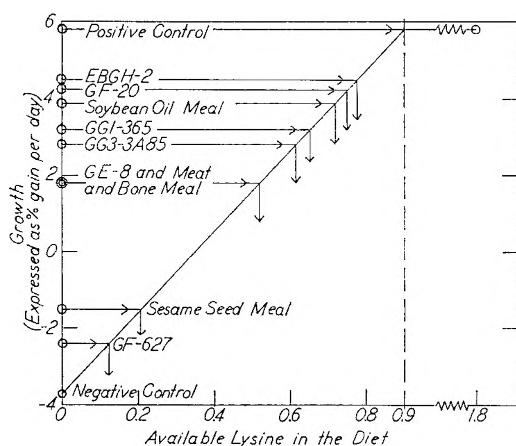

Fig. 1 Results of lysine availability analyses (experiment 5).

Disadvantages of the method include the expense of the diets, the necessity for the use of an assumed value for the requirement, and possible confusing interactions among amino acids and other materials present in the test food. These are considered later (see below).

EXPERIMENTAL METHOD

Sexed White Leghorn chicks used in these trials were raised for 11 days in electrically heated battery brooders on a commercial type chick starter mash, after which they were segregated on a basis of weight and sex into groups of 4 and placed in small cages in a room maintained at 85°F. Neither extremely large nor very small birds were used. After one or two more days on the starter mash, the chicks were placed on the experimental diets. Feed and water were supplied ad libitum, the chicks were weighed daily during the 6-day trial and were given 12 hours of light per day. All diets were duplicated. Growth rates were calculated by dividing the average gain per day per chick by the average of the initial and final body weights and multiplying the results by 100 to give per cent gain per day.

Diets included a mixture of amino acids which contributed the following to the final diets: DL-α-alanine, 0.4%; L-arginine·HCl, 1.3%; DL-aspartic acid, 0.4%; L-cystine, 0.5%; L-glutamic acid, 2.0%; glycine, 1.4%; L-histidine·HCl, 0.4%; DL-isoleucine or L-isoleucine with D-alloisoleucine, 1.2%; L-leucine, 1.4% or DL-leucine, 2.0%; L-lysine·HCl, 1.3% or DL-lysine·HCl, 2.6%; DL-methionine, 0.4%; DL-phenylalanine, 0.8%; DL-serine, 0.2%; DL-threonine, 1.0%; DL-tryptophan, 0.4%; L-tyrosine, 0.8%; and DL-valine, 1.5%. When an essential amino acid was being studied, it was omitted from the mixture and replaced with L-glutamic acid. All

amino acid sources were checked for possible toxic impurities, and some, notably leucine, were recrystallized before use.

The various foods were incorporated in the diet at levels that furnished 5, 6, or 10% of crude protein in the diet; the 10% level was used in most of the work reported here. This variation in level was necessary to provide less than the amino acid requirement, that is, to stay within the assay range. When a protein level lower than 10% was used, sufficient L-glutamic acid was included to bring the total protein equivalent to about 25%.

Vitamin and mineral mixtures were included to provide the following levels in the diet: thiamine·HCl, 0.001%; riboflavin, 0.001%; pyridoxine·HCl, 0.001%; niacin, 0.006%; calcium *d*-pantothenate, 0.003%; folic acid, 0.001%; biotin, 0.00001%; dry vitamin A concentrate (activity 10,000 units/gram), 0.1%; dry vitamin D concentrate (activity 1500 IC units/gram), 0.1%; dry vitamin E concentrate (20,000 units/lb.), 0.1%; vitamin B_{12} concentrate (0.1% trituration in mannitol), 0.0022%; choline chloride (25%), 0.2%; iodized salt, 0.5%; $MnSO_4 \cdot H_2O$, 0.03%; diphenyl - paraphenylendiamine (DPPD), 0.02%; ferric citrate, 0.074%; cobaltous acetate·$4H_2O$, 0.0002%; $CuSO_4$, 0.005%; $ZnSO_4 \cdot 7H_2O$, 0.0063%; $MgSO_4 \cdot 7H_2O$, 0.6%; KCl, 0.6%; $Al_2(SO_4)_3 \cdot 18H_2O$, 0.1%; $CaCO_3$, 1.92%; $Na_2SiO_4 \cdot 9H_2O$, 0.2% and $CaHPO_4$ 3.3%. The diets contained 5% of crude soybean oil and sufficient glucose (Cerelose) to make a total of 100%. The negative control contained additional L-glutamic acid instead of the protein tested. The positive control was one of the test diets to which an adequate level of the essential amino acid was added; thus it contained the protein under test and a full complement of crystalline essential amino acids.

Analyses for crude protein (N × 6.25) in the tested protein sources (see table 1) were made by the California State Feed Laboratory. Analyses for total content of the tested amino acids were made by the Wisconsin Alumni Research Foundation by microbiological methods of Henderson and Snell ('48) following acid or alkaline hydrolysis.

Description of fish meal samples

Sources of the protein materials including the fish meals employed in this work

TABLE 1
Protein meals used

Protein supplement	Crude protein	Value as a sole protein source[1]	Comments
	%		
Fishmeal GF-20	76.0	6.7	Herring meal
Fishmeal GE-8	48.6	2.7	Tuna meal[2]
Fishmeal GG1-365	62.4	5.8	Menhaden meal
Fishmeal GF 600	59.3	6.1	Menhaden meal
Fishmeal GG3-3A85	56.7	4.2	Menhaden meal
Fishmeal EBGH-2	59.6	6.6	Rosefish meal
Fishmeal GE 22	67.5	3.8	Peruvian fish meal (herring?)
Fishmeal GF 627	57.9	nil	Tuna meal laboratory processed—scorched
Sesame seed oil meal	43.7	3.0	Screw pressed
Corn gluten meal	47.7	1.2	Commercial meal
Cottonseed meal no. 346	42.0	4.8	Commercial meal prepress solvent
Cottonseed meal no. 440	41.0	4.2	Commercial meal screw pressed
Raw soy flakes	51.1	3.9	Hexane extracted without heat
Isolated soy protein	84.5	4.1	Commercial product[3]
Soybean oil meal	48.5	6.7	Commercial meal
Meat and bone meal	56.2	4.0	Commercial meal
Dried cottage cheese	79.9	4.0	Laboratory processed
Casein	85.0	—	Commercial, acid washed in laboratory
Dried brewers' yeast	45.6	4.8	Commercial meal

[1] Method of Grau and Williams ('55). Values given as % gain/day.
[2] This particular meal was made from bone, skin, and adhering flesh. Very little muscle protein present.
[3] Drackett C-1.

are listed in table 1. All fish meals except samples GF600 and GF627 were commercial meals, manufactured by the usual wet process employing flame-type driers.

The experimental meals were prepared in pilot plant equipment of the Seattle Fishery Technological Laboratory, U. S. Bureau of Commercial Fisheries. The experimental menhaden meal (GF600) was prepared by the wet process and dried in a small scale experimental steam-jacketed drier at about 230°F. The experimental tuna meal was prepared from the whole waste minus viscera. It also was prepared by the wet process and dried in the experimental small-scale drum flame-type drier described by McKee and Karrick ('56). This meal was deliberately burned by drying for three hours at a temperature of 395°F. The resulting meal was dark brown containing many charred particles. Commercial meals probably are never quite so badly charred as this experimental meal although occasionally overheating results in meals containing many charred particles.

RESULTS

Lysine. The first amino acid that we studied was lysine, both because it is so important in applied nutrition, and because its availability to the animal has been shown to be affected by processing and storage conditions (Lea et al., '58). The results of several lysine availability trials, presented in table 2, are based on the assumption that the lysine requirement is 0.9% of the diet. The results of a typical experiment are shown in figure 1.

Fish meals GF-20 and EBGH-2, which previous studies had shown to be of high quality, ranked highest in available lysine in each experiment. A fish meal scorched deliberately under laboratory conditions (GF 627) contained the lowest amount of available lysine. Of the other proteins tested, sesame seed oil meal, soybean oil meal, and the meat and bone meal showed the smallest differences between available and total lysine.[3] The lysine of the two samples of cottonseed meal appeared to be only partially available, probably because part of the lysine had reacted with gossypol during the manufacture of the meal.[4]

Arginine. Arginine, which is a dietary essential for the chick, was studied by the same method as that used for lysine with an assumed requirement of 0.9% of the diet. The availability of arginine, i.e., the ratio of available arginine to total arginine, was much more variable than that of any of the other amino acids studied (see table 2). For example, fish meal GF-20, which was an excellent source of all amino acids needed for normal growth (see table 1), appeared to contain only 3.0% available arginine in the protein, compared with a total of 5.7%. The arginine requirement is estimated to be satisfied by a protein containing 4.5% available arginine, if the protein is fed at 20% of the diet, therefore this availability estimate appears to be lower than indicated by data from other feeding experiments. This discrepancy cannot now be resolved, and at least some of the meals contain more available arginine than given in table 2.

The scorched fish meal protein (GF 627) contained 3.8% total arginine, but no available arginine was detectable by this method. As was previously mentioned, this meal also had the lowest total lysine; however, some lysine remained available despite scorching.

The wide variation in the percentage of arginine available to the chick explains the existing uncertainty concerning the arginine requirement of the chick. Published requirements fall between 1.1 and 1.9% of total arginine in the diet. Our experiments with amino acid mixtures indicate a requirement of approximately 0.9% of available arginine for the growing chick.

Methionine and cystine. Availability figures were determined for methionine alone and also for methionine plus cystine because of their combined importance as components of proteins. Results of the analyses are shown in table 2. The re-

[3] Estimates of total amino acid content based on microbiological assay are subject to errors similar to those present in a chick assay procedure; the use of the term "total" for such figures is a convenience.

[4] Other studies with these samples and with other cottonseed meals containing high levels of bound gossypol also indicated that lysine had reacted with gossypol, thus rendering both compounds unavailable to the animal. (See also Baliga and Lyman, '57).

quirement assumed for methionine was 0.3% of the ration.[5] For the combined analysis of methionine plus cystine, in which both are omitted from the basal amino acid mixture, the requirement was assumed to be 0.8%. The combined figure is subject to less variation and is more useful than the figure for methionine alone. Again in these analyses, the high quality fish meal (GF-20) contained more available methionine, and methionine plus cystine, than the lower quality fish meal (GGl-365), even though it contained a lower total amount. In contrast to arginine part of the sulfur-containing amino acids were available in the scorched fish meal (GF 627).

Tryptophan. In the analysis for available tryptophan, it was found that the levels of this amino acid in most feedstuffs are such that use of the test material at the 5% crude protein level gave the best results. From the data given in table 2, it appears that except in those meals severely damaged by heat, practically all of the tryptophan is available to the chick.

Valine. When the method was used for a valine assay, growth data and availability figures were much lower than had been expected. Other work (Benton et al., '56) has indicated that some complex interrelationships exist between the dietary levels of valine, leucine and isoleucine in their effects on growth. We believe that imbalances among those amino acids account for the observed discrepancies. At present, this analysis is not suitable for valine, leucine or isoleucine, but it is hoped that further study may reveal ways of estimating these also.

Other amino acids. The availability of a number of other essential amino acids was also studied and these data are also presented in table 2. For the phenylalanine analysis, sufficient protein supplement was added to provide 5% of crude protein; for the histidine analysis, 6% of crude protein; and for the threonine analysis, 10% of crude protein. The phenylalanine analysis could probably be improved if the combined availability of phenylalanine and tyrosine is used as the basis for assay.

Glycine is needed by the chick only for maximum growth, and its omission from the diet did not cause weight loss as did the omission of other essential amino acids. Thus, only relatively small differences were found between the negative control (without glycine) and the positive control (with glycine). At present this method is unsatisfactory for a glycine assay.

DISCUSSION

It has been recognized for many years that protein quality should preferably be expressed in terms of the essential amino acids which the protein source furnishes to the diet, rather than in more general terms. Data for the total amino acid content of proteins often fail to reveal differences that are demonstrated by animal experiments. Some of these discrepancies can be explained by the presence of nonprotein growth inhibitors (Lepkovsky, '55); others, however, clearly indicate variations among proteins as sources of individual amino acids. In other words, variations in the availability of the individual essential amino acids to the animal may explain discrepancies in the apparent quality of proteins, especially of those which have undergone extensive processing. Most of the methods used previously to determine amino acid availability (see Literature Cited, below), require a basal diet containing a feedstuff already naturally low in the amino acid being assayed, or one treated to make the amino acid unavailable. To this basal diet the test material is added and the growth response observed is compared with that obtained when graded levels of the pure amino acid are added to the same basal ration. Such a method, however, leaves unknown the availability of the amino acids in the basal ration. The unbalanced basal protein may affect the growth of the animal in many ways; for example, poor growth may result from the action of growth-depressing factors formed in the preparation of the basal protein, or at the other extreme, growth-promoting factors present in the test material may stimulate growth. Because the additions of test materials

[5] The published requirement for methionine of 0.45% (Almquist, '52) is based upon work using proteins of doubtful availability. Our recent work using amino acid mixtures indicates that the requirement is close to that suggested by Klain et al. ('58).

TABLE 2

Availability of amino acids in a number of protein supplements to chicks

Protein supplement	Lysine[1]		Arginine		Methionine		Methionine plus cystine	
	Total	Available	Total	Available	Total	Available	Total	Available
Fish meal GF-20	7.5	7.3 ± 0.4(5)[2]	5.7	3.0 ± 0.2(5)	2.6	2.7	3.5	3.5 ± 0.0(2)
Fish meal GE-8	5.7	5.2 ± 0.5(4)	6.7	4.8 ± 0.6(4)	1.9	2.4	2.5	2.6
Fish meal GG1-365	9.6	6.1 ± 0.3(5)	8.2	3.0 ± 0.7(5)	3.0	2.2	3.7	2.8 ± 0.2(3)
Fish meal GF-600	7.6	7.8	6.9	2.6	2.3	2.4		
Fish meal GG3-3A85	7.2	6.6 ± 0.4(2)	8.0	4.0 ± 1.0(2)	2.6	2.3	3.4	3.0
Fish meal EBGH-2	7.6	7.2 ± 0.5(2)	6.7	4.7 ± 0.1(2)	2.9	2.6	3.7	3.4
Fish meal GE-22		6.8		1.6				3.4
Fish meal GF-627	4.3[3]	1.2 ± 0.1(2)	3.8	0.0 ± 0.4(2)	1.7	0.7	2.1	0.7
Sesame seed oil meal	2.25[3]	2.6 ± 0.4(4)	12.7	8.3 ± 0.3(4)[4]	2.0	2.6	4.7	6.3
Corn gluten meal		1.6		0.9				4.4
Cottonseed meal no. 346	5.0	2.2 ± 0.3(4)	11.3	7.8[4]			2.6	2.5
Cottonseed meal no. 440	4.7	2.3 ± 0.1(4)	11.9	8.6[4]			2.5	2.0
Raw soy flakes		4.8		4.1				1.5
Isolated soy protein		4.5 ± 0.2(2)		4.4				1.6
Soybean oil meal	6.3	6.7 ± 0.5(4)	7.4	6.6 ± 0.6(4)	1.35	1.6	2.3	2.5
Meat and bone meal	5.3	5.5 ± 0.4(2)	7.7	5.5 ± 0.8(3)	1.4	1.9	2.4	1.9
Dried cottage cheese	9.8	6.1	4.1	0.7			2.8	3.6
Casein (acid washed)				1.5				
Dried brewers' yeast		3.7		2.9				1.6

Protein supplement	Tryptophan		Histidine		Threonine		Phenylalanine	
	Total	Available	Total	Available	Total	Available	Total	Available
Fish meal GF-20	1.1	1.3 ± 0.1(2)	2.3	2.0 ± 0.4(2)	4.2	3.7	4.1	4.5
Fish meal GE-8	0.5	0.6 ± 0.1(2)	2.4	1.9 ± 0.4(2)				
Fish meal GG1-365	0.8	1.0 ± 0.2(3)	2.2	1.6 ± 0.1(3)	5.7	3.1	4.3	2.4
Fish meal GF-600								
Fish meal GG3-3A85	0.9	1.1	1.7	1.9				
Fish meal EBGH-2	1.0	0.9	2.2	1.7				
Fish meal GE-22		1.4		2.1				
Fish meal GF-627	0.9	0.3	2.3	0.5				
Sesame seed oil meal	1.5	1.5	2.7	2.3				
Corn gluten meal		0.15		2.1				
Cottonseed meal no. 346	1.2	1.3	3.6	2.6				
Cottonseed meal no. 440	1.1	1.2	3.5	2.6				
Raw soy flakes		1.4		3.1				
Isolated soy protein		1.3		2.7				
Soybean oil meal	1.5	1.6	3.7	3.3	3.6	3.7	5.0	6.4
Meat and bone meal	0.8	0.5	2.0	1.5				
Dried cottage cheese	1.6	1.9	4.2	3.4				
Casein (acid washed)								
Dried brewers' yeast		0.6		1.5				

[1] All amino acid values are given as percentage of crude protein. Assumed amino acid requirements used for calculations (as % of diet): lysine 0.9, arginine 0.9, methionine 0.3, methionine plus cystine 0.8, tryptophan 0.15, histidine 0.3, threonine 0.6, and phenylalanine 0.4.

[2] Figures within parentheses are number of availability trials performed when more than one.

[3] Other analyses have given higher values.

[4] Growth rates here are beyond linear part of curve, availability is actually higher than indicated.

are often made at the expense of the energy source, thereby raising the protein level, the amino acid requirement may also be increased (Grau, '48). Thus differences in the performance of the diets may not be attributable solely to variation in the amino acid under study. The method presented here avoids most of the difficulties mentioned and yields data which help to explain observed variations in proteins subjected to processing or source variation.

It is true that for this method a chick requirement figure must be assumed for each amino acid tested. If too high a requirement is assumed, the available amino acid content of the protein will be unduly high; however, the relative availability of an amino acid in a series of proteins tested will not be affected. Thus, the necessity for this assumption does not seriously affect the usefulness of this method.

Interactions among dietary ingredients present the major problem in the use of the method. The complex and little known interrelationships of valine, leucine, and isoleucine (Benton et al., '56) prevent their assay by this method. The interactions of gossypol and the lysine of cottonseed meal are more pronounced in the test diets than in more commonly used rations where a similar effect is known (Baliga and Lyman, '57). An unexplained interaction between the arginine of some proteins and other dietary ingredients is also more pronounced in the test diets than in other diets. These interactions become apparent when amino acid availability data given by this method do not agree with data furnished by other types of feeding trials. Such discrepancies provide promising leads in the study of the interactions occurring in practical diets.

SUMMARY

A new chick assay method is proposed for the estimation of essential amino acids available to the animal from various protein sources. The material to be tested is fed as a supplement to a diet replete with crystalline forms of all essential amino acids except the one being studied; thus the source supplies this one as well as non-essential amino acid needs. The growth response to this diet, compared with that for the same diet plus the missing amino acid, allows estimation of the amino acids furnished by the protein source. The method has been used successfully for lysine, methionine and cystine, tryptophan, phenylalanine, histidine, and threonine. It has been less successful for arginine, and unsatisfactory for glycine, isoleucine, leucine, and valine. Results of tests with 19 protein sources are given for a number of amino acids, with special emphasis on fish meals of variable and known histories.

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Studies on the Ability of Sorbitol and Various Sugars to Enable Chicks and Rats to Survive Dietary Deficiencies of Single Vitamins[1]

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Morgan and Yudkin ('57) reported that the inclusion of 20% of sorbitol in a diet deficient in all the B complex vitamins enabled rats to live and make nearly normal growth. This surprising effect was attributed to promotion of intestinal synthesis of B complex vitamins by sorbitol. The present study was initiated to determine if sorbitol has a similar effect on the need of chicks for certain B complex vitamins and to determine the effect of varying the sugar component of the diet on the development of avitaminosis. Rat studies using some of the chick diets were conducted concurrently with the chick trials.

EXPERIMENTAL

The basal diet (table 1) used in all experiments was a modification of the 35% soybean protein[2] diet of Machlin and Gordon ('57). All components of the diet, except sorbitol or sugar and the three pre-mixes, were mixed in sufficient quantities to last for the duration of each experiment. This mixture and appropriate quantities of sugar or sorbitol and the pre-mixes were used to mix complete feeds weekly. All feed remaining in the feeders at the end of each week was discarded. Pre-mix C (table 1) was stored under refrigeration. The carrier for all pre-mixes was glucose and in making deficient diets glucose replaced the appropriate pre-mix.

Straight-run day-old chicks were used in the first and 6-week old chicks in the second chick experiment. Conventional, electrically heated starting batteries or metal developing batteries, equipped with raised wire floors housed the chicks. Duplicate lots of 20 chicks were used in experiment one and replicated lots of 10 in experiment F-54.

TABLE 1
Composition of diet

Ingredient	Quantity
	lbs.
Sorbitol or sugar	20.00
Soybean protein[1]	35.00
Corn oil	16.00
Glycine	1.00
Vitamin Pre-mix A[2]	10.00
Vitamin Pre-mix B[3] or glucose	5.00
Vitamin Pre-mix C[4] or glucose	5.00
Solkafloc	1.00
Minerals[5]	5.34
Glucose	0.80
DL-Methionine	0.59
	mg
Inositol	500.0
Vitamin B_{12}	0.6
Biotin	6.0
Menadione	54.0
Pyridoxine	195.0
Folic acid	37.0
Riboflavin	195.0
Ca pantothenate	630.0
Niacin	1.8
Total	99.63 lbs. +

[1] Drackett Assay Protein C-1.
[2] Ascorbic acid 10 gm, BHT 5.675 gm, vitamin E 500 I.U., vitamin D_3 12,000 I.C.U., vitamin A 300,000 I.U.
[3] Contained choline chloride 60 gm.
[4] Contained thiamine 80 mg.
[5] Glista ('51).

The Sprague-Dawley strain weanling rats used in these experiments were housed in conventional rat cages equipped with raised wire floors. Water and food were supplied *ad libitum* in both rat and chick studies. Individual animals were weighed at the beginning of each experiment and,

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[1] Presented in part at the 47th Annual Meeting of the Poultry Science Association, August 1958 (Wharton et al., '58).
[2] Drackett Assay Protein C-1.

with some exceptions, at the end of each week on test.

The basal diet was calculated to contain 39.7 μg of thiamine and 0.332 mg of choline chloride per pound. To those diets not intended to be deficient in choline or thiamine, these vitamins were added at levels of 600 and 0.8 mg per pound of feed respectively.

RESULTS

It was decided to study the effect of sorbitol, glucose, fructose and sucrose on growth and survival of chicks and rats deprived of thiamine or choline. Glucose is the sugar normally used in purified diets in our laboratory. Fructose was selected because sorbitol has been reported to follow the pathway of fructose metabolism (Seeberg et al., '55). Sucrose was chosen because it is composed of glucose and fructose, the two monosaccharides under investigation. Also, it has been suggested that sucrose does not stimulate, and indeed may retard, the development of intestinal micro flora. The sorbitol and sugars were added at a level of 20% of the total diet, replacing glucose.

Experiment 1. Neither sorbitol nor the sugars eliminated the need for dietary thiamine in chicks (table 2). In the absence of added thiamine, deficiency symptoms and almost a complete lack of growth were apparent by the 7th day. No chick survived 13 days of age. Growth was retarded in the absence of added dietary choline and a majority of the birds developed perosis (table 3). There was no mortality attributable to diet in the control and choline-deficient lots.

TABLE 2

Effect of sorbitol, sucrose, fructose and glucose on thiamine deficiency in the chick

Experiment 1

Treatment[1]	Gain/week in grams		Average days survival	
	1	2		
Control[2]	49.9	64.4		
Control	49.9	54.6		
Glucose	3.1	—	7.85	> 7.87
Glucose	7.0	—	7.90	
Sorbitol	0.0	—	6.55	> 7.00
Sorbitol	1.9	—	7.45	
Sucrose	0.9	—	6.50	> 6.75
Sucrose	2.1	—	7.00	
Fructose	1.6	—	7.40	> 7.02
Fructose	−0.3	—	6.65	

[1] Twenty chicks started in each lot.
[2] All diets, except the control, were deficient in thiamine.

TABLE 3

Effect of diet on choline deficiency

Experiment 1

Treatment	Av. 4 wk. gain[2]	Av. score perotic birds[3]	% perotic
Control	287.9	0	0
Glucose	251.1	2.1	80
Sorbitol	256.2	2.3	74
Sucrose	239.5	2.0	75
Fructose	273.1	2.6	80

[1] All diets, except the control, deficient in choline.
[2] Average of replicated lots of 20 chicks each.
[3] Perosis scored: 1 = slight in one leg, 6 = severe in both legs.

Experiment 1-A. The control and thiamine-deficient diets containing sorbitol or glucose were fed to lots of three male rats. After 6 weeks on these diets the animals receiving the thiamine-deficient glucose diet were changed to the control diet and the rats on the control diet to the thiamine-deficient fructose diet. The effect of this dietary regimen on body weight is shown in figure 1. The rats fed the thiamine-deficient sorbitol diet gained at essentially a normal rate throughout the 8-week test. Rats receiving the thiamine-deficient glucose diet made normal gains for the first two weeks but lost weight after the third week. These animals recovered rapidly when given the control diet. Rats fed the control ration made normal gains. However, when changed to the thiamine-deficient fructose diet rate of gain decreased during the first week with loss of body weight occurring the second week.

Fecal samples from rats receiving the control, thiamine-deficient sorbitol and thiamine-deficient fructose diets were collected over a period of 5 days in the following manner: all feces voided over night were discarded at 8 o'clock the next morning. Feces were collected at 10, 12, 2 and 4 during the day. Upon collection the fecal pellets were macerated in ethanol and refrigerated. The thiamine and riboflavin content of the feces were determined using the USP XV ('55) thiochrome and AOAC ('55) microbiological methods respectively. The feces samples of the control, sorbitol and fructose lots contained,

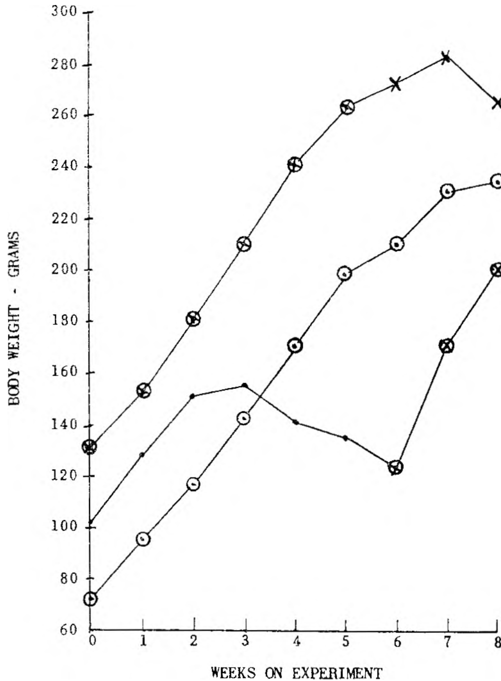

Fig. 1 The effect of sorbitol or sugars on growth of rats deprived of thiamine. ⊕, control, complete glucose diet; ●, thiamine-deficient sucrose diet; ⊙, thiamine-deficient sorbitol diet; ×, thiamine-deficient fructose diet.

respectively, 15.6, 4.63, and 3.04 μg of thiamine per gram (dry basis). Riboflavin on the same basis was 70.4, 19.3 and 11.6 μg per gram.

Experiment F-54. Replicated lots of 6-week-old chicks were fed diets with all of the carbohydrate contributed by glucose or with 20% of sorbitol or fructose replacing an equivalent quantity of glucose. All diets were fed with and without thiamine supplementation and the deficient diets with and without procaine penicillin at a level of 2 mg per pound of feed.

At the end of two weeks, one chick in each deficient group was given thiamine orally by syringe and plastic tubing into the crop, on alternate days, at a level of 30 μg per dose. The experiment was terminated at the end of 21 days.

Symptoms of thiamine avitaminosis were observed in chicks on the control diet after about 10 days on test. The thiamine pre-mix used in this series had been made about 4 months previous to the initiation of this experiment and stored in a closed container at room temperature. Apparently, thiamine was not stable under these conditions. Immediately upon noticing deficiency signs, all control diets were mixed anew with a fresh supply of thiamine. And in addition, thiamine was added to the drinking water for three days. This deficiency in the control diets accounts for the poor gains made by the control birds in the first two weeks on test (table 4, cerelose, fructose and sorbitol controls).

Regardless of dietary treatment only 6 out of 60 birds receiving a vitamin B_1-deficient diet survived the three-week test. Death in all instances appeared due to thiamine deficiency. The presence of penicillin in the ration did not appreciably increase livability, although weight losses of survivors were less when penicillin was included in the ration (table 5). Also, gains of birds given thiamine orally were slightly greater when the diet contained penicillin. Penicillin had no appreciable effect on survival time. In the absence of added thiamine, all survivors lost weight (table 4). The presence of 20% of either sorbitol or fructose in the diet significantly reduced gains of birds fed complete diets ($P < 1\%$). These results show that neither sorbitol nor fructose eliminated the need for dietary thiamine in the chick 6 weeks of age, and that this lack of effect was not influenced appreciably by the presence of penicillin in the diet.

Experiments F-59 A, C, D. The thiamine-deficient sorbitol diet, with and without penicillin, and the glucose control ration used in experiment F-59 were fed to lots of three male weanling rats weighing between 45 and 53 gm at the beginning of the test. All animals on the deficient diet lost weight during the first, and did not survive the second, week. There was no mortality among the controls which gained weight (table 6). Death of the animals on the deficient diet was attributed to a sharp decrease in temperature in the unheated room in which the animals were housed. Although no record of room temperature was kept the daily low recorded at a weather bureau station 15 miles from the laboratory ranged from 30 to 49°F. during the first 6 days the experiment was in progress.

The effect of a cool environment was explored in two experiments, employing three male weanling rats on each of the diets used in experiment F-59 A. In experiment F-59 D (table 6) none of the rats on deficient diets survived the first week. Room temperature was maintained at 40 to 50°F. during this time. The rats in experiment F-59 C were allowed two weeks in a warm room (75° F.) to adjust to their diets and then placed in the cool room. As shown in table 6 all the animals survived. The presence of penicillin in the diet appeared to reduce slightly the shock of this change in ambient temperature.

Experiment F-59 B. In this experiment three male rats were fed each of the following diets used in experiment F-59: glucose control and thiamine-deficient sorbitol diet with and without penicillin. In addition two female rats were fed each of the two sorbitol diets. These animals were maintained in a heated room for 6 weeks and then moved to a cool room for an additional 5 to 7 weeks. As will be noted in figure 2, the presence of penicillin in the thiamine-deficient sorbitol diet did not improve the growth rate of male rats. And the growth rate of rats on the thiamine-deficient sorbitol diet was less than that of

TABLE 4

Effect of penicillin on growth of chicks fed thiamine-deficient diets containing sorbitol, glucose, sucrose and fructose

Experiment F-54

Treatment	Penicillin	Av. change from initial wt.		Av. days to death
		2 weeks	3 weeks	
		gm	*gm*	
Control[1]				
Glucose	no	135.2(19)[2]	432.3(19)[2]	12.0
Sorbitol	no	72.3(18)	270.9(18)	2[3]
Fructose	no	52.6(19)	259.0(19)	[3]
Thiamine-deficient				
Glucose	no	– 30.3(8)	– 211.1(1)	16.3
Glucose	no	– 96.1(7)	*192.0*(5)	13.8
Sorbitol	no	– 49.3(7)	– 12.8(1)	13.8
Sorbitol	no	– 79.4(8)	*83.6*(4)	14.6
Fructose	no	– 90.0(7)	— (0)	16.2
Fructose	no	– 88.1(9)	*179.1*(6)	14.7
Glucose	yes	– 85.9(9)	32.0(1)	16.4
Glucose	yes	– 64.1(9)	226.3(6)	14.7
Sorbitol	yes	– 72.0(7)	– 139.2(2)	15.0
Sorbitol	yes	– 97.9(8)	*101.6*(8)	14.0
Fructose	yes	– 65.3(9)	– 38.3(1)	16.1
Fructose	yes	– 7.7(6)	220.7(6)	13.0

Groups whose weight change is shown in italic received thiamine orally after two weeks on experiment.

[1] Average of replicated lots of 10 chicks each, other treatments single lots of 10 chicks.

[2] Survivors within parentheses.

[3] One unaccountably missing.

TABLE 5

Summary of effect of penicillin on thiamine deficiency in the chick

Experiment F-54

Treatment	Av. change from initial wt.		Av. days to death
	2 weeks	3 weeks	
	gm	*gm*	
Thiamine-deficient diets			
Without penicillin	– 72.2 46/60[1]	– 111.9 2/30	15.4
With penicillin	– 65.4 47/60	– 48.5 4/30	15.8
Without penicillin + thiamine orally	—	157.5 15/24	14.9
With penicillin + thiamine orally	—	182.8 19/23	13.9

[1] Number of survivors/initial number.

the rats on the control diet. Penicillin appeared to improve the growth rate of females although the results were inconclusive because of the small number of animals used.

Moving these animals to a cool room had little effect on the characteristic of growth of the control rats and caused a decrease in growth rate of three weeks duration in the male rats fed the thiamine-deficient sorbitol diet without penicillin. The cool environment appeared to have no effect on the male rats fed the thiamine-deficient sorbitol diet with penicillin until the third week, when gains were drastically reduced. In both instances the rats recovered and continued growth in a manner not unlike that of the period prior to this decline. Female rats appeared to be affected during the third week in the cool room. They recovered to continue normal gains (lower two curves fig. 2).

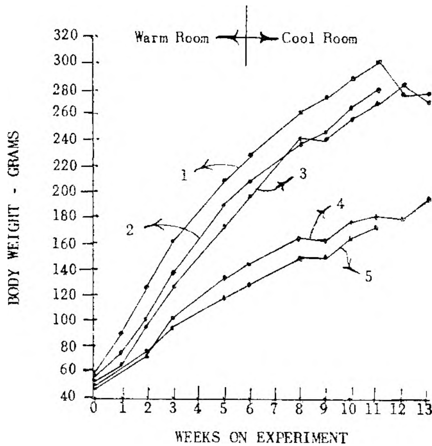

Fig. 2 Effect of presence or absence of penicillin on growth of rats fed a thiamine-deficient diet containing penicillin; 1, complete glucose diet; 2, thiamine-deficient sorbitol diet, no penicillin; 3, thiamine-deficient sorbitol diet with penicillin (three male rats on each of the foregoing treatments); 4, thiamine-deficient sorbitol diet, no penicillin; 5, thiamine-deficient sorbitol diet with penicillin (two female rats in each of treatments 4 and 5).

DISCUSSION

The evidence is quite conclusive that sorbitol at a level of 20% in the diet does not render chicks independent of a dietary need for either thiamine or choline. This is true whether the birds used are day-old chicks, without an established intestinal flora, or 6-week old birds, presumably with an established intestinal flora. The presence of penicillin at a level of 2 mg per pound of feed did not influence the lack of response to sorbitol. Equally lacking in effect were fructose and sucrose, replacing glucose, in a vitamin-deficient diet for chicks.

Rats were able to make near normal gains on thiamine-deficient diets containing 20% sorbitol, provided they were maintained in an adequately heated room during the first two weeks on such a dietary regimen. There was no apparent benefit from the addition of penicillin to the thiamine-deficient sorbitol diet for rats. This is somewhat at variance with the work of Jones and Baumann ('58) who found a supplementary action of penicillin on the effectiveness of sorbitol in enabling rats to overcome a thiamine-deficient diet. Un-

TABLE 6

Effect of environment on response of rats to a thiamine-deficient diet containing sorbitol

Treatment	Experiment F-59A[1] Av. weight change 1st week	Experiment F-59A[1] Av. weight change 2nd week	Experiment F-59D[2] Av. weight change 1st week	Experiment F-59C[3] Av. weight change 3rd week	Experiment F-59C[3] Av. weight change 4th week	Experiment F-59C[3] Av. weight change 5th week
	gm	*gm*	*gm*	*gm*	*gm*	*gm*
Control	18.5	36.0	20.0	26	27	15
Thiamine-deficient (Sorbitol)	– 4.0	(9.3)[4]	(3.5)	3	20	17
Thiamine-deficient(Sorbitol) + penicillin	– 6.3	(10.3)	(3.0)	18	27	12

[1] Low temperatures at official weather station 15 miles from laboratory 30, 38, 40, 49, 41, 60, 59, 57, 43 and 36°F. for first 10 days of test.

[2] Temperature in room at 8 A.M. 50, 50, 48, 42 and 40°F. first 5 days of test.

[3] Animals on diets two weeks prior to being placed in a cool room with range in temperature at 8 A.M. of 40 to 50°F.

[4] Average number of days to death within parentheses.

der our conditions, rats receiving thiamine-deficient diets containing sorbitol continued to grow at a rate similar to that of the control animals over a 13-week period. The data of Jones and Baumann showed that after three weeks sorbitol ceased to promote growth on the deficient diet in the absence of penicillin. Differences in diet may account for this. The diet used in our experiment contained 16% of corn oil, whereas the diet employed by Jones and Morgan contained 4%. Morgan and Yudkin ('57) used a diet containing 30% of casein and 15% of arachis oil which, although considerably higher in protein, was similar to our diet in regard to Calories contributed by fat. Also, the diet used by Jones and Baumann contained penicillin at a much higher level than ours, 22.7 mg per pound vs. 2.

That penicillin increases the thiamine available to the rat was shown by Mameesh and Johnson ('58). In their studies, using C^{14}-labeled thiamine, the presence of 50 mg of penicillin per kilogram of diet promoted increased microbial synthesis of physiologically available thiamine. No growth data were reported so that no direct comparison with our divergent result is possible.

The sorbitol-fed rats had much less riboflavin per gram of dry feces than the control rats, although both groups grew at a comparable rate. Also, the fructose-fed rats had a fecal riboflavin content only slightly less than that of the sorbitol-fed rats, although at the time of sampling the fructose-fed animals were losing weight. The thiamine content of feces from the sorbitol- and fructose-fed rats was similar and considerably less than for the control animals. These findings were surprising in view of the hypothesis that sorbitol mediates its effect through stimulation of intestinal synthesis. Since total fecal excretion was not determined there exists the possibility that both the fructose and sorbitol animals excreted more fecal matter than the control animals. However, this would suggest a similarity in mode of action which the growth data do not support. Since the diets of all three groups contained the same quantity of riboflavin, on a per unit weight basis, it is somewhat surprising that both the fructose and sorbitol groups excreted considerably less riboflavin than the control. The lower riboflavin excretion by the fructose animals might be explained on the basis of reduced food intake, since they were losing weight at the time the feces were collected. However, the sorbitol animals were gaining weight and should have been consuming feed in a manner similar to the control animals. The significance of this difference in vitamin excretion pattern is, at the moment, obscure.

The inability of weanling rats to survive when placed in a cool room concomitant with feeding the thiamine-deficient sorbitol diet invites speculation. We have observed, as was reported by Morgan and Yudkin ('57), that a 6- to 10-day growth lag occurs when rats are fed diets containing sorbitol. It has been suggested that during this period the microflora is undergoing a change and adjustment. This pre-supposes that the population is changing from one providing little thiamine to the host animal to a microbial population supplying enough thiamine to meet body needs. Since the requirement for thiamine increases as environmental temperature is reduced (Kline et al., '45), one might postulate that the reduced temperature caused a greater demand for thiamine than was available from the yet unestablished microbial population, causing death to the animal due to acute thiamine avitaminosis.

Hill ('47) reported that internal control of body temperature in white rats was not achieved until 18 days of age, and that development of this control is rapid from 18 to 30 days of age. The rats which did not survive on a sorbitol thiamine-deficient diet in our studies were between 18 and 30 days of age, the period when temperature regulation control should have been developing rapidly. Our data show that those rats which might be expected to have an established sorbitol-type of intestinal microflora, or a developed temperature control mechanism, could adjust to a cold environment when restricted to a thiamine-deficient diet containing sorbitol.

SUMMARY

The need of the chick for a dietary source of choline and thiamine was not

eliminated by the substitution of 20% of sorbitol, sucrose or fructose for glucose in a purified chick diet deficient in these vitamins. This lack of influence was not affected by the presence or absence of penicillin in the diet and was observed in both chicks started on test at one day and at 6 weeks of age.

Rats fed a diet deficient in thiamine and raised in a warm environment grew in a normal manner when 20% of sorbitol was included in the diet. Substitution of fructose or sucrose for sorbitol resulted in a diminution in weight gains within one or two weeks and weight losses thereafter.

Weanling rats fed a thiamine-deficient diet containing sorbitol in a cool environment were unable to survive a two-week period. Feeding the vitamin B_1-deficient sorbitol diet for two weeks prior to exposure in the cool room enabled rats to live and after an adjustment period to continue a normal growth rate.

Fecal excretion of riboflavin and thiamine by rats receiving thiamine-deficient diets containing sorbitol or fructose was not appreciably different and was considerably lower than excretion of these vitamins by animals receiving the vitamin B_1-supplemented control diet containing only glucose as the carbohydrate.

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The Role of Coprophagy in the Availability of Vitamins Synthesized in the Intestinal Tract with Antibiotic Feeding[1]

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It has been reported that some antibiotics stimulate the growth of rats fed diets limiting in certain B vitamins (Lih and Baumann, '51; Sauberlich, '52; Braude et al., '53; Guggenheim et al., '53; Johnson et al., '53). Although the mechanism by which the autibiotic effects this "sparing" action was not fully understood, it was generally agreed that the antibiotic increased the amount of the limiting vitamin available to the rat. Mameesh and Johnson ('58), using carbon-14-labeled thiamine, showed that penicillin increased the amount of thiamine contributed by intestinal microbial synthesis and calculated its amount. Support of this finding has been reported recently by Wostmann et al. ('59), who found that penicillin did not "spare" thiamine in germ-free rats.

In view of the report by Barnes et al. ('57) that the rat normally consumes and recycles a large portion of its feces, it became important, as a continuation of the studies on the mechanism of the growth-promoting action of antibiotics, to determine whether the rat obtains the products of intestinal microbial synthesis on their first passage through the intestinal tract or by coprophagy. It is the purpose of this report to evaluate the role of coprophagy in the pantothenate and thiamine "sparing" action of oxytetracycline and penicillin, respectively, in the rat.

METHODS

Two studies, each consisting of two experiments, were conducted, using weanling male albino rats of the Sprague-Dawley strain. The rats were housed individually in wire-bottom cages in a temperature-controlled laboratory. Food and water were provided ad libitum. Individual data on daily food consumption were kept, and the animals were weighed at weekly intervals.

Pantothenic acid vs. oxytetracycline. The diet was composed of the following ingredients: sucrose, 72.6; "vitamin-free" casein,[2] 18.0; mineral mixture 446 (Schendel and Johnson, '54), 4.0; choline dry mixture (25% choline), 0.4%. To 1 kg of this diet the following vitamins, premixed with 10 gm of cerelose, were added: nicotinic acid, 100 mg; riboflavin, 16 mg; thiamine·HCl, 40 mg; pyridoxine·HCl, 6 mg; folic acid, 4 mg; biotin, 0.6 mg; vitamin B_{12}, 50 μg; vitamin A, 20,000 I.U., vitamin D, 2000 I.U.; α-tocopherol, 120 mg; and 2-methyl-1,4-naphthoquinone, 1.0 mg. Calcium pantothenate was mixed into the diet at two levels, i.e., 1.0 mg per kilogram of diet (pantothenate-limiting) and 20 mg per kilogram of diet (pantothenate-adequate). To the diets of rats receiving the antibiotic, 100 mg of oxytetracycline per kilogram of diet were added. Six rats were used per group in experiment 1 and 5 per group in experiment 2. Both experiments were terminated at 5 weeks.

The method of Barnes et al. ('57) of preventing and estimating coprophagy by the use of tail cups was used. No tail cups were placed on rats in groups in which coprophagy was allowed.

Thiamine vs. penicillin. The composition of the diet was the same as used in the first study, with the exception that 40 mg of calcium pantothenate replaced the thiamine in the vitamin premix. To avoid losses due to storage of the diet, the thi-

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[2] Labco.

amine was administered by pipetting an aqueous solution of thiamine hydrochloride containing 1% of glycerol onto the daily feed (Kandutsch and Baumann, '53; Waibel, Bird and Baumann, '54; Mameesh et al., '56). The vitamin was added at two levels, i.e., 0.5 μg per gram of diet (thiamine-limiting) and 10 μg per gram of diet (thiamine-adequate). The concentrations of the thiamine solutions were such that 0.5 ml per gram of diet gave the desired level. The solutions were prepared fresh each week and, along with all diets used in the two studies, were kept in the cold. To the diet of rats receiving the antibiotic, 50 mg of crystallin sodium penicillin G were added per kilogram of diet.

In experiment 3, 4 rats were used per group, whereas 5 rats were used per group in experiment 4. All rats were on experiment 4 weeks.

In this study also, coprophagy was prevented by the use of tail cups. However, in this study all rats had tail cups and coprophagy was allowed in certain groups by offering to each, on a free-choice basis, his own weighed feces which had been collected in the tail cup on the previous day. The weight of feces consumed, expressed as a percentage of the feces excreted, was taken as the percentage of coprophagy.

RESULTS

Table 1 gives the results from the experiments on pantothenic acid and oxytetracycline. Experiments 1 and 2 are seen to be in substantial agreement. Oxytetracycline stimulated the growth of rats fed the pantothenate-limiting diet, but was without effect when the vitamin was adequately supplied. The stimulating effect of the antibiotic in rats fed the diet low in pantothenic acid was observed whether coprophagy was allowed or prevented. Statistical analysis of the data from these experiments revealed that coprophagy prevention significantly depressed growth, independent of other treatments. This effect was probably due to the physical stress of the collection cups on the rats.

When coprophagy was measured according to the method of Barnes et al. ('57), it was found that the rats fed the pantothenate-limiting and adequate diets consumed about 39 and 35% of their feces, respectively. When these diets were supplemented with oxytetracycline, coprophagy was approximately 87% on the limiting, and 50% on the adequate diet.

Table 2 gives the results from the experiments on thiamine and penicillin. Again the two experiments (3 and 4) were in substantial agreement. It can be noted, however, that pencillin alleviated thiamine deficiency *only* when coprophagy was allowed, but not when it was prevented. Since tail cups were placed on all the rats in this series of experiments, the growth increment of the coprophagic rats, compared to the non-coprophagic rats fed the thiamine-limiting diet, could not be attributed to the relief of the physical stress from the tail cups, but rather reflects the ability of the rats fed the unsupplemented diet to obtain thiamine by consuming their feces. In these experiments, coprophagy was approximately the same in all groups, the rats consuming between 75 and 85% of their daily fecal output.

TABLE 1

The effect of coprophagy prevention and oxytetracycline supplementation of the diet on gains at 5 weeks of rats fed diets limiting and adequate in pantothenic acid[1]

	Coprophagy allowed		Coprophagy prevented	
	Control	Antibiotic	Control	Antibiotic
	1 μg calcium pantothenate/gm diet			
Experiment 1	69 ± 9.1	87 ± 5.9	55 ± 5.3	90 ± 7.9
Experiment 2	63 ± 8.2	78 ± 6.1	44 ± 7.4	62 ± 7.8
	20 μg calcium pantothenate/gm diet			
Experiment 1	206 ± 7.0	220 ± 5.8	207 ± 9.3	187 ± 7.4
Experiment 2	210 ± 12.3	199 ± 6.2	164 ± 5.3	166 ± 5.7

[1] All figures are average gains in grams ± standard error of the mean.

TABLE 2

The effect of coprophagy prevention and penicillin supplementation of the diet on gains at 4 weeks of rats fed diets limiting and adequate in thiamine[1]

	Coprophagy allowed		Coprophagy prevented	
	Control	Antibiotic	Control	Antibiotic
	0.5 μg thiamine·HCl/gm diet			
Experiment 3	46 ± 8.4	60 ± 2.5	24 ± 4.2	20 ± 2.2
Experiment 4	39 ± 2.2	69 ± 6.4	19 ± 4.3	23 ± 1.9
	10 μg thiamine·HCl/gm diet			
Experiment 3	—	—	142 ± 4.8	132 ± 6.2
Experiment 4	113 ± 3.8	105 ± 8.1	125 ± 6.4	136 ± 9.3

[1] All figures are average gains in grams ± standard error of the mean.

DISCUSSION

The results in table 1 show that oxytetracycline exerted a sparing effect on pantothenic acid-deficient rats both in the presence and the absence of coprophagy. In terms of the mechanism of this effect, the data indicate that the microbially synthesized pantothenate was absorbed directly from the intestinal tract during its first passage through. It could not be known, however, whether oxytetracycline acted by increasing the production of the vitamin by the intestinal microflora or by improving the absorption of the pantothenate thus produced. However, reasoning by analogy with thiamine where increased intestinal synthesis was proved (Mameesh et al., '58), it would appear that pantothenic acid is probably also made at an increased rate by the microflora, and that in the case of pantothenic acid it is probably extra-cellular (with respect to the bacteria) and thus absorbable.

In the experiments with thiamine and penicillin (table 2), penicillin did not stimulate the growth of rats fed a diet limiting in thiamine when coprophagy was prevented, while the usual growth-stimulating effect was obtained when the rats were given access to their feces. It has been reported by Mameesh and Johnson ('58), and later confirmed by Wostmann et al. ('59), that penicillin increases the amount of microbially synthesized thiamine available to the rat. These reports did not show whether the effect of the antibiotic was to increase the production or the absorption of the extra microbially synthesized thiamine. The results in table 2 indicate that this thiamine synthesized by the intestinal microflora was not absorbed to any appreciable extent on its first passage through the intestines and that the animals had to obtain this thiamine by coprophagy. These results may indicate that the bacterially synthesized thiamine is intra-cellular with respect to the bacteria.

From these results it appears that antibiotics stimulate growth in animals fed diets limiting in certain B-vitamins by increasing the production of these vitamins by the intestinal microflora. Some of the vitamins produced under such conditions are available and absorbed directly, e.g., pantothenic acid, while others are not available for absorption and must be obtained by coprophagy, e.g., thiamine.

SUMMARY

Oxytetracycline (100 mg/kg diet) alleviated pantothenic acid deficiency in normal as well as non-coprophagic rats, while penicillin (50 mg/kg diet) improved growth in rats fed a thiamine-limiting diet only when coprophagy was allowed, but not when it was prevented. Obviously, the production, rather than the absorption, of thiamine synthesized by the intestinal microflora was stimulated by penicillin; the thiamine thus produced was not available to the rat except by means of coprophagy. Under similar conditions, pantothenic acid was absorbed on its first passage through the intestinal tract.

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The Effect of Nitrogen Intake upon the Urinary Riboflavin Excretion of Young Male Adults

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The amount of riboflavin excreted in the urine has been reported as being influenced inversely by the quantity of protein ingested. Thus, Oldham et al. ('47) found that women consuming a diet containing 18 gm of nitrogen excreted less urinary riboflavin than when the food provided approximately 5 gm of nitrogen. The same report reviewed further evidence for this inverse relationship between dietary nitrogen and urinary riboflavin as shown by studies on humans and other animals. Pollack and Bookman ('51) reported high levels of urinary riboflavin when hospital patients were in negative nitrogen balance. These authors state: "evidence is presented that nutritionally normal individuals will usually retain more than 50 percent of the ingested riboflavin when in nitrogen equilibrium, and less than 50 per cent when in negative balance." Bro-Rasmussen ('58) has presented a critical review of the above-mentioned papers and others concerning the interdependence of riboflavin and nitrogen.

In the study herein reported previous findings are extended in that data are given on urinary riboflavin excretion of human subjects maintained on a nearly nitrogen-free diet as well as a below-equilibrium level of nitrogen, a near-equilibrium level, and a diet of ample nitrogen. The two highest intakes closely simulated the quantities studied by Oldham et al. ('47). Riboflavin intake was maintained constant throughout the 4 nitrogen variations, so that variable riboflavin intake was not a complicating factor in interpretation of the findings. It was possible, therefore, to test within the same subject the sensitivity of the nitrogen-riboflavin relationship over a range of 4 levels of nitrogen intake.

EXPERIMENTAL PROCEDURE

Subjects

Seven healthy men served as subjects in each of two studies—the first in April and May, 1955, and the second, a repeat of the first, in February and March of the following year. One subject (RI) participated in both groups, and was counted twice in figuring the total of 14 subjects for both years. The men were 22 to 27 years of age, and at the beginning of the studies weighed between 56.1 and 80.6 kg (table 1). The subjects lived together in a house maintained by the University, and carried out their customary activities as students. A medical examination given both at the beginning and at the end of the controlled study, was the basis of their selection as participants, and served as a check on the adequacy of the dietary regime.

Experimental design

Each experimental study was divided into 4 consecutive parts of 12–, 10–, 10– and 10-days' duration during which the diets were designed to supply zero (as nearly as possible), 2, 4 and 17 gm of nitrogen, respectively, and 1.6 mg of riboflavin throughout all periods. The intake of the vitamin was identical for all subjects and was not individually adjusted to body weight or to protein intake. The 1.6 mg was considered a generous intake by the authors as it was the allowance recommended by the National Research

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TABLE 1

Age, height, initial weight and average weight per period (of both studies) for 14 subjects

Subject	Age	Height	Initial weight	Average weight per period				
				I	II	III	IV	
	yr.	cm	kg	kg	kg	kg	kg	
1955								
TC	25	185.4	80.6	80.8	80.6	80.9	81.3	
AC	25	181.6	80.5	80.4	80.2	79.8	79.6	
DF	24	195.6	79.3	78.1	77.2	76.9	77.0	
NG	22	177.8	70.9	69.8	68.9	68.4	68.5[1]	
RI	24	175.3	68.0	67.4	67.0	66.9	66.2[1]	
MT	27	172.7	66.9	66.5	66.2	66.2	66.5	
JO	23	171.4	56.1	55.6	54.9	55.2	55.4	
1956				I-2	II-2	III-2	IV-2	V-2
HG	22	182.9	77.9	77.3	77.0	77.2	76.9	77.8
SD	24	174.0	75.6	74.2	73.7	74.0	74.4	75.2
ED	24	180.3	74.8	73.4	72.5	72.3	72.8	73.8
OR	23	193.0	73.2	72.7	72.2	72.2	72.8	74.0
DCF	25	177.8	67.8	66.8	66.7	67.2	67.5	68.7
RI	25	175.3	67.4	67.1	66.5	66.5	66.2	67.4
TF	23	179.1	64.9	64.5	64.1	64.1	64.5	64.8

[1] Average for only 6 days.

Council ('53) for men weighing 65 kg. However, had the recommended procedure (NRC, '53) been used of determining the riboflavin intake by multiplying the grams of protein by the factor 0.025, 2.8 mg instead of 1.6 mg of riboflavin would have been given during the periods of highest nitrogen intake.

The last period (17.5 gm of nitrogen) was extended to 15 days in the second study in order to give some indication as to whether equilibrium in the vitamin excretion had been reached within 10 days, or whether the amounts excreted would continue to increase as was suggested by the trend in the first study.

In future references, the periods in the two studies will be designated as periods I to IV and I-2 to V-2 for the studies in 1955 and 1956, respectively. In view of the similarity of the mean values for nitrogen and riboflavin excretion in each of the two studies, all data might have been combined into means representing the 14 cases and thus treated as one study. The authors believe, however, that by showing the agreement between the two separate studies, the validity of the findings is strengthened.

Dietary regime

The nitrogen-free diet used in periods I and I-2 was, of necessity, very plain. It consisted of cornstarch cookies, lemon juice, washed butter fat and sugar (table 2). Foods added to this basic menu during the last three periods were applesauce, oatmeal, lettuce, French dressing, potato, lean ground round of beef and 20% cream. White bread was added during the second and third periods. In succeeding periods, the cornstarch cookies were discontinued, whole milk, casein bread and preserves were added to furnish the additional protein and to maintain the calories constant.

The cornstarch cookies were based on the formula used by Bricker et al. ('49), with small increases in sucrose, butter fat, lemon juice and baking powder which made the cookies more palatable. Daily vitamin supplements were divided equally between noon and evening meals. Details concerning other daily supplements are also itemized in table 2. When the cornstarch cookies, and thus the salt mix, were omitted, calcium carbonate was supplied by capsule. The iron intake was lower during these periods than in the first three, but, even so, it remained above the recommended allowance for that nutrient. For all nutrients listed in the table, the combined intake from food and supplements met the National Research Council's recommended allowances ('53). Food nutrients were calculated according to Bowes

TABLE 2

Daily foods and vitamin and mineral supplementation

	Period			
	I, I-2	II, II-2	III, III-2	IV, IV-2, V-2
	gm	*gm*	*gm*	*gm*
Foods				
Cornstarch cookies	350	350	350	—
Lemon juice	80 ml	80 ml	80 ml	100 ml
Applesauce		100	100	180
Oatmeal, raw wt.		8	16	40
Lettuce		30	30	60
French dressing		20 ml	20 ml	40 ml
Potato		11	21	100
Beef, raw wt.		9	18	300
Cream, 20%		51	102	50
Bread, white		73	145	—
Bread, casein		—	—	150
Preserves		—	—	150
Milk		—	—	183
Washed butter fat[1]	21–61	10–46	5–49	33–87
Sugar[1]	175–350	90–322	37–274	46–260
Supplementation				
Ascorbic acid	50	50	50	50
CaCo$_3$	—	—	—	1643
Ca pantothenate	12.0	11.5	11.1	5.9
Niacinamide	16.0	14.5	12.4	—
Pyridoxine·HCl	2.0	2.0	1.6	1.7
Salt mix[2]	yes	yes	yes	—
Thiamine·HCl	1.6	1.4	1.3	0.8
Vitamin A[3] (I.U.)	5000	5000	5000	5000
Vitamin D[3] (I.U.)	1000	1000	1000	1000

[1] Butter fat and sugar were individually adjusted to furnish calculated caloric intake. Fat supplied one-third of daily calories.

[2] Prepared according to Rose ('50) and included in cornstarch cookies.

[3] Vitamins A and D supplied by 5 drops of Natola, Parke-Davis.

and Church ('46), Bradley ('42) and Taylor and MacLeod ('48).

In order to maintain the body weight of the subjects, the amounts of butter fat and sugar included in the diet were individually adjusted. The amount of fat was so regulated throughout the study that it supplied 33% of the daily intake of calories. The caloric intake for each subject was determined initially by allowing 45 Cal./kg of body weight. In the case of some individuals, especially in the second study, it became necessary to raise the caloric intake to 48, 50 and even 53 Cal./kg in order to maintain weight. Individual daily caloric intakes for both studies and all subjects thus ranged from a minimum of 2500 for JO to a maximum of 3975 for SD.

Laboratory Procedures

Twenty-four-hour urine samples were collected in brown bottles containing 60 ml of glacial acetic acid. After adjusting to volume, a portion of the total daily collection was taken for immediate determination of riboflavin. Two other portions were held under refrigeration until analyzed for nitrogen and creatinine. These three assays were made daily in both studies, except that during the first 5 days of periods I-2, II-2 and III-2 only nitrogen and creatinine were determined. The decision to omit the riboflavin analyses was made on the assumption that the excretion pattern of this material had been established during the first study. Thus, since the values were to be omitted from the means for the periods, the analyses were not deemed justifiable. Creatinine determinations were a means of indicating complete urinary collections.

Feces marked with ferric oxide were combined into 4-day pools for periods I and I-2, and into 5-day pools for the remainder of the study. Nitrogen determi-

TABLE 3

Mean daily intake of riboflavin and nitrogen (analyzed values)

Period	Days	Daily intake			
		Riboflavin			Nitrogen
		Food	Supplement	Total	
		mg	*mg*	*mg*	*gm*
1955					
I	12	0.03	1.50	1.53	0.2
II	10	0.23	1.36	1.59	2.5
III	10	0.40	1.24	1.64	4.4
IV	10	1.17	0.45	1.62	17.2
1956					
I-2	12	0.03	1.57	1.60	0.2
II-2	10	0.22	1.45	1.67	2.4
III-2	10	0.41	1.23	1.64	4.6
IV-2	10	1.14	0.43	1.57	17.5
V-2	5	1.14	0.43	1.57	17.5

nations only were made on the fecal material.

As foods were prepared for the subjects, an additional serving was set aside for laboratory analyses. Food pools corresponded to those mentioned above for the feces. For the pools, the foods were thoroughly mixed in a Waring Blendor. Prior to determination of riboflavin in the foods, clarase and papain were added to aliquots which were then incubated at 37°C for 16 to 18 hours.

The fluorometric procedure as described by Burch et al. ('48) was used for all riboflavin assays and the Kjeldahl method for nitrogen determinations. Analytical values for riboflavin and nitrogen content of the diets during the 9 periods of the two studies are summarized in table 3. All further references to intake of these nutrients are based on analyzed rather than calculated values.

With the few exceptions noted in table 4, urinary nitrogen and riboflavin, and fecal nitrogen values, are means of data collected during the last 5 days of each period. This selection was made on the assumption that the first 5 days should be discarded as exhibiting adjustment to the different levels of dietary protein and that excretions during the last part were more representative of the effect of the dietary level in question.

RESULTS

In summary of the nitrogen metabolism data (tables 4 and 5), all subjects were in negative balance during the first two periods, in near equilibrium during the third period, and in positive balance during the 4th. By the end of the 4th period, however, the total of accumulated negative balances was still greater for each subject than the total of the positive nitrogen balances. The possible adequacy of the various nitrogen intakes for all subjects during each of the studies can be assessed from the nitrogen balance data. From these mean data it is apparent that, except for subjects TF and DCF an intake somewhat above 4 gm of nitrogen was required for equilibrium. The individual nitrogen requirements can be calculated from these data by extrapolation but such information is not the subject of this paper.

During nitrogen-free periods I and I-2, subject averages of urinary riboflavin spread from 557 to 997 μg per day. The means of 742 and 723 μg for the two studies result in an overall mean of 732 μg for both years. This mean was more than 200 μg higher than comparable ones during the next two levels of dietary nitrogen, and almost 500 μg greater than the means of periods IV and IV-2. For each study the lowest daily excretion values of the vitamin for individual subjects occurred between the second and the 5th days of the 4th period (see figs. 1 and 2 for mean daily values). Three of the 14 subjects reached a low of 105 μg but only for one day. The mean excretion values of 217 and 277 μg during periods IV and IV-2,

respectively, are low in comparison with values reported by other laboratories for studies in which comparable intakes of riboflavin were used. Thus, Keys et al. ('44) reported that 4 men consuming 1.3 mg of riboflavin daily excreted an average of 308 μg (range of 213 to 422). Brewer et al. ('46) found that 9 college women on a daily intake of 1.6 mg of riboflavin excreted about 320 μg (range of 170 to 440) whereas Horwitt et al. ('48) reported an excretion of 434 ± 185 μg for 39 persons ingesting approximately 1.6 mg of the vitamin for 100 days. Oldham et al. ('47) reported immediately-occurring low values of 73, 76, and 97 μg for urinary riboflavin as three subjects changed from a low to a high intake of nitrogen when daily riboflavin intake was slightly more than 1 mg.

Visual examination of figures 1 and 2 suggests that significant differences in urinary levels of riboflavin might be expected between the first period and each succeeding one as well as between the 4th and the two preceding periods. When the "t" test was used to take into account the day to day individual variation, the statistical findings (table 6) gave support to these observations. The difference between the second and third periods was significant in less than half of the cases.

The design of the first and second studies was identical up through the 4th period of high nitrogen intake. Period V-2 is peculiar to the second study only, hence, the data collected during those 5 days were summarized in table 5 apart from the first 4 periods.

Each subject was in positive nitrogen balance each day of period V-2. The total of the positive nitrogen balances for the entire 5 periods exceeded the sum of the negative balances for only two of the subjects as evidenced by the data on nitrogen deficit in table 5. According to this cri-

TABLE 4

Subject means[1] for riboflavin excretion and nitrogen balance during eight periods

Subject	Urinary riboflavin				Nitrogen balance			
	Period				Period			
	I	II	III	IV	I	II	III	IV
	μg	μg	μg	μg	gm	gm	gm	gm
1955								
TC	608	362	421	174	−2.797	−0.898	−0.126	+2.992
AC	997	438	569	248	−3.177	−1.214	−0.540	+0.044
DF	757	473	471	250	−3.067	−1.390	−0.819	+1.025
NG	750	460	415	162[2]	−3.416	−1.510	−0.821	+4.264[2]
RI	777	494	269	138[2]	−2.468	−0.857	−0.013	+4.926[2]
MT	588	429[3]	329	168	−2.805	−0.938[3]	−0.172	+3.060
JO	717	581[3]	597	246	−2.329	−0.719[3]	−0.448	+2.621
Mean	742	462	439	217	−2.866	−1.075	−0.420	+1.948
SD	± 134.7	± 67.1	± 113.9	± 42.3	±0.3862	±0.2978	±0.3288	±1.3469
	I-2	II-2	III-2	IV-2	I-2	II-2	III-2	IV-2
1956								
SD	668	560	427	239	−2.992	−1.251	−0.043	+3.521
SD	660	550	483	320	−3.293	−1.595	−0.299	+3.557
ED	557	462	391	178	−3.574	−1.670	−0.628	+4.472
OR	781	530	515	344	−2.615	−0.949	−0.229	+4.477
DCF	728	487	421	193	−3.059	−1.216	+0.019	+3.899
RI	803	546	498	280	−2.759	−1.471	−0.052	+2.887
TF	867	548	470	383	−2.635	−0.901	+0.559	+3.450
Mean	723	526	459	277	−2.990	−1.293	−0.096	+3.752
SD	± 104.1	± 37.1	± 46.2	± 77.3	±0.3559	±0.3013	±0.3626	±0.5768

[1] Means are for last 5 days in each period except IV, in which only days 6 to 9 were used, as a load dose was given on day 10. Data for V-2 appear in table 5.

[2] Mean of days 1 to 5 only as subjects did not complete the period; values were not included in the period mean.

[3] Mean of days 7 to 10 only as collection bottles were accidently mixed on day 6.

TABLE 5

Riboflavin excretion, nitrogen balance and deficit for seven subjects during periods IV-2 and V-2

Subject	Urinary riboflavin			Nitrogen balance			Nitrogen deficit[1]
	IV-2		V-2	IV-2		V-2	
	Days 1–5	Days 6–10	Days 1–5	Days 1–5	Days 6–10	Days 1–5	
	μg	μg	μg	gm	gm	gm	
HG	172	239	247	+5.272	+3.521	+4.563	−11.156
SD	226	320	366	+5.498	+3.557	+3.451	−29.678
ED	136	178	149	+6.426	+4.472	+4.765	−34.445
OR	274	344	373	+5.470	+4.477	+3.943	− 0.492
DCF	147	193	234	+4.833	+3.899	+3.611	+ 0.974
RI	213	280	285	+4.734	+2.887	+3.197	− 8.814
TF	254	383	408	+6.364	+3.450	+3.545	+ 1.484
Mean	203	277	295	+5.514	+3.752	+3.868	
SD	± 53.0	± 77.3	± 92.5	±0.6692	±0.5768	±0.5899	

[1] Figures obtained by totaling daily nitrogen balances of entire study.

TABLE 6

Significance according to "t" test of period differences in urinary riboflavin excretion

Period comparison[1]	Subject						
	TC	AC	DF	NG	RI	MT	JO
1955							
I–II	**[2]	**	**	**	*	**	**
II–III	—	*	—	—	**	**	—
III–IV (days 1–5)	**	**	**	**	**	**	**
III–IV (days 6–9)	**	**	**	inc.[3]	inc.[3]	**	**
IV (days 1–5)–IV (days 6–9)	**	—	—	inc.[3]	inc.[3]	**	—
	HG	SD	ED	OR	DCF	RI	TF
1956							
I-2–II-2	*	*	—	**	**	**	**
II-2–III-2	**	**	—	—	**	—	—
III-2–IV-2 (days 1–5)	**	**	**	**	**	**	**
III-2–IV-2	**	**	**	**	**	**	—
IV-2 (days 1–5)–IV-2	**	**	*	*	—	**	**
III-2–V-2	**	**	**	**	**	**	—
IV-2 (days 1–5)–V-2	**	**	—	**	**	**	**

[1] Unless specified, calculations were based on days 6 to 10 except in period II, only days 7 to 10 were used for JO and MT.

[2] Code: — not significant. * significant, 0.05 level. **highly significant, 0.01 level.

[3] Incomplete collection as subject did not finish period IV.

terion, 5 of the men had not fully replaced the nitrogen losses incurred earlier in the study.

During the 5 additional days on the intake of 17.5 gm of nitrogen (period V-2), 6 of the 7 subjects were still excreting riboflavin in amounts that were significantly less than those found during the third period on 4.6 gm nitrogen (table 6), but since excretion during the 5 days of period V-2 had shown a significant increase over that of days 1 to 5 of period IV-2, one cannot help wondering whether with further time on the same diet the urinary riboflavin excretion might not have risen to considerably higher levels.

DISCUSSION

In considering the results of the study as a whole (figs. 1 and 2), two areas stand out prominently — the extremely high riboflavin excretion values at the beginning of the study, and the abrupt drop in urinary riboflavin occurring simultaneously with the administration of 17 gm of nitrogen.

The design of the study does not permit conclusive evidence concerning reasons for the exceptionally large amounts of riboflavin excreted during the first few days of nitrogen-free consumption. A high riboflavin content in pre-study diets is undoubtedly a partial explanation. Records kept by the subjects in 1956 of self-selected diets for the two days immediately preceding the study, showed that the average intake of the vitamin was 2.6 mg with a minimum of 1.2 mg (DCF) and a maximum of 4.8 mg (RI). The calculated intake of protein also was high, averaging 110 gm (60 to 150 gm). Thus, when the subjects started the low-nitrogen diet there was a sudden marked reduction in protein intake which may have accounted in part for the elevated riboflavin values. A comparable situation with regard to change in protein level exists between the third and 4th periods; in the latter, however, the sequence was reversed in that the low-nitrogen diet preceded the high intake. In

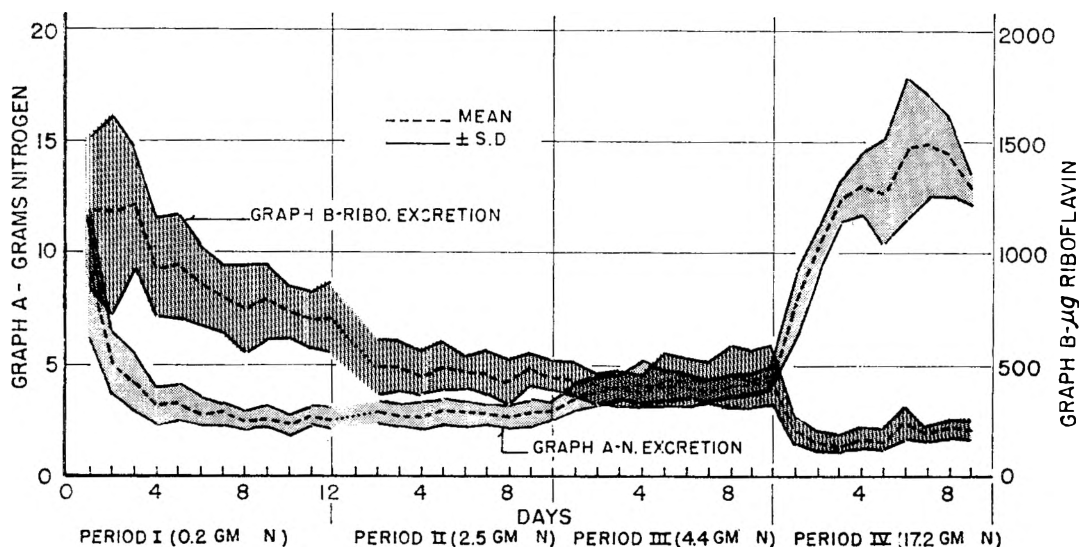

Fig. 1 Daily urinary riboflavin and nitrogen excretion values, average ± SD for 7 subjects —1955 study. Data are not plotted for the first day of period II as initiation of daylight saving time cut this to 23 hours.

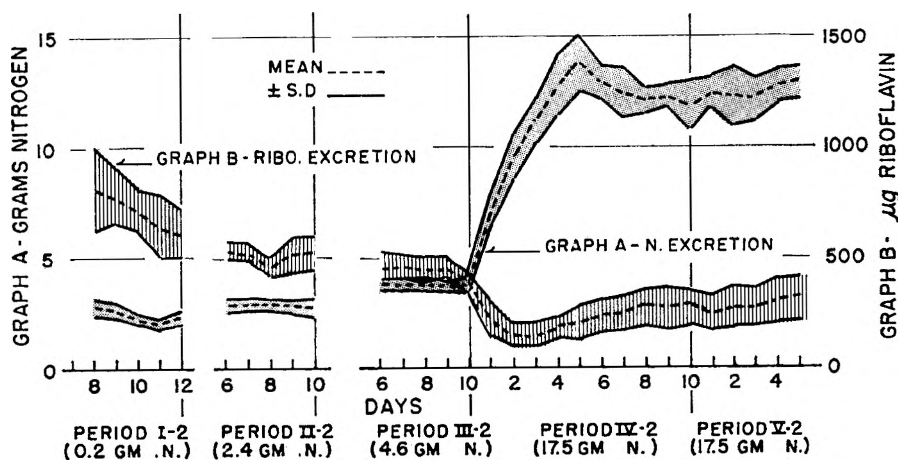

Fig. 2 Daily urinary riboflavin and nitrogen excretion values, average ± SD for 7 subjects —1956 study. As explained in the text, urinary riboflavin was not assayed during the initial days of periods I-2 to III-2.

this instance, as the dietary nitrogen was increased, the urinary excretion of riboflavin decreased immediately. Oldham et al. ('47) reported increases in urinary riboflavin of 380, 169 and 163 μg in three subjects 24 hours after nitrogen intake had dropped from 17.7 to 20.1 gm to 4.8 to 5.2 gm. In a review of nitrogen storage in the adult animal, Kosterlitz and Campbell ('45) stated that upon going from a normal protein diet to a protein-free diet, there was an immediate rapid loss of liver cytoplasm, and thus, of nitrogen. According to Sarett and Perlzweig ('43) and Oldham et al. ('47), in periods of negative balance, high levels of urinary riboflavin may be due to a release of the vitamin stored in conjunction with nitrogen as well as the inability of the liver to store dietary riboflavin without a sufficient amount of nitrogen.

The immediacy and extent of the decrease in urinary riboflavin resulting after a change from low nitrogen (4.5 gm) to high nitrogen (17.5) can be illustrated by the fact that the mean daily riboflavin for all subjects in 1955 was 447 ± 136.6 μg on the last day of the third period and 220 ± 77.6 on the first day of the 4th period. Similar values for 1956 were 413 ± 42.8 and 248 ± 83.5, respectively. These mean daily riboflavin values decreased to a minimum of 151 ± 43.1 and 172 ± 54.5 on the third day of the 4th period for the 1955 and 1956 studies, respectively.

In considering negative nitrogen balances and a return to more positive balances in relation to the amount of riboflavin excreted in the urine, Pollack and Bookman ('51) discussed labile and stable proteins. Thus, according to their concept, labile proteins, which include the flavoproteins, increase or decrease rapidly as the body shifts from a negative to a positive nitrogen balance or from a positive to a negative balance, respectively. The amount of riboflavin stored in conjunction with these proteins would then likewise change rapidly.

Acceptance of and evidence recorded in the literature of labile and stable division of proteins does not appear to be as precise and positive as presented by Pollack and Bookman. Some investigators have questioned any qualitative differences in protein movement (Luck, '36). However, Pollack and Bookman's interpretation neatly fits the data of the present study and other published data reviewed in this paper.

It is possible, then, according to this theory that, while the subjects in the studies herein being reported were in high negative balance, labile proteins were being used in counteracting the lack of dietary protein; thus, riboflavin was released. It is not known at what time in the study labile protein reserves were exhausted (if they were), but it is possible that by the second and third periods only stable protein reserves remained to be used from which there would be little or no release of riboflavin. The riboflavin excretion was found to be somewhat constant during these two periods. In the 4th period, as shown by the positive nitrogen balances, protein reserves were being replenished. As the labile portion filled rapidly, riboflavin, therefore, was simultaneously incorporated within the body.

The sequence found within the periods on 17 gm of dietary nitrogen can also be explained according to Pollack and Bookman ('51). Immediately with the offering of 17 gm of nitrogen in the 4th period, the riboflavin decreased in the urine and the subjects reached their highest positive nitrogen balances. As the period progressed, the urinary riboflavin increased slowly and the nitrogen balances tended toward equilibrium. By the end of the studies, however, only two subjects had overcome nitrogen deficits; that is, the sum total of positive balances equaled or exceeded the totaled negative balances for the entire study. Pollack and Bookman ('51) reported that "labile reserves can be replenished long before the stable proteins are replaced. Thus it is possible to have a high riboflavin retention during one phase of positive nitrogen balance and a normal or poor riboflavin retention in the subsequent periods of positive nitrogen balance."

Oldham et al. ('47), in their study on three young women, fed two different levels of protein (4.76 to 5.85 gm of nitrogen and 17.68 to 20.14 gm of nitrogen) over three 10-day periods, beginning and ending with the lower level of nitrogen. The

intake of riboflavin was 859 to 1060 μg and 1237 to 1519 μg on the low and the high levels of nitrogen, respectively. They concluded that "an inverse relationship exists between urinary riboflavin excretions and nitrogen balances." The relationship found in the studies herein reported was very similar to that of the above-mentioned paper and was found to apply with 22 days on two lower levels of nitrogen intake as well as when the highest intake was continued for 5 days.

As the present study did not give sufficient data to allow the authors to conclude as to specific mechanisms involved in the nitrogen-riboflavin relationship, the investigators can only suggest possible solutions. In addition to the effect of proposed labile and stable proteins, there is the possibility that differences in the ratio of synthetic to natural sources of riboflavin during the 4 periods may have had some role in affecting the observed relationship between urinary protein and riboflavin. Thus, as is shown in table 3, almost all of the riboflavin of the nitrogen-free diet was in synthetic form whereas during the period of high nitrogen, the greatest portion of riboflavin was in natural foodstuffs. Everson et al. ('48, '52) found the synthetic form of the vitamin much more available to the body than the riboflavin in some natural foods, as evidenced by improved retention of synthetic forms, digestibility being the main factor. In the present studies, the quantity of urinary riboflavin decreased as more of the dietary riboflavin was given in natural foods. In spite of these data the change of riboflavin, from synthetic to natural as the study progressed, was not considered by the authors to be an important factor in observed changes in urinary riboflavin.

SUMMARY AND CONCLUSIONS

During the spring seasons of 1955 and 1956, 14 healthy male subjects, 22 to 27 years of age, were maintained on diets containing approximately zero, 2.5, 4.5, and 17 gm of nitrogen during consecutive periods of 12, 10, 10 and 10 to 15 days, respectively. Riboflavin intake was held constant at 1.6 mg. Urinary riboflavin and urinary and fecal nitrogen were determined.

As the nitrogen balance became more positive, and as dietary and urinary nitrogen increased throughout the study, the urinary riboflavin decreased. For example, in 1956, the average nitrogen balances for 7 subjects were −2.990 ± 0.3559; −1.293 ± 0.3013; −0.096 ± 0.3626; +3.752 ± 0.5768; and 3.868 ± 0.5899 gm for the first through the 5th periods, respectively. The riboflavin excretion values (in micrograms) for comparable periods were 723 ± 104.1; 526 ± 37.1; 459 ± 46.2; 277 ± 77.3; and 295 ± 92.5. The data on the relationship between urinary riboflavin and nitrogen were interpreted on the basis of labile and stable protein reserves with the assumption that the flavoproteins may be part of the labile rather than stable protein.

Unusually large amounts of riboflavin (mean value, 1186 ± 341.3 μg) excreted in the urine during the first three days of the study may have been influenced by pre-study diets and severe changes in nitrogen intake.

This study again stresses the point that, in attempting to set a requirement for riboflavin on the basis of urinary riboflavin excretion, the intake of protein and the nitrogen balance must be considered in the experimental plan and in the conclusions drawn.

ACKNOWLEDGMENTS

The authors wish to acknowledge the cooperation of Dr. Floyd Boys in this project and to express their appreciation to the students who served as subjects and to Sally Parsons. Patricia Lowy and Marcia Manring for their technical assistance.

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

Convention Hall, Atlantic City, New Jersey
April 13–17, 1959

COUNCIL MEETINGS

Council meetings were held at the Traymore Hotel, Atlantic City, on Saturday evening, April 11, Sunday morning and evening, April 12, Monday morning, April 13, and at various times throughout the week. Formal actions of the Council reported at the Society business meetings are included in the following minutes.

SCIENTIFIC SESSIONS

There were a total of 151 scientific papers submitted by members (26 more than previous year). A total of 16 of these were transferred to the programs of other Societies and 26 transferred to intersociety sessions. With the 12 papers received by transfer from other Societies, a nutrition program of 12 half-day sessions was arranged. In addition, three half-day symposia were scheduled with invited speakers. These included a symposium arranged by Dr. Olaf Mickelsen on "Effects of High Calcium Intakes" (cosponsored by the Metabolism and Nutrition Study Section, National Institutes of Health), one arranged by Dr. W. H. Sebrell, Jr. and Dr. A. E. Schaefer on "Nutritional Appraisal —Haiti, Alaska, and Ethiopia," and the principal symposium on "Protein Requirement and its Assessment in Man," a panel discussion arranged and moderated by Dr. Paul György. All scientific sessions were well attended with the various symposia drawing up to 600 persons.

In addition, 76 abstracts from various Societies, including the American Institute of Nutrition, were divided into 7 intersociety sessions on "Atherosclerosis" under the direction and sponsorship of the Nutrition Society. These sessions were well attended.

BUSINESS MEETINGS

Dr. W. J. Darby, President, presided at the two business meetings held on April 14 and 16 in Room E. Approximately 140 members were present on Tuesday and 100 members on Thursday. The following items were acted upon:

I. *Minutes of 1958*

The minutes of the 1958 meeting, as published in The Journal of Nutrition, 66: 139, September, 1958, were approved.

II. *Election*

The Secretary transmitted the sealed ballots to the Tellers' Committee, Dr. C. D. Tolle and Dr. Elsa O. Keiles. At the second business meeting, the Committee reported election results on 313 ballots as follows:

Effective July 1, 1959:
President: D. Wayne Woolley
President-Elect: Floyd S. Daft
Treasurer: J. B. Allison
Councilor: Wendell H. Griffith

Effective May 1, 1959:
Associate Editors (4-year term):
Douglas V. Frost
Alfred E. Harper
Olaf Mickelsen

(See list of all officers on page 102).

III. *Constitutional Amendments*

By over two-thirds of all votes cast, the following constitutional amendments were adopted (please refer to the September issue of *Federation Proceedings*, Vol. 17, 814, 1958 for the former wording of the Constitution and Bylaws):

A. *Eligibility for Membership*: By vote of 263 for to 11 against Article I, Section 1 as changed, now reads:

Section 1. *Eligibility for membership.* Any person who has conducted and published meritorius original investigations in some phase of nutrition and who is presently professionally active in the field of nutrition shall be eligible for membership in the Society.

B. *Honorary Members.* By vote of 268 for to 6 against, a new section of Article 1 now states:

Section 6. *Honorary members.* Distinguished individuals of any country who are not members of the American Institute of Nutrition and who have contributed to the advance of the science of nutrition shall be eligible for proposal as Honorary Members of the Society. Proposals for Honorary Members shall be made by at least two members of the Society to the Secretary in writing 6 weeks before the annual meeting with statements of their qualifications. The Council may, from the candidates so proposed, make nominations to the Society at the spring meeting. A two-thirds majority of the ballots cast shall elect. Honorary Members shall pay no membership fees but shall be eligible to subscribe to the official journal(s) at member's rates. They may attend the business sessions of the Institute but without voting privileges.

(Note: The complete Constitution of the Society is printed each year in the September issue of *Federation Proceedings.*)

IV. *Membership Status*

The Secretary reported that as of April 1, 1959, there were 450 active members and 53 retired members, or a total of 503 members in the American Institute of Nutrition. This is a net increase of 50 members since last year. There were no resignations.

Members present at the meeting stood for a moment of silence in memory of the following 3 members who passed away since the last meeting:

William H. Adolph, September 23, 1958
Elmer M. Nelson, December 24, 1958
Eugene F. DuBois, February 12, 1959

Appropriate resolutions which had been received relative to the deceased members were approved and copies are on file in the Secretary's office. Resolutions received for Dr. DuBois and Dr. Nelson, past presidents of the Society, are given below:

RESOLVED, That the American Institute of Nutrition, assembled at Atlantic City, New Jersey, in its Annual Meeting, April 14, 1959, place in its minutes for permanent record this statement of deep regret and sorrow at the passing of the distinguished scientist, Eugene F. DuBois, and further

RESOLVED, That high tribute be paid to Dr. DuBois for his outstanding scientific accomplishments in the science of nutritional physiology, particularly those of defining the metabolic consequences of a variety of diseases, the caloric demands of hypermetabolic states, and the mechanisms of heat loss and heat conservation under normal conditions and in fever. Dr. DuBois was particularly fond of the American Institute of Nutrition which he helped to found as President of the Organizing Group in 1928–1930. He was President of the present American Institute of Nutrition in 1935. He served the Institute as a Member of the Editorial Board of the *Journal of Nutrition* for a number of years and as a Councilor in 1933, the first year of the present organization. In 1958 Dr. DuBois was selected in the first group of Fellows of the American Institute of Nutrition in honor of his distinguished career in the science of nutrition. He was held in great esteem and respect by all of his associates in this Society and elsewhere.

RESOLVED, That the American Institute of Nutrition, assembled at Atlantic City, New Jersey, in its Annual Meeting, April 14, 1959, place in its minutes for permanent record this statement of deep regret and sorrow at the passing of past president Elmer M. Nelson, and further

RESOLVED, That high tribute be paid to Dr. Nelson for his outstanding scientific accomplishments in nutrition science, for his many valuable contributions to the literature on food and nutrition, for his long and effective career as a representative of the Food and Drug Administration in connection with enforcement problems in the field of nutrition, for his able assistance in the establishment of international standards for vitamins, for his devoted service to the American Institute of Nutrition as Treasurer for the period 1945 to 1948, as President in 1948, and as a member of the Editorial Board for two terms, and for the great respect and high esteem accorded to him by all of his associates in this Society and elsewhere.

V. *New Members*

The Council received 79 nominations for membership. The following 76 candidates were approved by members at the business meeting and have accepted membership in the Society.

NEW MEMBERS* — 1959

Clemens J. Ackerman	Nestor W. Flodin
Edward H. Ahrens, Jr.	Richard H. Follis, Jr.
Jay O. Anderson	Martin Forbes
James T. Baldini	Robert S. Goodhart
Lewis A. Barness	Paul Griminger
Marvin C. Bell	Wesley A. Hardison
Charles H. Best	John F. Herndon, Sr.
Anne M. Briscoe	David C. Herting
Helen B. Brown	Eldon G. Hill
Edward C. Bubl	William G. Hoekstra
John J. Burns	Hartley W. Howard
Charles W. Carlson	Eugene E. Howe
Helen E. Clark	Jeng M. Hsu
Nicholas F. Colovos	Lucille S. Hurley
Robert L. Cowan	Norman L. Jacobson
Carl D. Douglass	William H. James
Harold H. Draper	Leo S. Jensen
Hardy M. Edwards, Jr.	Elton L. Johnson
Thomas M. Ferguson	Kendall W. King
Lloyd J. Filer	Morton P. Lamden

Stanley M. Levenson	Dan A. Richert
Hellen M. Linkswiler	Thomas R. Riggs
Richard L. Lyman	Catharine S. Rose
Jose Mendez	Morris H. Ross
Alvin L. Moxon	Robert E. Shank
Frank R. Mraz	Damon C. Shelton
Harold S. Olcott	Thomas R. Sisson
Theodore C. Panos	Leon Swell
Herbert E. Parker	Samuel B. Tove
Henry S. Perdue	Paul E. Waibel
Charlie F. Peterson	Sholon O. Waife
Clyde E. Poling	William M. Wallace
Oscar W. Portman	Richard G. Warner
Martha Potgieter	Paul H. Weswig
Isidor S. Ravdin	Ferdinand Wharton
Elwood F. Reber	Mary Ann Williams
J. Thomas Reid	Harold Yacowitz
Jonathan E. Rhoads	Norman Zamcheck

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

The Secretary announced that new membership nomination forms may be obtained from the Secretary's office any time after August 1. Many eligible nutritionists still exist who are not members of the Society. It is every members' duty to nominate such persons in the event they know of them.

VI. *Treasurer's Report*

The report of Treasurer J. B. Brown from April 1, 1958, to April 1, 1959, as summarized below, was read and approved.

The Auditing Committee, Mary Brown Patton and Fred A. Hitchcock, submitted a report that the Treasurer's account has been found in good order. Their report was approved.

Dues of $2.00 for the coming year were approved (no change from the preceding year). A special assessment of $2.00 per year until 1960 for the International Congress on Nutrition was approved in 1958.

A motion was passed thanking Dr. Brown for the very efficient services he has given as Treasurer over the past 3 years.

VII. *Editor's Report*
Journal of Nutrition

The Editor, Dr. George R. Cowgill, submitted an annual report for 1958, a summary of which follows:

Volumes of *Journal of Nutrition*, 1957	64, 65, 67
Pages published	1862
Papers published	150
Papers submitted	201
Papers rejected	34
Pages per paper	12.4
Supplements	—
Bibliographies (P. C. Jeans, H. J. Deuel, Jr., and H. A. Mattill)	3

Dr. Cowgill, who retires as Editor on July 1, 1959, after serving since 1939, paid

Balance brought forward		$ 1,455.43
Receipts		
455 subscriptions to *Journal of Nutrition* @ $6.50	$2,892.50	
461 Assessments for Fifth International Congress on Nutrition @ $2.00	922.00	
Grants and Contributions to Fifth International Congress on Nutrition	6,997.00	
460 Institute dues	919.85	
Interest on bond	13.80	
Nutrition Dinner — 1958	62.97	
460 Federation assessments	1,840.00	$13,648.12
Total received		$15,103.55
Expenditures		
Wistar Institute	2,892.50	
Federation Office	1,840.00	
Secretary's Office	354.95	
Treasurer's Office	115.00	
Fifth International Congress on Nutrition Fund	7,919.00	
Career Leaflet printing	42.00	
Deficit in Biochemistry and Nutrition Smoker	37.50	
Bank charges	.35	$13,201.30
Balance on hand April 8, 1959		1,902.25
Bond (U. S. Savings)		500.00
TOTAL BALANCE		$ 2,402.25

personal tribute to the many members of the Society who have served, or are now serving, on the Editorial Board. (Sixty-six Society members have served on the Editorial Board while Dr. Cowgill was Editor). Dr. Cowgill also gave tribute to his editorial assistant, Dr. Rebecca B. Hubbell.

The report of the Editor was approved and the following resolution honoring Dr. Cowgill was unanimously accepted:

"The American Institute of Nutrition wishes to express its great appreciation to Dr. George R. Cowgill, Editor of *The Journal of Nutrition* for the past 20 years. The members of the Society are indebted to Dr. Cowgill for his great contributions throughout these 20 years during which he has edited 49 volumes of the Journal. His patience, meticulous care, kindly guidance, and scholarly leadership have been a major influence within our Society. For these, and more, we are deeply grateful."

A motion was also unanimously approved expressing the Society's sincere appreciation to Dr. Rebecca Hubbell for her excellent services as editorial assistant.

VIII. *Selection of New Editor*

The election by the Editorial Board of Dr. Richard H. Barnes, Dean of the Cornell University Graduate School of Nutrition, Ithaca, New York, as the new Editor of *The Journal of Nutrition*, beginning July 1, 1959, was announced by Dr. H. A. Schneider, temporary Chairman of the Board. A meeting of the Editorial Board, with Dr. Barnes, is scheduled for June 19.

IX. *Reports of Committees and Representatives*

A. *Ad Hoc Committee on Journal of Nutrition*: D. M. Hegsted, Chairman; R. W. Engel, O. L. Kline, M. O. Lee, C. V. Moore, B. L. Oser, E. E. Snell, and Charlotte M. Young.

This Committee presented its report at the Council meeting on November 1, 1958, at which time its work was completed.

In summary, their report made the following recommendations:

1. That the format of the Journal should be improved.
2. That the American Institute of Nutrition recover ownership of the Journal of Nutrition and publish it as its official Journal.
3. That profits from the Journal should be used for the support and improvement of the Journal.
4. That the greatest improvement which can be made in the Journal at this time would be the prompt appearance of the monthly issues.
5. That the office of the Editor be satisfactorily financed and that he be provided adequate assistance.
6. The aim of the Journal should be to publish papers from anywhere in the world which contribute fundamental information in the broad area of nutrition, including nutritional biochemistry and the basic aspects of clinical nutrition.
7. That a new and smaller committee be formed to carry on negotiations with the Wistar Institute and to establish future publishing policies.

This interim report was unanimously approved at a special meeting of the Council on November 1, 1958, with special thanks to Dr. Hegsted and his Committee for the excellent work they have done in a short time.

On the basis of this report, a new ad hoc Committee on Publication Policies was formed in November to negotiate with the Wistar Institute (see next item).

B. *Ad Hoc Committee on Publication Policies:* W. J. Darby, Chairman; P. György, O. L. Kline, M. O. Lee, and G. M. Briggs (ex officio).

Dr. Darby reported that it became clear at the onset that The Wistar Institute was desirous of keeping *The Journal of Nutrition* and building up the Wistar press. Therefore, negotiations were started in November to follow the recommendations of Dr. Hegsted's Committee as much as possible. As a result of these negotiations an agreement with The Wistar Institute has been signed. In summary, it has been agreed that for a two-year trial period, beginning July 1, 1959, The Wistar Institute will:

1. Provide a new format for the Journal beginning September, 1959. This will provide opportunity for approximately one-third increase in the numbers of papers published and space for reports, symposia, book reviews, etc., if desired.
2. Publish all issues and supplements within three months from the time manuscripts are received by the printer (on a "best efforts" basis). Similarly, for short material not requiring return of proof for revision, there would be a 6-weeks publication time.
3. Pay the expenses for editorial costs and supplies for the Secretary's office, up to $9,000 per year (formerly, the amount paid was approximately $2,400 plus $500 for stationary supplies or a total of $2,900).
4. Provide promotional services at an estimated amount of $1,000 per year.

5. Supply relevant financial information concerning costs of the Journal.
6. Welcome suggestions from the editorial board or American Institute of Nutrition members as to the development of the Journal.

For the two-year trial period, the American Institute of Nutrition has agreed to:

1. Continue to provide editorial management of the *Journal of Nutrition.*
2. Take a more sympathetic attitude toward an advertising policy which would increase revenues from this source. Disagreements as to suitability of proposed advertising copy would be settled by a Joint Committee of AIN and Wistar.
3. Continue the present policy of requiring all active members to subscribe to the Journal.
4. Increase annual subscription rates for members from $6.50 to $8.50.
5. Establish a joint policy committee of the Editorial Board and the Council of AIN for the two-year period.

At the end of the two-year trial period, a reappraisal of the relationship will be undertaken and at that time if either party is dissatisfied, the possibility of transfer of the ownership of the Journal to American Institute of Nutrition will be discussed. Also, at that time, each party will be free to dissolve its relationship with the other if it so chooses.

It was also understood that if, during the contract period, Wistar does not meet its cash commitments, the society is automatically released from this agreement.

This report was unanimously approved by the Council and by the Society membership present at the meeting.

C. *Public Information Committee:* R. W. Engel, Chairman; A. E. Schaefer, L. Voris, and G. M. Briggs (ex officio).

Dr. Engel reported that two meetings of the Committee were held during the year and that the following actions were taken:

1. Career leaflet. Two thousand additional copies of the leaflet "Career Opportunities in Nutrition" were printed in answer to many requests. Some suggestions for revising this leaflet have been received and are under study. The Committee is seeking advice of the Council on the advisability of printing and mailing the leaflet to the approximately 60,000 high schools in the United States and on the advisability of including the addresses of other societies, such as the American Dietetic Association, on the back of the leaflet as a possible source of additional information. No action taken on this point.
2. The Committee has prepared a mimeographed list of sources of good educational material in nutrition from reliable Boards, Associations, Foundations, etc. in response to a number of inquiries for such material.
3. The Committee made arrangements with Mr. William Rubin of the National Vitamin Foundation to assist in giving more effective news coverage of the American Institute of Nutrition program at the Federation Meetings this year. Several press conferences are planned as well as a press briefing on Sunday night, April 12.
4. On the advice of the Council, the Committee has recommended that the Federation Office obtain a full-time public relations person.

The report was approved.

D. *Representative to various National Research Council Boards and Divisions:* N. R. Ellis.

Mr. Ellis's complete report as approved is on file. A number of meetings were attended during the year in connection with these duties. Various special requests were handled, including nominations, suggestions for program items, and other advice. Mr. Ellis, as American Institute of Nutrition representative, has accepted appointment as a member of the Governing Board of the Agricultural Research Institute (sponsored by the Agricultural Board, a unit of the Division of Biology and Agriculture).

A large number of publications in the area of nutrition are available from the National Research Council-National Academy of Science of interest to Society members. Lists of such publications are available on request from the Academy and include the 1958 revision of "Recommended Dietary Allowances," and booklets on "Food Packaging Materials," "Commercial Sources of Animals for Research," and "Recommendations on Undergraduate Curricula in the Biological Sciences."

E. *U. S. National Committee—IUNS:* Paul György, Chairman; R. W. Engel, Secretary.

The first official meeting of this Committee was held in Washington on October 30, 1958. Plans are being completed for the affiliation of the IUNS with appropriate international organizations in order to most effectively carry out its purposes, particularly the International Council of Scientific Unions—ICSU. The purposes of the Committee and its Constitution were published in the September 1958 *Journal of Nutrition.*

F. *Fellows Committee.* Dr. Jukes, Chairman, recommended that the present system of electing Fellows be continued. He urged that individual members send

in nominations of Fellows to the new Chairman.

G. *Organizing Committee of the Fifth International Congress on Nutrition, September 1–7, 1960, Washington, D. C.* Dr. György, Chairman, reported that good progress has been made on plans for the Congress. Several meetings of the entire Committee and a number of subcommittee meetings have been held this past year. The continued need for funds and other sources of support was stressed by Dr. György. (Full information on the Congress and on travel awards may be obtained from Dr. M. O. Lee, Federation Office, 9650 Wisconsin Avenue, Washington 14, D. C.)

X. *Actions of Federation Board*

AIN representatives are R. R. Williams, W. J. Darby, and D. W. Woolley.

The complete record of actions of the Federation Board will be published in the *Federation Proceedings*. The following items approved by the Board, will be of interest to AIN members:

A. Appointed Dr. Philip Handler to appear as a representative of the Federation before a Senate Committee in support of the National Institutes of Health's budget for research and training in the biological sciences.

B. Recommended that the registration fee at the next Federation be increased to $10.00. (The next meeting will be held in Chicago, April 11–15, 1960.)

C. Announced that the size of *Federation Proceedings* will be increased to $8\frac{1}{2}'' \times 11''$.

D. Approved a change in the Constitution and Bylaws of the Federation so that Advisory Committee members from each Federated Society shall be appointed for a three year term. (In conformity with this rule, the AIN Council has recommended that in the future the President-Elect elected in 1959, 1962, 1965, etc. be designated as our representative to the Advisory Board for a three year term.)

E. Recommended that $6,000 be spent to establish a Federation public information program on a professional level, its activity to be centered around the Annual Meeting.

F. Announced that the Advisory Committee had recommended that the Federation offer financial assistance and publication services to AIN in recovering the *Journal of Nutrition*.

G. Accepted National Associate membership of the Federation in the Council of International Organizations in the Medical Sciences (CIOMS).

H. Approved the refunding of $1.00 of the registration fee to each Society for each member registered (in proportion to the number of members registered from each Society thus allowing for dual membership).

XI. *Report of Council Actions*

Dr. Darby reported several other actions of the AIN Council of interest to the membership, as follows:

A. The Council recommended the formation of an ad hoc Committee on *Journal of Nutrition* Management composed of representatives from the Council and the Editorial Board to:

1. Within the Society, plan the appropriate structure of relationship between the Editor, the Council, and the Editorial Board and suggest constitutional changes as necessary.
2. To evaluate the trial agreement with the Wistar Institute regarding the *Journal of Nutrition*.
3. To study all other aspects of Journal management and ownership during the next two years.

B. The Council recommended the formation of an AIN committee to screen scientific abstracts submitted to the Fifth International Congress on Nutrition. This Committee will screen all non-invited papers from the U. S. and abroad.

C. The Council enthusiastically thanked the National Vitamin Foundation and Mr. William Rubin for their excellent help in publicity work in connection with the Nutrition Program of the 1959 Annual Meeting.

D. The following three names were nominated by the Council for three year terms on the National Committee of IUNS:

Gladys Emerson
J. M. Hundley
A. E. Schaefer

E. In conformity with past regulations, the Council reconfirmed the right of the Program Committee, chaired by the Secretary, to select only those abstracts submitted for the annual meeting which contribute to the highest possible quality program.

F. In response to suggestions from members regarding the AIN balloting procedure, the Council recommended that three names be placed on the ballot for position of Councilor in the future.

XII. *Acknowledgments*

The Secretary wishes to acknowledge the excellent secretarial assistance of Mrs. Diana Ingram and Mrs. Nancy J. Hess and the entire staff of the nearby Federation Office for many hours of devoted help in connection with Society activities.

Thanks also are due to The Wistar Institute, particularly Mr. James S. Ream, for excellent cooperation in supplying various printing needs, usually with short deadlines.

The assistance of Dr. John G. Bieri in helping with the arranging of the Nutrition program of the Federation Meeting is also gratefully acknowledged. In fact, all

members of the Society, particularly Dr. Darby, Dr. Kline, Dr. Engel, and Council members who have been called on to do special tasks have been most cooperative.

ANNIVERSARY BANQUET AND PRESENTATION OF FELLOWS AND AWARDS

The annual banquet was held on April 15 at the Traymore Hotel with 325 members and guests in attendance. Dr. W. J. Darby, as Toastmaster, introduced the special guests and awardees.

Fellow certificates were presented to the 1958 awardees as follows:

1958 Fellows

Thorne M. Carpenter	Elmer V. McCollum*
George R. Cowgill*	Harold H. Mitchell
Eugene F. DuBois**	John R. Murlin
Ernest B. Forbes	Harry Steenbock
Casimir Funk	R. R. Williams

*Present to receive certificate
**Deceased

Dr. T. H. Jukes, Chairman, Committee on Fellows, presented certificates of Fellow to the following persons selected in 1959 who have had distinguished careers in nutrition:

AGNES FAY MORGAN

"Tireless research worker; inspirer of young women in the search for scientific knowledge. For her discoveries that show the relation of nutritional deficiencies to abnormal physiology, and for her outstanding investigations of the nutritional qualities of foods."

WILLIAM C. ROSE

"Distinguished teacher of biochemistry. For his discovery of threonine and for his studies of the requirement of laboratory animals and man for amino acids. The modern concept of the role of proteins in nutrition is based on his research."

ALBERT G. HOGAN

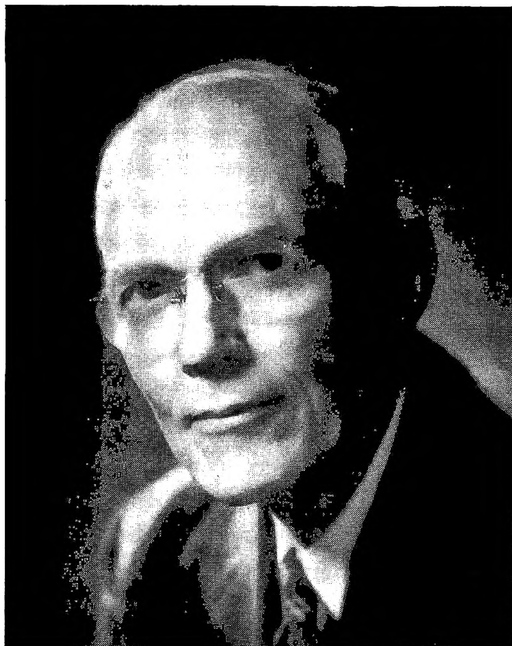
"Pioneer of the newer knowledge of nutrition. For his discoveries of vitamin deficiencies in animals and for his studies with purified diets that have illuminated the broad concept of nutrition."

The *1959 Borden Award* of $1,000 and a gold medal was presented to Dr. Harry Steenbock (in absentia). "For long and continued investigation in the field of mineral metabolism, the relation of ultraviolet light to anti-rachitic activity, and the physiological chemistry of Vitamin A and Vitamin D."

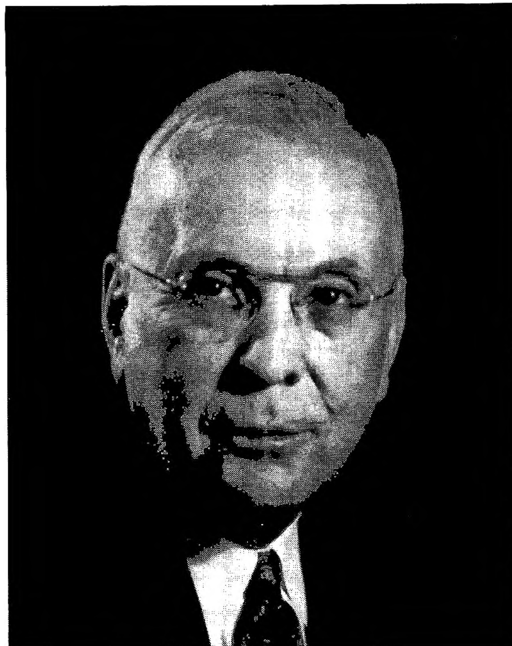
The *1959 Osborne-Mendel Award* of $1,000 and a scroll was presented to Dr. Grace A. Goldsmith with the following ci-

AGNES FAY MORGAN

WILLIAM C. ROSE



ALBERT G. HOGAN



DR. GEORGE R. COWGILL

tation: "For her contributions in the field of macrocytic anemias (especially studies on the specific roles of folic acid and vitamin B_{12}) and the clarification of the interrelationship of tryptophan and niacin in human nutrition and in the field of protein malnutrition."

Dr. Goldsmith responded with a few interesting remarks of acknowledgment and thanks.

A special highlight of the banquet was in special recognition of Dr. George R. Cowgill, retiring Editor of *The Journal of Nutrition*. An attractive gift in appreciation of his valuable services was presented to Dr. Cowgill from all present and past members of the Editorial Board who have served under his editorship over the past 20 years.

This same group also presented a check to Dr. Rebecca B. Hubbell as a token of appreciation of her past services as assistant to Dr. Cowgill.

After the banquet, the joint Biochemistry-Nutrition Smoker was held at the Hotel Ambassador with approximately 1,000 present. The very efficient work of Dr. R. E. Olson in making the Smoker a success is gratefully acknowledged.

OFFICERS AND COMMITTEES — AMERICAN INSTITUTE OF NUTRITION

July 1, 1959 — June 30, 1960

Council

President: D. W. Woolley, Rockefeller Institute for Medical Research, New York, New York

President-Elect: F. S. Daft, NIAMD, National Institutes of Health, Bethesda, Maryland

Past-President: W. J. Darby, Vanderbilt University School of Medicine, Nashville, Tennessee

Secretary: G. M. Briggs, National Institutes of Health, Bethesda, Maryland (1960)

Treasurer: J. B. Allison, Rutgers, the State University, New Brunswick, New Jersey (1962)

Councilors: Paul György (1960), J. H. Roe (1961), W. H. Griffith (1962)

Committees

Nominating Committee: Gladys Emerson (Chairman), H. H. Williams, C. A. Baumann, R. W. Engel, and J. M. Hundley

Committee on Nomenclature (joint with American Society of Biological Chemists): O. L. Kline (1961), P. L. White (1960)

Nominating Committee — Borden Award: C. A. Baumann (Chairman) (1960), G. V. Mann (1961), and E. E. Snell (1962)

Nominating Committee—Osborne-Mendel Award: D. M. Hegsted (Chairman) (1960), J. S. Dinning (1961), and Grace A. Goldsmith (1962)

Fellows Committee: Cosmo Mackenzie (Chairman) (1960), Paul György (1960), W. D. Salmon (1961), Karl Folkers (1961), and E. W. McHenry (1962)

Public Information Committee: R. W. Engel (Chairman) (1960), L. Voris (1960), A. E. Schaefer (1960), and G. M. Briggs (Ex officio) (1960)

Committee on Membership (ad hoc): A. E. Harper (Chairman), W. J. Darby, Gladys Emerson, P. György, D. M. Hegsted, A. E. Schaefer, and L. D. Wright

Committee on Journal of Nutrition Management (ad hoc): W. J. Darby (Chairman), F. S. Daft, O. L. Kline, and H. A. Schneider

Tellers Committee: H. R. Bird (Chairman) (1960), M. K. Horwitt (1960)

Auditing Committee: M. W. Taylor (Chairman) (1960), W. H. Ott (1960)

U. S. National Committee — IUNS

Paul György (Chairman) (1961), W. H. Sebrell, Jr., (Vice Chairman) (1961), L. Voris (1960), M. O. Lee (1960), R. W. Engel (Secretary) (1960), E. L. Severinghaus (1961), Gladys Emerson (1962), J. M. Hundley (1962), and A. E. Schaefer (1962)

Also, ex officio (voting) members are D. W. Woolley (1960), Grace Goldsmith, C. G. King (1961); and ex officio (non-voting) members are H. B. Steinbach, R. K. Cannan, W. W. Atwood, Jr., and E. V. McCollum

Representatives to other organizations

Federation Board: W. J. Darby (1960), D. W. Woolley (1961), and F. S. Daft (1962)

Federation Advisory Committee: F. S. Daft (1963)

National Research Council Boards and Divisions: N. R. Ellis (1960)

American Association for the Advancement of Science Council: P. L. Day (Section N —Medical) (1961); E. L. Severinghaus (Section C — Chemistry) (1960)

Food and Agriculture Organization: J. M. Hundley (1960)

Editorial Board of Federation Proceedings and Federation Secretaries Committee: G. M. Briggs (1960)

Officers and Committees of the Fifth International Congress on Nutrition, September 1–7, 1960, Washington, D. C. (Organized by American Institute of Nutrition and U. S. National Committee of IUNS) E. V. McCollum, Honorary President, C. G. King, President

Organizing Committee:

Paul György, Chairman

M. O. Lee, General Secretary

W. H. Sebrell, Jr., Chairman of Program Committee

E. L. Severinghaus, Chairman of Finance and Budget Committee

F. S. Daft, Chairman of Hospitality Committee (and Co-Chairman of Finance and Budget Committee)

W. J. Darby, Chairman of Lectureships and Travel Assistance Committee

Hazel Stiebeling, Chairman of Publications Committee

LeRoy Voris, Chairman of Public Information Committee

G. M. Briggs, Recording Secretary

Other Members: C. H. Best, C. A. Elvehjem, Gladys Emerson, R. W. Engel, Grace Goldsmith, F. Gomez-S, J. M. Hundley, L. A. Maynard, L. B. Pett, H. E. Robinson, F. J. Stare, R. R. Williams

Editorial Board — Journal of Nutrition: R. H. Barnes, Editor (1964), C. P. Berg (1960), C. G. Mackenzie (1960), H. R. Bird (1960), R. W. Engel (1961), P. L. Harris (1961), H. A. Schneider (1961), O. L. Kline (1962), E. S. Nasset (1962), H. Pollack (1962), D. V. Frost (1963), A. E. Harper (1963), and O. Mickelsen (1963)

Respectfully submitted,
GEORGE M. BRIGGS, *Secretary*
American Institute of Nutrition

INVITATIONS FOR NOMINATIONS FOR 1960 AMERICAN INSTITUTE OF NUTRITION AWARDS AND FELLOWS

Nominations are now being invited for the 1960 A. I. N. awards and fellowships.

Nominations for the *1960 Borden Award in Nutrition* must be submitted by December 1, 1959, to Dr. C. A. Baumann, Department of Biochemistry, University of Wisconsin, Madison.

Nominations for the *1960 Osborne and Mendel Award* are due also by December 1, 1959, and should be sent to Dr. D. M. Hegsted, Harvard School of Public Health, One Shattuck Street, Boston, Massachusetts.

The deadline for receipt of nominations for *A. I. N. Fellows* is January 1, 1960. These should be sent to Dr. Cosmo G. Mackenzie, University of Colorado School of Medicine, Denver, Colorado.

Full details of the rules for these awards and lists of former recipients are given in the August 1959 issue of *The Journal of Nutrition*.

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