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RUSSELL M. WILDER

(1885–1959)

RUSSELL M. WILDER

Russell M. Wilder

— *A Biographical Sketch*

(November, 1885 – December, 1959)

Dr. Russell M. Wilder, distinguished clinician, scientist and teacher died on December 16th, 1959 at the age of 74 years. He was widely known for his many contributions to knowledge of diseases of metabolism and nutrition; and at the time of his death he was an emeritus member of the medical staff at the Mayo Clinic and emeritus professor of medicine in the Mayo Foundation, Graduate School, University of Minnesota.

Dr. Wilder was born on November 24th, 1885, in Cincinnati, Ohio. He was of colonial American stock. One of his ancestors, Edward Wilder, was an early planter who served in King Phillip's War in 1675. Many of Dr. Wilder's forebears were physicians. His father, Dr. William Hamlin Wilder, was head of the Department of Ophthalmology of Rush Medical College of the University of Chicago. He helped to found, and was secretary of, the first of the specialty examining boards of Ophthalmology. Russell Wilder's mother was Ella Taylor, a granddaughter of Dr. Thomas Carroll, a distinguished physician and professor of medicine in Cincinnati. The Taylors were a Quaker family from New Jersey. One of the physicians of this family was Dr. Edward Taylor, a pupil of Dr. Benjamin Rush, and who became superintendent of the Friends' Hospital in Philadelphia where he and his wife were amongst the first to put into practice in America the more humane treatment of the insane. Also of this family were Dr. Joseph Wright Taylor, the founder of Bryn Mawr College, and Dr. H. Longstreet Taylor whose interest in tuberculosis led to the organization and effective management of public sanatoriums and preventoriums in Minnesota.

Dr. Wilder attended school in Chicago and received his degree of bachelor of science from the University of Chicago in 1907. His undergraduate studies included one year in Heidelberg, Germany. While Dr. Wilder was a medical student, he became interested in the Islands of Langerhans. He told how this came about, in a paper presented in August, 1959, at the Annual Meeting of the American Dietetic Association: "My adventurous nature led me in 1908 in the direction of these islands. At the end of the first two years of medicine at the University of Chicago, my fellow student Morris Pincoffs was assigned with me to the task of presenting to the class of physiology a review of the anatomy and physiology of the pancreas. Pincoffs took the anatomy and I the physiology but we worked together and came up with two lectures which the salty old professor A. J. Carlson said were very good. Carlson was young then but old to our still youngish eyes."[1] As a result of this experience Dr. Wilder decided to spend more time in basic science before continuing his clinical studies. He was appointed an instructor in the Department of Anatomy and worked under Dr. Robert Bensley who was professor and head of the department. Dr. Bensley was a chemist as well as an anatomist, and by special methods which he developed for staining pancreatic tissues, he concluded that without a doubt the cells of the Islands of Langerhans represented an organ with a function independent of the rest of the pancreas.

In 1910, while still a medical student, Dr. Wilder went to Mexico with Dr. Howard Taylor Ricketts to study typhus fever. Little was known about typhus at this time although Ricketts had found bipolar organisms in the blood of persons sick with Rocky Mountain spotted fever and in ticks which transmitted the disease. The suspected relationship between these organisms and typhus fever later proved

to be correct. Ricketts died of typhus fever in May 1910 but Dr. Wilder returned to Mexico to complete the studies which were underway.

In 1911, Dr. Wilder married Lucy Elizabeth Beeler, the daughter of John M. and Mary Crawford Beeler of Hamilton, Ohio. The following year, he received the degree of doctor of medicine from the University of Chicago and the degree of doctor of philosophy *magna cum laude* in the same year from the same institution. He also received the Benjamin Rush gold medal for standing first in his class. Dr. Wilder served an internship in Chicago under Dr. Roland T. Woodyatt and Dr. Frank Billings. He also spent a year in the laboratory of the famous sugar chemist, John Ulrich Nef, a pioneer investigator of unsaturated carbon compounds. Study with Professor Nef was at the prompting of Dr. Woodyatt who was investigating the behavior of various derivatives of glucose when injected into diabetic animals and human beings. Woodyatt had studied with the illustrious physician, Friedrich von Müller of Munich.

In 1914, Dr. Wilder spent eight months in Vienna at the first medical clinic studying under Dr. Müller Deham who had been one of Dr. Carl von Noorden's assistants. During this period, Dr. Wilder's interest in diabetes became even more firmly established. The stay in Vienna was terminated by the outbreak of the first World War. Mrs. Wilder and an infant son were with him in Vienna and the family experienced considerable difficulty in getting home by way of Germany, Holland and England.

Dr. Wilder returned to Chicago where he had been offered the post of resident physician in the Presbyterian Hospital by Dr. Frank Billings. This was the first residency created at any hospital west of the eastern seaboard. In association with this position, research facilities were available at the Otho S. A. Sprague Memorial Institute of Rush Medical College of the University of Chicago. In this Institute, Dr. Wilder worked with Dr. Woodyatt on the utilization of glucose and on metabolites of fatty acid oxidation. Dr. Woodyatt hoped to bypass that step in the breakdown of sugar in the body which appeared to be obstructed when diabetes mellitus was present. In reminiscing about these years Dr. Wilder stated: "We had no luck with any of the derivatives of glucose but were adding basic knowledge to the general problem and a collateral investigation of glucose tolerance was remunerative from the standpoint of research. It depended on a perfectly timed intravenous injection made with a machine designed by the boss himself. Also of some value was the study of the rate of utilization of the ketone bodies which are responsible for diabetic acidosis."

Dr. Wilder's teaching and research activities at Rush Medical College were interrupted by service in the first World War. He was commissioned a first lieutenant in the Medical Reserve Corps of the Army in 1914 and in 1917 was ordered to active service. He went to France in January, 1918, as a captain and chief of the medical service of Evacuation Hospital no. 2. In this capacity he served in several campaigns including the offensives of Saint Mihiel and Argonne Woods. Subsequently, Dr. Wilder became a member of the headquarter's staff of Colonel Charles R. Reynolds serving as medical chief of war gas defense of the Second Field Army. He left the Army in June, 1919, with a citation by the commander in chief of the American Expeditionary Forces for "exceptionally meritorious and conspicuous service."

In the fall of 1919, Dr. Wilder joined the staff of the Mayo Clinic as an associate in the Division of Medicine and assistant professor of medicine in the Mayo Foundation. In his memoirs,[2] Dr. Wilder indicated that one of the reasons leading to his employment at the clinic was to extend facilities for bedside teaching and clinical investigation and to provide for the training of graduate physicians in the several specialties of medicine. Dr. Wilder was placed in charge of all diabetic patients, and divided his time between the study of these patients and general diagnosis at the clinic. He initiated clinical investigative studies and secured the services of one technician who was housed in an improvised laboratory in a tiny room on the floor of the hospital which was devoted to the diabetic service.

The first project was carried out with the assistance of Miss Carroll Beeler and dealt with chlorides and edema in diabetes mellitus. The retinitis of diabetes was investigated with Dr. Henry P. Wagner who was then a fellow in Ophthalmology. Dr. Wagner later became renowned for his knowledge of the retina and Dr. Wilder indicated in his memoirs: "I take some pleasure in having had a part in arousing his interest in the manifestations of systemic disease of the retina." This remark is typical of Dr. Wilder whose keen interest and enthusiasm stimulated the many young men whose privilege it was to work with him. He derived great pleasure and satisfaction from their accomplishments. He always gave more than due credit to his collaborators and in his modesty kept little for himself.

The staff of the Mayo Clinic was enlarged considerably in the years following Dr. Wilder's appointment. In 1920, Miss Mary Foley was appointed director of dietetics and spent much of her early time in the preparation of special diets for patients with diabetes who were under Dr. Wilder's supervision. The dietary arrangement so impressed Dr. Will Mayo that he asked whether similar facilities could be provided for other physicians of the clinic and their patients. As a result, the Rochester Diet Kitchen was established. This included a restaurant with a seating capacity of 100 and a number of rooms where individual and group dietary instruction could be given. This provided a unique service for patients and physicians as well as for teaching.

Dr. Wilder's interest and enthusiasm in research are illustrated by his remarks concerning the discovery of insulin: "Suddenly from Toronto in the fall of 1921 came the news by word of mouth of the discovery of insulin. What a strike that was and how welcome the results: the end of the long trail and final proof at last of the importance of the part played in diabetes by the pancreatic islands." Shortly after insulin was discovered, Professor MacLeod called together a small group of experts to undertake an extensive clinical evaluation of insulin. Concerning this meeting Dr. Wilder writes: "Never again was I to experience the thrill equal to that of being invited to attend the meeting in Toronto of the small committee of experts to undertake an extensive clinical investigation of insulin." At this meeting, plans were adopted whereby insulin was to be distributed at first only to this group of specialists. It was felt that the most reliable information could be gained in this way and the least danger encountered until problems of industrial production had been solved, and a potent, stable and standardized form of insulin could be marketed. Members of this committee studied independently but published their results jointly in a single issue of the *Journal of Metabolic Research*. Dr. Wilder and his colleagues contributed significant observations to this report.

The first studies of insulin were carried out in a metabolic unit which had been set up at the Mayo Clinic by Dr. W. M. Boothby. To quote Dr. Wilder again: "By 1923 we had given insulin to 150 diabetic patients with results that were exhilarating." In speaking of one of the attractive features of making something of a specialty of diabetes, Dr. Wilder wrote that "the area of one's medical interest is as wide as general practice. Diabetic patients are subject to all of the diseases of mankind and unless the disease is treated wisely the diabetes cannot be well managed." He also said that the specialist in diseases of metabolism comes in intimate contact with all the other specialties and his knowledge grows accordingly.

Dr. Wilder (in collaboration with Mary A. Foley and Daisy Ellithorpe) wrote a primer for diabetic patients which was published first in 1922 and was followed by eight subsequent editions. In 1927, a new disease was discovered, hyperinsulism, the antithesis of diabetes, characterized by chronic hypoglycemia arising from hyperplasia of the islet tissue of the pancreas and production of excessive amounts of insulin.

In the spring of 1929, Dr. Wilder was invited to become the chairman of the Department of Medicine at the University of Chicago. In writing about this, he indicated that in his student days it had

been his ambition to occupy such a post but that on two previous occasions Dr. Will Mayo had talked him out of it. In this instance, however, Dr. Mayo gave his blessing and Dr. Wilder spent the next two years at Chicago. During this time he worked on problems related to osteitis fibrosa cystica, obesity, Addison's disease and epilepsy. In the fall of 1931, Dr. Wilder returned to the Mayo Clinic to fill the place left vacant by Dr. Leonard Rowntree's resignation, that of professor of medicine in the Mayo Foundation and head of the Department of Medicine. At this time, developments in endocrinology led Dr. Wilder to believe that the activities of the goiter service and those of the diabetic service should be combined. He felt that "by this means one closely knit group would be responsible for diseases of metabolism, endocrinology and nutrition which have become so closely interwoven."

In 1933, Dr. Wilder established a new teaching procedure for Fellows in the Department of Medicine, a more formal type of instruction which consisted of review courses for each of the three years of fellowship. The juniors covered laboratory diagnosis, the "middlers" reviewed clinical aspects of disease and the seniors reviewed physiology and biochemistry of morbid processes. The senior group met with Dr. Wilder at his home where they sat around the fire one evening every week for discussion accompanied by beer and pretzels. The topic had been assigned prior to the meeting and one of the Fellows had been designated to cover its most important aspects. These meetings were highlights in the experience of medical Fellows at the Mayo Clinic as I can attest as it was my privilege to attend some of these sessions. In commenting on these meetings, Dr. Wilder had an amusing word to say: "The Wilder family owned a great Dane back in those days and his name was Thor. At these evening seminars, Thor would lie under the piano and if the essayist was long-winded would protest with an atrocious yawn."

The principle areas of research in which Dr. Wilder and the metabolic section of the Mayo Clinic were engaged during the 1930's and 1940's were diseases of the thyroid gland, diabetes mellitus with special reference to the use of newer insulins (protamine zinc and NPH), diseases of the pituitary and adrenal glands, and nutrition with particular attention to obesity and thiamine deficiency. In association with Dr. Ray D. Williams, an isolated metabolic ward, diet kitchen and laboratory were set up in one of the wings of Rochester State Hospital. Here Drs. Wilder and Williams carried out studies on deficiency of thiamine and the human requirement for this vitamin and for riboflavin. In these studies the principal investigators were assisted by Drs. Mason and Powers of the Division of Biochemistry and Dr. Higgins of the Institute of Experimental Medicine.

Among some of the important findings during these years were demonstration that certain of the symptoms of acromegaly due to pituitary tumor could be suppressed by administration of female sex hormones. Methods were developed for treatment of diabetes with long-acting insulins. It was demonstrated that the severity of Addison's disease could be influenced favorably by limiting the amount of potassium in the diet. Studies of electrolytes in Addison's disease led to the development of two diagnostic tests for this condition, one involving restriction of sodium and administration of potassium and the other the so-called "water" test. Dr. Wilder studied the effects of ultraviolet irradiation of rachitic chickens with Dr. G. M. Higgins and Dr. Charles Sheard. There were also investigations of the suprarenal cortical syndrome.

Dr. Wilder's appreciation of the importance of nutrition in medicine was indicated in his chairman's address to the section of Pharmacology and Therapeutics of the American Medical Association in 1930. He stated that although knowledge of nutrition was increasing rapidly in the laboratories of biochemistry and physiology, the medical profession as a whole gave the subject scant attention. He pleaded "for the thoughtful attention of the members of the profession to this important field of therapeutics, for greater intellectual application on the part of practitioners to quantitative features of dietetics and for a greater amount of in-

struction in nutrition by medical schools." He indicated in his memoirs that "this became my theme song for the balance of my professional career."

Dr. Wilder was appointed a member of the Council on Foods and Nutrition of the American Medical Association in 1931. In December, 1938, at a meeting of this Council, he proposed that the addition of thiamine to white flour be encouraged. From that time on, Dr. Wilder became an active proponent of the enrichment program and assisted greatly in accomplishing enrichment of white flour and bread in this country.

Dr. Wilder was a member of the Committee on Medicine of the National Research Council from 1940 to 1946 and chairman of this committee in 1940. In 1941, he organized and became the first chairman of the Food and Nutrition Board of the National Research Council serving on the Board from 1941 to 1947. During this time, the Board developed its early policy concerning enrichment. In 1956, the American Bakers' Association gave Dr. Wilder an award in recognition of his activities in promoting enrichment of white flour and bread with vitamins and iron.

In the late 1930's an additional medical pavilion was constructed at St. Mary's Hospital in Rochester and space was made available for a metabolic unit which was called a nutrition unit hoping that this name would carry a connotation which would be more acceptable than metabolic to the patients. Dr. Wilder had little opportunity to use this unit as he was called to Washington for war activities and was away from Rochester much of the time after June, 1940. However, he took great satisfaction in the excellent work which was carried out in the new facility by his colleagues. In 1943, Dr. Wilder was made chief of the Civilian Food Requirements Branch of the War Food Administration. He also served as a member of one of the first of the study sections of the Office of Research Grants and Fellowships, United States Public Health Service. In 1944 and 1948, he participated in nutrition surveys in Newfoundland with a group of Canadian and American scientists.

Dr. Wilder became a senior consultant in the Division of Medicine at the Mayo Clinic in July, 1946, and retired from the Clinic and Foundation on December 31, 1950. He then became director of the National Institute of Arthritis and Metabolic Diseases of the U. S. Public Health Service. He held this position for only a short period due to the development of coronary artery disease and several episodes of coronary thrombosis. He returned to Rochester in July, 1953, where he remained actively interested in nutrition and metabolism and continued to participate in numerous medical and scientific organizations.

Dr. Wilder was a Fellow of the American Medical Association and of the American College of Physicians, a member of the Association of American Physicians, the American Society for Clinical Investigation, the American Physiological Society, the American Society for Experimental Pathology, the American Institute of Nutrition, Central Society for Clinical Research, the Central Interurban Clinical Club, the Minnesota Society for Internal Medicine, the Institute of Medicine of Chicago and the Academies of Medicine of Minnesota and Washington, D. C. He was also a member of the Society of Sigma Xi, Alpha Omega Alpha, Nu Sigma Nu and Delta Kappa Epsilon. He served on the Editorial Boards of the Journal of Nutrition and the Archives of Internal Medicine and on the Editorial Committee of Nutrition Reviews. He was an associate editor of Public Health Reports.

Dr. Wilder was a recipient of many honors and special awards. He was president of the American Diabetes Association in 1946–47 and this group presented the Banting medal to him in 1947. He was president of the National Vitamin Foundation in 1956. In 1957, he was made a Master of the American College of Physicians. The University of Chicago gave him the Distinguished Service Medal in 1941 and the Howard Taylor Ricketts Award in 1949. The Medical Alumni Association of the University of Chicago presented him with the honorary Gold Key in 1955. He received the Joseph Goldberger Award in Clinical Nutrition from the American

Medical Association in 1954. It was appropriate that this award was presented at a meeting of the Food and Nutrition Board of the National Research Council which he had been instrumental in establishing. It was my great privilege to have the honor of presenting this award to him on behalf of the Council on Foods and Nutrition of the American Medical Association. In 1959 Dr. Wilder was made an honorary member of the American Dietetic Association.

Dr. Wilder made many contributions to scientific knowledge. He published a textbook, *Clinical Diabetes Mellitus and Hyperinsulism,* in 1940. He contributed more than 250 papers to medical and scientific journals and was a contributor to several text books of medicine and to the *Encyclopedia Britannica.* His great interest in nutrition is illustrated by his bibliography. Subjects of his papers included experimental thiamine deficiency and minimal daily requirements of this vitamin, nutritional problems as related to national defense, the enrichment program, nutrition surveys, the diagnosis and pathology of nutritional deficiency, the misinterpretation and misuse of Recommended Dietary Allowances of the National Research Council, the importance of research and nutrition in public health. Dr. Wilder contributed to a number of publications of the National Research Council and to the *Handbook of Nutrition* prepared under the auspices of the Council on Foods and Nutrition of the American Medical Association.

In one of his last addresses which was given at the American Dietetic Association in 1959, Dr. Wilder stated, "I can lay no claim to any great discovery but I was a member of the crew and several of the ships engaged in exploration of the Islands of Langerhans and I must admit to a degree of pleasure in recalling these adventures." This exemplifies Dr. Wilder's modesty in his scientific accomplishments. He suggested many research problems to his graduate students and was known for his generosity in giving credit to his collaborators.

In addition to his many medical and scientific activities, Dr. Wilder was an inveterate reader. His interest in literature was shared by his wife, Lucy, who for a number of years conducted a book shop in Rochester which became a meeting place for the readers of the city. Interest in the Mayo Clinic shown by the many visitors to the shop led Mrs. Wilder to publish a description of the Mayo Clinic and the Mayo Foundation in 1936, a book which has been published in a number of subsequent editions.

During World War II when Dr. Wilder's two sons were in service in the Navy, the doctor developed an active interest in religion. He became a member of the Episcopal church and later served as vestryman and senior warden in Calvary Episcopal Church in Rochester. He was also a lay reader and member of the Bishop's Council of the Diocese of Minnesota. The Wilders' two sons are carrying on in the family tradition of medicine. Both are physicians, one is a surgeon and the other an internist.

Dr. Wilder was interested in the out-of-doors and in sports. During his student days he mentioned "a summer's tramp in the high Sierra" and for many years at the Mayo Clinic he was an active member of the tennis club. His cheerful personality and interest in his fellow men won him a host of friends. His optimism and enthusiasm never left him in spite of the restrictions imposed on his activities in his last years due to coronary heart disease.

Dr. Wilder will long be remembered for his enthusiasm in research and his scientific accomplishments, his stimulation as a teacher and his keen interest in the development of his students, his clinical ability and his concern for the comfort and morale of his patients as well as for their medical welfare, and his loyalty and devotion to his friends and colleagues.

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Effect of Methyl Arachidonate Supplementation on the Fatty Acid Composition of Livers of Pyridoxine-Deficient Rats[1,2]

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The failure of methyl arachidonate to improve body weight or skin lesions of vitamin B_6-deficient rats fed a 20% casein–10% cottonseed oil diet (Williams and Hincenbergs, '59) indicated that, under these conditions a secondary deficiency of archidonate did not exist in the vitamin B_6-deficient rat although vitamin B_6 is concerned in the formation of arachidonate from linoleate (Witten and Holman, '52). Tissue arachidonate levels, however, were not measured in the study of Williams and Hincenbergs. Hence, the possibility still existed that a secondary deficiency of arachidonate might not have been alleviated by the arachidonate supplement if the vitamin B_6-deficient rats were unable to retain the administered arachidonate. The following experiment was undertaken to test the effect of vitamin B_6 deficiency on the retention of dietary arachidonate by the liver and to study the effect of vitamin B_6 and/or fat deficiencies on the pattern of liver fatty acids.

EXPERIMENTAL

Male weanling (21-day-old) Long-Evans rats bred in our colony were fed ad libitum a purified fat-free diet of the following composition per 100 gm: vitamin-free casein, 20.0 gm; salts (U.S.P. 14, '50), 4.0 gm; choline bitartrate, 0.18 gm; vitamin A, 1000 I.U.;[4] vitamin D, 100 I.U.; vitamin E, 5.3 I.U.; sucrose, 75.82 gm. The B vitamins and menadione were fed separately in 20% ethanol (Williams and Hata, '59). Vitamin B_6 deficiency was created by the omission of pyridoxine from the B-vitamin mix. The rats were considered vitamin B_6-deficient when they reached a plateau in body weight (failure to gain more than 3 gm in one week).

Twelve rats were selected carefully according to body weight and time required for vitamin B_6 depletion. Three rats were sacrificed without treatment (group 1). The remaining 9 were divided into groups of three and given the following supplements daily for 6 days: (a) 3 µg of pyridoxine plus 360 mg of cottonseed oil (group 2); (b) 40 mg of methyl arachidonate dissolved in 360 mg of cottonseed oil (group 3); (c) 3 µg of pyridoxine plus 40 mg of methyl arachidonate in 360 mg of cottonseed oil (group 4). The experimental plan is outlined in table 1. After 6 days the rats were fasted overnight, decapitated, and the livers prepared for analysis. The liver lipid patterns for group 1 are assumed to represent the liver lipid composition of groups 2, 3, and 4 before supplementation.

The rats in group 5, which received 50 µg of pyridoxine per day, were littermates of the rats in group 1. These rats were pair-fed and sacrificed with their littermates in group 1. The results for group 5 show how the liver lipid composition of

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[2] Presented before the Pacific Slope Biochemical Conference, Davis, California, 1960.

[3] Recipient for 1960 of a Mead Johnson and Company award for graduate education in dietetics.

[4] Supplies of vitamin A (Nopcay 30: 30,000 I.U./gm), vitamin D (Supernopdex 15: 15,000 I.U./gm), and vitamin E (Nopvite: 44 I.U./gm) were kindly furnished by the Nopco Chemical Company, Richmond, California. The concentrated preparations were diluted with sucrose to the desired concentrations. The vitamin E preparation is coated with gelatin. The "microtized" vitamin A and vitamin D preparations are coated with a wax, of which the gross energy is 2.6 Cal./gm.

TABLE 1
Experimental treatment of rats

Group	Depletion period (18–22 days post weaning)		Supplementation period (6 days)		
	Pyridoxine	Cottonseed oil	Pyridoxine	Cottonseed oil	Methyl arachidonate
	µg/day	*mg/day*	*µg/day*	*mg/day*	*mg/day*
1	0	0	—	—	—
2	0	0	3	360	0
3	0	0	0	360	40
4	0	0	3	360	40
5	50	0	—	—	—

the young rat was affected by fat depletion for 18 to 22 days.

The oil supplements were fed by stomach tube. The methyl arachidonate (98 to 99% pure)[5] was dissolved in cottonseed oil just before feeding. Cottonseed oil was used to retard the oxidation and to facilitate the transfer and administration of the arachidonate. The vitamin supplement was supplied in a castor cup. The low level of 3 µg of pyridoxine per day, which is one third of the amount needed for maximum growth, was selected to limit the weight gains of the pyridoxine-supplemented rats.

The livers were homogenized in redistilled 95% ethanol. The homogenates were filtered after extraction for two hours at room temperature. The homogenate residues were re-extracted twice with 95% ethanol under the same conditions as the original homogenate. The residues were then extracted in a Soxhlet apparatus for 24 hours with freshly redistilled ethyl ether. The solvents from the combined alcohol-ether extracts were evaporated *in vacuo* at a temperature below 60°C. The remaining material was then extracted into a known volume of redistilled petroleum ether (b.p. 40 to 60°C) and stored in the dark at 0°C. All lipid analyses were performed on aliquots of the petroleum ether extract.

Methyl esters of the fatty acids were prepared and analyzed by gas-liquid chromatography by the procedure of Okey et al. ('60). Total lipids were estimated by a modification of the dichromate oxidation method of Bloor ('28). Lipid phosphorus was determined by the method of Sumner ('44). Cholesterol was determined by a modification (Okey and Lyman, '54) of the method of Sperry and Webb ('50). The use of the petroleum ether extract, instead of the alcohol-ether extract, for the cholesterol determinations decreases both the apparent "free" cholesterol values and the ratio of "free" cholesterol to total cholesterol. These decreases reflect the petroleum-ether insolubility of noncholesterol substances which react positively in the determination of free cholesterol directly from the alcohol-ether extract.

The amount of each fatty acid per liver was estimated by multiplying the calculated percentage of that fatty acid by the amount in milligrams of total noncholesterol lipid per liver. The latter value is the difference between the total crude lipid, measured by the Bloor oxidation procedure ('28), and the total cholesterol measured by the Sperry-Webb procedure ('50). It is realized that the chromatographic results represent only the ratios of the fatty acids eluted from the columns, and that the percentage recovery of fatty acids applied to the columns was not determined. We believe, however, that these estimations are at least roughly quantitative and have comparative value.

RESULTS

Data for body weight changes during the depletion and supplementation periods, liver size, and liver lipids of all groups are presented in table 2. The time required for vitamin B_6 depletion ranged from 18 to 22 days. Supplementation of the vitamin B_6-deficient rats with arachidonate and cottonseed oil (group 3) did not improve body weight. Supplementation with

[5] Hormel Foundation, Austin, Minnesota.

TABLE 2

Growth and liver lipid data

Group	Body weight			Weight change after treatment	Liver weight	Liver lipids, % wet weight			
	Initial	Plateau	Post treatment			Total lipid	Cholesterol Total	Cholesterol Free	Phospholipid (% P × 25)
	gm	*gm*	*gm*	*gm*	*gm*	%	%	%	%
1 Minus vitamin B_6, minus fat	55	64	—	—	1.87	3.84	0.33	0.32	3.58
	48	60	—	—	2.15	4.66	0.33	0.27	4.12
	48	53	—	—	1.87	3.92	0.32	0.28	3.92
2 Plus vitamin B_6 (3 μg/day) plus cottonseed oil	58	79	80	+ 1	3.18	4.60	0.33	0.31	4.10
	48	58	61	+ 3	2.20	4.68	0.32	0.32	4.55
	50	61	72	+11	3.51	4.13	0.26	0.23	3.15
3 Minus vitamin B_6 plus cottonseed oil plus arachidonate	46	67	63	− 4	2.17	3.91	0.32	0.30	4.45
	47	63	60	− 3	2.26	4.08	0.30	0.28	4.42
	50	66	62	− 4	2.24	4.24	0.36	0.30	4.20
4 Plus vitamin B_6 (3 μg/day) plus cottonseed oil plus arachidonate	53	78	90	+12	3.10	4.42	0.30	0.28	3.98
	50	58	70	+12	2.57	7.87	0.32	0.31	4.65
	47	60	66	+ 6	2.03	4.63	0.32	0.28	4.75
5 Plus vitamin B_6 (50 μg/day), minus fat	60	92	—	—	2.68	4.16	0.38	0.32	4.10
	59	92	—	—	2.61	4.23	0.37	0.30	4.42
	59	88	—	—	3.08	4.36	0.33	0.28	4.40

the low level of pyridoxine increased body weight or halted weight loss (groups 2 and 4) as would be expected. Total liver lipids, expressed as a percentage of wet or dry weight, showed no consistent variations between groups. The highest percentage of total lipid, 7.9%, occurred in the group supplemented with pyridoxine, cottonseed oil, and arachidonate (group 4), but the other values in this group were similar to the values in the other groups.

The liver fatty acid patterns of group 1 (minus vitamin B_6, minus fat) and group 5 (plus vitamin B_6, minus fat) show the development of fat deficiency symptoms in the presence or absence of pyridoxine (table 3). In comparison with the groups which received both arachidonate and cottonseed oil (groups 3 and 4), groups 1 and 5 showed elevated levels of palmitoleate and oleate and decreased levels of linoleate and arachidonate. Patterns of groups 1 and 5 also showed an unidentified fatty acid which appeared on the chromatogram just ahead of arachidonate. From the position on the chromatogram, this "fraction x" may be a dienoic or trienoic C_{20}-acid, perhaps 5, 8, 11-eicosatrienoic acid which Mead and Slaton ('56) and Fulco and Mead ('59) have identified in rats deficient in essential fatty acids. The decreases in the percentages of linoleate and arachidonate and the increase in "fraction x" were greater in group 5, which received pyridoxine, than in group 1. Both groups were similar with respect to the total amount of linoleate or arachidonate per liver, but the total amount of "fraction x" was much greater in the pyridoxine-supplemented rats (group 5). There were no marked differences between the two groups in the percentages of other fatty acids, phospholipids, total cholesterol or free cholesterol (table 2). Also, cholesterol and phospholipid apparently constituted all of the lipid present since the sum of the percentages of phospholipid and cholesterol was slightly greater than the percentage of total crude lipid. This discrepancy most probably is due to the estimation of phospholipid values from the phosphorus content of the petroleum ether extract rather than from the oxidation procedure used to estimate total crude lipid.

Comparison of group 3 (minus vitamin B_6, plus cottonseed oil plus arachidonate) and group 1 (minus vitamin B_6, minus fat) shows the effect of essential fatty acid supplementation on the liver fatty acid pattern of rats depleted in both fat and pyridoxine. In group 3, the percentage of arachidonate increased by 40 to 50%, the total amount of arachidonate per liver nearly doubled, "fraction x" was not detected, and the percentage and total amounts of oleate and palmitoleate were greatly reduced. The total amount of stearate per liver also increased in group 3.

Comparison of group 3 (minus vitamin B_6, plus cottonseed oil plus arachidonate) and group 4 (plus vitamin B_6, plus cottonseed oil plus arachidonate) shows the effect of pyridoxine supplementation on the utilization of cottonseed oil and arachidonate by rats depleted in both fat and vitamin B_6. The amount of each fatty acid per liver tended to be greater in group 5 because of the larger livers, but in both groups, the fatty acid patterns on a percentage basis were very similar, and "fraction x" was not detected. The percentages of cholesterol and phospholipid were similar also. Thus, vitamin B_6 deficiency appeared to affect arachidonate and cottonseed oil utilization only by limiting growth and therefore the net retention of the fat supplement.

Comparison of group 2 (plus vitamin B_6, plus cottonseed oil) with group 1 (minus vitamin B_6, minus fat) shows the effect of pyridoxine and cottonseed oil supplementation on the liver fatty acid pattern of rats depleted of both vitamin B_6 and essential fatty acids. In group 2, the percentage of linoleate was doubled, but the percentage of arachidonate did not increase although the total amount of arachidonate per liver increased nearly twofold (10 to 16 mg per liver). "Fraction x" was not detected, and the percentage of oleate was reduced in two of the three rats in group 2. The percentages of cholesterol and phospholipid were similar in both groups.

Comparison of groups 4 and 2 indicates the more rapid effect of the greater essential fatty acid intake in group 4 (plus vitamin B_6, plus cottonseed oil plus arachidonate) in remedying the effects of fat deficiency, although the liver fatty acid

TABLE 3

Fatty acid composition of livers

Group	C_{14} and below		Palmitic		Palmitoleic		Stearic		Oleic		Linoleic		Fraction "x"		Arachidonic	
	%	*mg*	%	*mg*	%	*mg*	%	*mg*	%	*mg*	%	*mg*	%	*mg*	%	*mg*
1 Minus vitamin B_6, minus fat	2.2	1.4	19.0	12.5	8.1	5.3	12.6	8.3	24.1	15.9	7.8	5.1	3.1	2.0	23.1	15.2
•	1.5	1.4	22.5	21.1	5.9	5.5	13.6	12.7	21.0	19.7	7.8	7.3	5.6	5.2	22.1	20.7
	0.7	0.5	17.5	11.9	3.5	2.4	19.7	13.4	18.6	12.7	9.2	6.3	3.6	2.5	27.2	18.6
2 Plus vitamin B_6 (3 μg/day) plus cottonseed oil	1.0	1.4	23.6	32.2	3.4	4.6	15.7	21.4	17.7	24.2	15.3	20.9	—	—	23.3	31.8
	1.0	1.0	16.7	16.0	3.5	3.4	14.5	13.9	17.2	16.5	14.6	14.0	—	—	32.5	31.2
	1.3	1.7	25.9	34.4	4.7	6.3	13.3	17.7	18.7	24.9	12.8	17.0	—	—	23.2	30.9
3 Minus vitamin B_6, plus cottonseed oil plus arachidonate	0.3	0.2	18.2	14.3	1.0	0.8	22.6	17.7	8.6	6.7	14.1	11.1	—	—	35.3	27.7
	0.3	0.3	18.8	16.2	1.4	1.2	21.5	18.5	10.2	8.8	15.1	13.0	—	—	32.7	28.1
	0.4	0.4	15.4	13.6	2.1	1.8	20.4	18.0	12.2	10.8	13.8	12.2	—	—	35.7	31.5
4 Plus vitamin B_6 (3 μg/day) plus cottonseed oil plus arachidonate	0.2	0.3	18.9	24.2	1.8	2.3	21.7	27.8	11.9	15.3	12.3	15.8	—	—	33.2	42.6
	1.3	2.5	24.4	47.4	4.2	8.2	12.2	23.7	20.3	39.5	15.1	29.4	—	—	22.3	43.4
	0.5	0.4	19.5	17.2	2.1	1.9	21.0	18.5	11.0	9.7	12.6	11.1	—	—	33.6	29.7
5 Plus vitamin B_6 (50 μg/day), minus fat	0.7	0.7	19.5	20.1	4.6	4.7	16.6	17.1	21.5	22.1	4.8	4.9	13.0	13.4	19.3	19.9
	1.0	1.0	18.8	19.3	5.2	5.3	15.5	15.9	24.3	24.9	5.9	6.1	9.9	10.2	19.3	19.8
	0.6	0.7	21.4	26.8	4.1	5.1	17.7	22.2	20.9	26.2	8.2	10.3	9.5	11.9	17.5	21.9

pattern of one rat in group 4 resembles the patterns of group 2.

DISCUSSION

The vitamin B_6-deficient rat was able to retain sufficient of the arachidonate and cottonseed oil supplement to restore liver fatty acids to the levels observed in the pyridoxine-supplemented rats fed arachidonate and cottonseed oil (cf. groups 3 and 4). The vitamin B_6-deficient rats in group 3 deposited 7 to 15 mg of arachidonate and two of the three rats in group 4 deposited 20 to 25 mg of arachidonate. Thus, in both groups, arachidonate constituted 30 to 35% of the fatty acids determined by gas-liquid chromatography. Consequently, the failure of methyl arachidonate to improve the body weight or acrodynia of vitamin B_6-deficient rats (Williams and Hincenbergs, '59) did not result from an inability to retain dietary arachidonate. Data for group 3, however, show that the vitamin B_6-deficient rat did not retain arachidonate in excess of the amount needed to maintain a "normal" level, if the arachidonate levels in group 4 are called "normal" for this experiment. Thus, vitamin B_6 deficiency limited the net retention of arachidonate in the liver indirectly by limiting liver size, which determines largely the total amount of arachidonate in the liver.

Comparison of group 1 (minus vitamin B_6, minus fat) and group 5 (plus vitamin B_6, minus fat) shows the extent to which the rate of fat depletion increased as the rate of growth increased. In the animals in group 5, which grew more because of pyridoxine supplementation, the percentage of arachidonate was approximately 5 to 10% lower and the percentage of "fraction x" was approximately 5 to 10% higher than that observed in group 1. On this basis, the pyridoxine-supplemented rats which showed no visible dermal symptoms of fat deficiency were more severely deficient in arachidonic acid than were the vitamin B_6-deficient rats. This observation supports the suggestion that arachidonic acid is not directly implicated in the acrodynia of vitamin B_6 deficiency. In comparison with the groups which received fat, however, the decreases in the percentages of arachidonate and linoleate, the increases in the percentages of palmitoleate and oleate, and the appearance of "fraction x" in both groups 1 and 5 reflect fat depletion (Hilditch, '56; Mead, '57; Fulco and Mead, '59).

SUMMARY

The effect of vitamin B_6 deficiency on the ability of the rat to deposit dietary arachidonate in the liver has been investigated. Vitamin B_6-deficient young male rats, in which the liver arachidonate had been depleted by feeding a fat-free diet, were fed daily for 6 days a supplement of 40 mg of methyl arachidonate and 360 mg of cottonseed oil, with and without the addition of 3 μg of pyridoxine per day. The lipid supplement increased the *percentage* of liver arachidonate equally in the vitamin B_6-deficient and the vitamin B_6-supplemented groups although the *net gain* in liver arachidonate by the former group was less because of the smaller liver size. The lipid supplement had no effect on the body weight of the vitamin B_6-deficient rats. Thus, vitamin B_6-deficient rats fed arachidonate were able to maintain the same percentage of liver arachidonate as the pyridoxine-supplemented rats. Vitamin B_6 deficiency appeared to affect liver retention of dietary arachidonate only indirectly by restricting liver size, which, in turn, determines to a large extent the total amount of arachidonate per liver.

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Influence of Dietary Zein on the Concentration of Amino Acids in the Plasma of Chicks

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Klain et al. ('60) observed that, when a diet containing 8% of soybean protein and 15% of zein was fed to chicks, poorly pigmented feathers were produced which were characteristic of a dietary lysine deficiency. The diet was comparable in protein level to a practical chick starter diet but was severely deficient in several amino acids, including lysine, for promotion of normal growth. It was also observed by these workers, but not reported, that the inclusion of zein in the diet containing 8% of protein resulted in a striking reduction in the level of lysine in the blood plasma.

The present studies are concerned with this depression of lysine level and also with changes in the plasma level of certain other amino acids induced by feeding a diet containing zein.

MATERIALS AND METHODS

All experiments were conducted with Barred Plymouth Rock chicks obtained from a commercial hatchery. The chicks were housed in battery brooders and assigned at random to the various pens, 12 to 25 birds per pen depending on the experiment.

Blood was taken from the carotid artery using heparin as an anticoagulant. Equal quantities of blood were collected from each bird and pooled for each experimental group. The blood was centrifuged and the resulting plasma deproteinized by the use of tungstic acid according to the method of Hier and Bergeim ('45).

Amino acids were determined by microbiological assay using the organism *Lactobacillus plantarum* for valine assay, *Streptococcus faecalis* 9790 for arginine, leucine, threonine and tryptophan and *Leuconostoc mesenteroides* P60 for glycine, histidine, isoleucine and lysine. Further details of the preparation of the deproteinized plasma and the amino acid assays are given in a previous publication (Gray et al., '60).

The composition of the low protein diet is shown in table 1.[1] This diet will be referred to hereafter as diet A. The diet differed somewhat from that used by Klain et al. ('60) who used soybean oil meal (50%

TABLE 1
Composition of diets

Ingredients	A	B
	%	%
Soybean protein[1]	9.5	9.5
Zein	—	15.0
DL-Methionine	0.29	0.29
Glycine	0.19	0.19
Corn oil	4.0	4.0
Cellulose[2]	2.0	2.0
Salts 5[3]	6.0	6.0
Choline chloride[4]	0.44	0.44
Vitamin premix[5]	2.0	2.0
Dextrose	75.58	60.58

[1] Purified protein purchased from Archer-Daniels-Midland Company, Cincinnati.
[2] Alphacel, Nutritional Biochemicals Corporation, Cleveland.
[3] Briggs et al. ('43).
[4] An equal-parts mixture of pure choline chloride and a 25% choline chloride concentrate.
[5] Contained in grams per kilogram: vitamin E concentrate (20,000 I.U./gm), 40.8; vitamin A concentrate (250,000 I.U./gm), 2.12; vitamin D_3 concentrate (16,500 I.C.U./gm), 2.40; riboflavin premix (1 gm riboflavin/oz), 16.3; Ca pantothenate, 2.03; niacin, 5.29; biotin, 0.0176; folic acid, 0.11; menadione, 1.01; thiamine·HCl, 0.40; pyridoxine·HCl, 0.70; vitamin B_{12} (0.1% triturate), 1.01; *p*-aminobenzoic acid, 1.00; and inositol, 55.1.

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[1] We are indebted to Merck and Co. Ltd., and Pfizer Canada, both of Montreal, Quebec, and Distillation Products Industries, Rochester, New York, for several of the vitamins used in the experimental diets.

protein) and a different mineral and vitamin mix.

Fifteen per cent of zein was incorporated into diet A replacing an equivalent amount of dextrose to prepare the diet hereafter referred to as diet B. The zein[2] contained by analysis, 14.6% of nitrogen, 1.1% of glycine, 0.08% of lysine and 0.06% of tryptophan.

Gelatin, rather than zein, was used in two experiments and was incorporated into diet A in the same manner as zein.

Glycine, DL-methionine, L-arginine hydrochloride, L-histidine hydrochloride and L-leucine, used as supplements, were C.P. grade. The L-lysine used was a commercial product containing 95% of L-lysine hydrochloride.[3] The quantities reported in the table refer to the L- or DL-form provided by the supplement.

The experimental procedures used in the different experiments were not identical in every case. In a few experiments female birds were used, and in others male birds. In some experiments birds were fed the experimental diets from one day to 4 weeks of age; in others, birds were fed diet A from one day to three weeks of age and then diet A and diet B were compared over the 4th week. Furthermore, the experiments did not involve the same number of chicks in each case. In some experiments the average values represent pooled samples from single pens of 20 to 25 birds each, in others the average of replicated groups of 12 to 15 birds for each diet. An examination of all our data, however, did not reveal any evidence that these variables influenced the effect of zein on plasma amino acid values observed in samples taken from birds of 4 weeks of age.

RESULTS AND DISCUSSION

Influence of zein on plasma amino acids. The average effect of feeding zein, for all experiments conducted, on the plasma levels of 9 amino acids is shown in table 2. The statistical significance of the percentage changes was evaluated by the "t" test using paired comparisons for the various experiments.

It is important, in evaluating the effect of feeding zein on plasma amino acids, to bear in mind that the growth rate of birds receiving diet B is equal to or generally less than that of birds receiving diet A. Accordingly, the amino acids which are contributed by zein are an excess supply which the birds must metabolize or otherwise eliminate. The problem of the relationship of growth and feed consumption to plasma amino acid levels will be discussed in a later section.

Apart from the reduction in the level of lysine in the plasma, an average of 64%, several other changes are recorded in table 2. The large increase in the level of threonine was not unexpected. It was found previously (Gray et al., '60) that

[2] Purchased from Nutritional Biochemicals Corporation, Cleveland.

[3] We are indebted to Dr. James Waddell of E. I. duPont de Nemours and Company, Wilmington, Del., for L-lysine hydrochloride.

TABLE 2

Effect of including 15% of zein in a low-protein diet on plasma amino acid levels

Amino acid	No. of experiments	Amino acid concentration in diet B[1]		Amino acids in plasma		Difference in level
		From soy protein	From zein	Diet A	Diet B[1]	
		% of diet	*% of diet*	*mg/100 ml*	*mg/100 ml*	*%*
Arginine	10	0.64	0.25	3.0	2.9	−3
Glycine	10	0.55	0.17	6.8	6.0	−12[2]
Histidine	8	0.20	0.17	2.6	2.4	−8
Isoleucine	8	0.42	0.68	1.1	1.4	+27[2]
Leucine	10	0.63	2.98	1.6	5.0	+213[2]
Lysine	10	0.49	0.01	2.2	0.8	−64[2]
Threonine	7	0.33	0.39	1.0	12.2	+1120[2]
Tryptophan	10	0.08	0.01	1.3	1.2	−8
Valine	7	0.44	0.58	1.6	2.3	+44[2]

[1] Diet B is diet A with zein included.

[2] Difference statistically significant ($P < 0.01$).

there was a relatively high threonine level in the plasma of chicks fed a lysine-deficient diet formulated from natural ingredients, and also in chicks which had fasted for 24 hours. It has been suggested that threonine is relatively resistant to deamination by the chick and accumulates in the plasma when conditions are conducive to its entering the plasma but not to its utilization for new tissue synthesis (Charkey et al., '53, '54). It will also be noted that isoleucine, leucine and valine increased significantly, whereas arginine, glycine, histidine and tryptophan decreased. Since the amino acids in the latter group were contributed in lesser amounts by the zein, the possibility is suggested that during the disposal of the excess amino acids contributed by the zein, additional amino acids are lost by deamination or excretion. On the other hand, zein may interfere with the absorption of amino acids from the intestinal tract. Whatever the explanation may be, however, of the amino acids assayed, lysine was disproportionately affected by the inclusion of zein in the diet.

Effect of added amino acids. It seemed important to investigate the effect of adding L-glutamic acid and L-leucine to diet A, since 26.0 and 20.0%, respectively, of these amino acids were found in zein by analysis. L-Histidine and L-arginine were also tested. Each amino acid was included in diet A at the level contributed by 15% of zein. However, since the degree of digestibility of the zein must be considered, this procedure did not insure that the contributions, as pure amino acids or in the form of zein, were equivalent. The results are given in table 3. The addition of each amino acid caused a corresponding increase in the plasma level. The level of leucine in the plasma was about the same as that when diet B was fed but the levels of arginine and histidine were considerably higher. When L-leucine was fed, the birds grew slowly and plasma levels of arginine, histidine and tryptophan decreased. When L-histidine was fed the arginine level was decreased. None of the amino acids decreased the lysine level.

Therefore, the effects obtained by adding glutamic acid, leucine, arginine and histidine singly to diet A do not parallel those found by adding equivalent amounts of these in the form of zein. Furthermore, it seems unlikely that the relatively low level of lysine observed in chicks fed diet B can be attributed to the addition of any single constituent amino acid of zein.

Amino acids limiting for growth. In earlier experiments it was observed[4] that when supplemental lysine was fed with diet B the growth of chicks was considerably increased and a reduction occurred in the level of tryptophan in the plasma. These effects were not noted when diet A was similarly supplemented. Such observations provided strong evidence that lysine was the first amino acid limiting to growth when diet B was fed and suggested

[4] Unpublished data.

TABLE 3

Effect of adding amino acids to a low-protein diet on plasma amino acid levels

Diet[1]	Experiment 1			Experiment 2					
	Av. weight at 4 weeks[2]	Lysine	Tryptophan	Av. weight at 4 weeks[3]	Arginine	Histidine	Leucine	Lysine	Tryptophan
	gm	*mg/100 ml in plasma*		*gm*		*mg/100 ml in plasma*			
Diet A	94(19)	2.4	1.0	107(22)	3.0	2.5	1.7	1.8	1.2
Diet B	89(21)	0.9	1.0	112(23)	2.9	2.2	5.3	0.7	1.1
Diet A + 4% of glutamic acid	93(18)	3.1	1.0						
Diet A + 0.25% of L-arginine				108(25)	5.2	2.6	1.7	2.2	1.1
Diet A + 0.24% of L-histidine				100(25)	1.7	10.0	1.6	2.1	1.0
Diet A + 3.5% of L-leucine				72(22)	2.2	1.8	4.3	1.9	0.8

[1] Diet B is diet A with zein included.

[2] Number of birds is indicated in parentheses. Each diet was fed to a single pen of birds from one day of age.

[3] Number of birds is indicated in parentheses. Each diet was fed to duplicate pens, starting the experiment with 13 birds each, from one day of age.

also that the degree to which an amino acid was decreased in the plasma might be related to the sequence in which it was limiting for growth. The data presented in table 4 are a test of this hypothesis. They also give results of experiments in which gelatin was included in diet A to produce a diet for which tryptophan was the amino acid limiting growth.

In the three experiments birds were fed diet A from one day to three weeks of age. They were then divided among the pens so that the average weight of the birds in each pen was approximately equal. The experimental diets were then assigned at random to the pens.

From experiment 1 it is clear that lysine and tryptophan in that order were limiting to growth when diet B was fed. Also, tryptophan, to a greater degree than other amino acids studied, was decreased in the plasma (1.2 to 0.5 mg per 100 ml) when diet B was supplemented with lysine. Furthermore, in experiment 2 it was found that a deficiency of tryptophan was limiting to growth when diet A plus gelatin was fed, and that the tryptophan level in the plasma showed a marked decrease.

Because it was noted in experiment 1 that apart from lysine and tryptophan, arginine was decreased in the plasma to the greatest extent when supplemental lysine was added to diet B, supplementation with arginine was studied in experiment 3. In addition treatments identical to those used in experiment 2 were included.

The pattern of changes shown by the data of experiment 3 was similar to that in experiments 1 and 2. Also, on the basis of the average gains in weight the sequence of limiting amino acids was lysine, tryptophan and arginine.

It is difficult to find a completely satisfactory explanation of the results. One possibility is that a metabolic mechanism operates to maintain at all times within the tissues a mixture of amino acids which is as close as possible to optimal proportions for the synthesis of proteins. If this balance is disturbed by adding an excess of one or more amino acids to the diet, the amino acids which are thus rendered disproportionately low in the diet will be drawn into the tissues from the plasma at an increased rate. It would have to be assumed that the tissue could draw in additional quantities of an amino acid when the need arose but could not prevent the entrance of excess quantities. If this hypothesis is correct then the decreases observed in the concentration of lysine, tryptophan and arginine in the plasma represent an attempt by the tissues to maintain, as nearly as possible, an optimal balance of amino acids in the face of a changed pattern offered by the plasma.

In addition, other factors in these experiments influenced the concentration of amino acids in the plasma. In experiment 3, when diet B plus lysine was fed, the level of threonine was much lower than when diet B alone was fed. The evidence showed, however, that a deficiency of threonine is not limiting to growth of chicks fed diet B ahead of lysine and tryptophan. The probable explanation is that threonine accumulated in the plasma when diet B was fed, whereas it was used at an increased rate for the synthesis of new tissue when the addition of the lysine supplement stimulated growth.

The high concentration of glycine found in the plasma when gelatin was fed in experiment 3 is also of interest. Presumably the 15% of gelatin was contributing glycine in such quantities that the pathways of elimination were not able to maintain its concentration in the plasma within a normal range. When the gelatin diet was supplemented with tryptophan, the concentration became still greater (41.7 mg/100 ml) because the increased feed consumption, which paralleled the greater rate of gain, added to the excess of glycine.

Rate of gain and feed consumption. The average gains from 3 to 4 weeks, of birds fed diet B, were considerably less than those of birds fed diet A (table 4). It is difficult to determine how much influence such differences had on the concentration of amino acids in the plasma in these investigations. It is reasonable to believe, however, that the rate of synthesis of new tissue, as represented by rate of growth, and the inflow of amino acids, as represented by feed intake, would be among the factors influencing the concentration of amino acids present in the

TABLE 4

Sequence in which amino acids are required for growth and their concentration in the plasma

Diet[1]	Av. gain 3–4 weeks[2]	Concentration in plasma								
		Arginine	Glycine	Histidine	Isoleucine	Leucine	Lysine	Threonine	Tryptophan	Valine
	gm	*mg/100 ml*								
Experiment 1										
Diet A	22.3	2.3	7.4	—	—	—	1.8	—	1.1	—
Diet B	14.6	2.6	6.3	—	1.3	4.2	1.0	—	1.2	2.1
Diet B + 0.5% of L-lysine	26.2	1.4	6.1	—	1.2	4.5	3.5	—	0.5	1.7
Diet B + 0.5% of L-lysine + 0.12% L-tryptophan	33.6	1.1	6.0	—	—	4.9	4.4	—	1.8	—
Experiment 2										
Diet A	37.1	—	—	—	—	—	2.1	—	1.6	—
Diet A + 15% of gelatin	29.6	—	—	—	—	—	6.0	—	0.3	—
Diet A + 15% of gelatin + 0.12% L-tryptophan	73.7	—	—	—	—	—	6.2	—	1.8	—
Experiment 3										
Diet A	24.1	2.9	6.9	2.3	0.9	1.6	2.6	0.8	1.2	1.8
Diet B	17.6	3.1	7.2	2.5	1.4	4.4	1.0	12.6	1.2	2.1
Diet B + 0.5% L-lysine	28.5	1.9	5.5	2.1	1.3	4.6	4.3	7.2	0.6	1.8
Diet B + 0.5% L-lysine + 0.12% L-tryptophan	58.5	1.7	6.5	2.5	1.7	5.9	6.5	7.8	1.7	2.2
Diet B + 0.5% L-lysine + 0.12% L-tryptophan + 0.4% L-arginine	71.7	5.9	4.9	1.0	1.4	5.3	3.3	3.3	1.6	1.7
Diet A + 15% of gelatin	23.7[3]	8.2	29.3	1.4	1.4	2.1	8.4	5.6	0.4	2.7
Diet A + 15% of gelatin + 0.12% L-tryptophan	49.4	11.3	41.7	1.2	1.0	2.0	7.3	2.4	1.3	2.6

[1] Diet B is diet A with zein included.

[2] In experiment 1 each diet was fed to a single pen of 25 birds, in experiment 2, to 4 pens of 11 birds each, in experiment 3, to 2 pens of 15 birds each.

[3] Average of duplicate pens which averaged 13.7 and 33.7 gm.

plasma. Since feed consumption and growth are positively correlated they should partially counteract each other in their effects, and their combined effect, if any, should be related to the value of the feed: gain ratio.

In one experiment, chicks were distributed at random into 14 pens of 13 each, fed diet A from one day of age, and feed consumption and gains from three to 4 weeks of age were recorded as well as lysine concentration in the plasma at 4 weeks. Analysis of the data showed a correlation of +0.46 between lysine concentration and feed: gain ratio. This correlation was not statistically significant.

In table 5 are shown gains, feed consumption and plasma lysine concentrations for two experiments in which there was sufficient replication for useful statistical analysis. Differences between the effects of the two diets with respect to gains, feed consumption and lysine concentration were highly significant ($P < 0.01$). Only small differences were recorded between the feed: gain ratios. Moreover, if lysine concentration versus feed: gain ratio is positively correlated, as suggested above, any increase in the feed: gain ratio should tend to a more elevated level of lysine in the plasma rather than a lower level.

The reduced growth and feed consumption which occurred when diet B was fed could be attributed to the creation of an amino acid imbalance (Harper, '58; Kumta and Harper, '60). A similar statement could be made with respect to the gelatin diet (table 4, exp. 2 and 3). In experiment 3 the average gains in weight of the duplicate pens of birds fed gelatin was 23.7 gm as compared with 24.1 gm for birds fed diet A. However, there is reason to suspect the recorded value of 33.7 gm for one of the pens because the gains of chicks in the duplicate pens receiving the other diets in this experiment checked closely, and also, the feed consumptions, considerably lower than for diet A, were similar for both pens of birds fed gelatin. Moreover, the results of experiment 2 support the view that birds receiving diet A plus gelatin will grow more slowly and eat less feed.

It is not possible to say that the decrease in the concentration of lysine in the plasma in the case of zein, and of tryptophan in the case of gelatin, was a direct or indirect cause of the depression in growth and feed consumption. Sauberlich and Salmon ('55) found with rats that, when gelatin was added to a diet containing 7% of casein, in addition to a depression in growth, there was a decrease in the concentration of tryptophan in the plasma, and also some increase in the urinary excretion of tryptophan. These workers suggested that the decrease in tryptophan level in the plasma might be responsible for the growth depression.

A careful review of all our experiments in which diet A and diet B were fed suggests that perhaps with respect to rate of weight gain, birds adjust to diet B over a period of time, or respond equally well to diets A and B if both diets are fed from the time the birds are one day old. For 5 experiments in which diet A and diet B were fed from one day to 4 weeks of age, the unweighted average weights at 4 weeks were 101 and 104 gm, respectively. On

TABLE 5

Effect of zein in the diet on gain in weight, feed consumption and lysine concentration in the plasma

Experiment	Diet[1]	No. of pens[2]	Av. gain/ chick 3–4 weeks	Av. feed consumption/ chick 3–4 weeks	Feed/gain	Lysine in plasma
			gm	*gm*		*mg/100 ml*
1 (♂ Chicks)	A	7	31.5	107.0	3.40	2.2
	B	4	21.6	74.5	3.45	0.7
2 (♀ Chicks)	A	7	28.5	110.4	3.87	2.3
	B	7	22.0	89.6	4.07	0.7

[1] Diet B is diet A with zein included.

[2] Experiment started with 14 chicks per pen and ended with not less than 11 chicks per pen.

the other hand, for 6 experiments in which diet A was fed for the first two or three weeks to all birds and then diet A and diet B from two to 4 weeks or from three to 4 weeks, the unweighted average gains for three to 4 weeks of age were 30 and 21 gm, respectively. However, regardless of whether diet B was fed one week, two weeks or 4 weeks, its depressing effect on the lysine level in the plasma was evident.

SUMMARY

The addition of 15% of zein to a diet containing 9.5% of soybean protein resulted in a significant reduction in weight gain and feed consumption, a marked decrease in the level of lysine in the deproteinized plasma, considerably smaller average decreases in the levels of arginine, glycine, histidine and tryptophan and increases in the levels of isoleucine, leucine, threonine and valine.

It is suggested that the reduction in the level of plasma lysine may be related to the fact that a deficiency of lysine was limiting growth when the diet containing zein was fed. This conclusion is supported by the observation that the levels of tryptophan and arginine, which appeared to be the second and third amino acids limiting to growth, were reduced when the diet containing zein was supplemented with excess lysine. Also, a reduction in the level of tryptophan in the plasma was found when 15% of gelatin was added to the low-protein diet, rather than zein, thus producing a diet for which a deficiency of tryptophan was limiting growth.

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Nutrition and Longevity in the Rat[1]

III. FOOD RESTRICTION BEYOND 800 DAYS

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In previous work it was shown that rats receiving a moderately restricted diet lived longer and had a lower incidence of degenerative diseases than animals fed ad libitum (Berg, '60; Berg and Simms, '60). Under the limitations imposed on spontaneous activity by prolonged cage confinement, the lean and slightly smaller rat maintained with a restricted food intake adapted to its environment better than the obese animal fed ad libitum. Also, in the restricted rat, life expectancy was lengthened by avoiding overfeeding, without the severe retardation of growth and sexual immaturity produced in McCay's animals by drastic underfeeding (McCay et al., '43). In our experiments food restriction did not connote undernutrition but rather a dietary intake that kept adult rats in a nutritional state short of developing excess body fat. Since our previously reported observations were limited to rats up to 800 days old, the present study was designed to extend 1200 days (using one level of dietary restriction tested previously). Besides mortality rate and onset of disease in these older animals, related observations were made on body and organ weights, skeletal measurements, the proximal tibial epiphysis, as well as appearance and behavior as compared with unrestricted rats.

METHODS AND MATERIALS

A detailed description of rat selection, colony conditions, body weight curves and body measurements of rats receiving different levels of food intake, and pathological technique was reported previously (Berg, '60; Berg and Simms, '60). The diet consisted of vitamin D-free pellets[2] and was supplied at weaning when rats were 4 weeks old. The pellets contained all the nutritional elements necessary for growth, maintenance and fertility, and were composed of natural foods containing 24.3% of crude protein, 4.0% of fat, and 54.2% of carbohydrate. Caloric values per 100 gm of diet were: protein, 78; fat, 36; carbohydrate, 217.

Food was supplied at two levels of intake: (1) ad libitum (unrestricted), and (2) 54% of the ad libitum intake, designated as "46% restriction," a level which kept the weight of males equal to that of unrestricted females and the weight of restricted adult females constant and equal to that of 56-day-old unrestricted females. All rats were free from lung infection, a condition that is endemic in most colonies and interferes seriously with the validity of nutrition experiments.

Cages measured 9½ inches in height, 14½ inches in width, and 18½ inches from back to front, and had wire mesh windows to preclude isolation from neighbors. Shavings were used in the cages, and drinking water was present at all times. The restricted rats were caged singly, whereas most of the unrestricted rats were kept together, three or 4 in a cage. No significant difference was observed in skeletal size of unrestricted rats caged singly or together, but food intake and male body weight were slightly lower in singly caged animals (Berg, '60).

When the animals were about 800 days old, the building in which our colony was located was demolished. At that time all of the surviving unrestricted rats (31

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[2] Rockland "D-Free" Pellets, A. E. Staley Manufacturing Company, Decatur, Illinois.

males and 58 females) and also 55 of the 46%-restricted rats (38 males and 17 females) were killed. Twenty-six surviving males and 20 females that received the 46%-restricted diet were transferred to temporary, but less favorable, quarters for observations beyond 800 days. The data on mortality and onset of lesions for these rats were compared with those of a group of unrestricted rats that had completed their lifespan or were killed before the change to new quarters. These totaled 89 males and 79 females. Because the survival rate of restricted rats was greater than that of unrestricted rats, most of the data on onset of lesions in restricted rats over 800 days old were obtained from animals killed while still in good condition, whereas the findings in ad libitum-fed rats were obtained when they were moribund or dead from disease.

Diseases studied

Four major diseases developed in our Sprague-Dawley rat strain. Three of these conditions, chronic glomerulonephritis, periarteritis, and myocardial degeneration were described previously (Simms and Berg, '57; Berg and Simms, '60), together with the system of grading of lesions according to severity.

Muscular degeneration. A 4th condition, described by Berg ('56) and by Simms and Berg ('57) is muscular dystrophy, characterized by weakness or paralysis of the hind legs. Its description was not included in the earlier report (Berg and Simms, '60) because the disease rarely appeared before 800 days. Early microscopic changes consisted of loss of cross striations of muscle fibers and an increased number of nuclei. Lesions rated as moderate involved more fibers, and showed fragmentation and replacement by fat cells. In severe lesions there was marked distortion due to necrosis and disappearance of sarcoplasm leaving collapsed sarcolemma sheaths. At this advanced stage of degeneration, clusters of nuclei resembling giant cells and fatty infiltration were usually present. The skeletal muscle changes occurring in old rats resembled those seen in vitamin E deficiency, but were unaffected by large doses of α-tocopherol (Berg, '59). Chronic glomerulonephritis, periarteritis and muscular dystrophy were primary causes of death, whereas myocardial degeneration was a contributory cause.

Tumors. A wide variety of spontaneous tumors developed in our rat strain. Neoplasms were chiefly of the benign type, the more common being fibroadenoma of the female breast, adenoma of the thyroid, chromophobe adenoma of the pituitary, pheochromocytoma of the adrenal, and islet adenoma of the pancreas. Malignant tumors included unclassified neoplasms of the central nervous system, carcinomas, sarcomas and lymphomas.

RESULTS

Body weight of unrestricted and restricted rats. Restricted rats of both sexes weighed about 40% less than their ad libitum-fed counterparts (table 1). Final weight of restricted males in good condition and killed at 800 or 1100 days of age, was about equal to that of 800-day-old unrestricted females.

Unrestricted rats of both sexes, in good condition and killed at 800 days, weighed about 20% more than unrestricted animals that died or were moribund at older ages. Also, healthy restricted males killed at 800 or 1100 days of age were heavier than restricted males moribund or dead at 900 days. Lower final body weights, in these instances, were the result of greater incidence and severity of disease (Berg and Harmison, '57).

When McCay and associates ('43) supplied a severely restricted diet, their male rats weighed 40% less than our restricted males, but the final weights of restricted females were about equal in the two studies. Our female rats, however, attained at 100 days of age (Berg, '60) the weight that McCay's greatly retarded females reached when they were over 1000 days old.

Tibia length and body length. Little difference was noted between the skeletal measurements of rats at 800 days and at older ages. Tibia length of restricted rats as compared with unrestricted rats was less by 5 to 8% and body length was less by 8 to 13% (table 1). Earlier studies (Berg, '60) showed that measurements of tibia and body length were proportional,

TABLE 1

Measurements and body weights of rats fed unrestricted and restricted diets

Age		No. of rats	Mean body weight	Mean body length	Mean tibia length
Mean	Range				
days	*days*		*gm*	*cm*	*cm*
		Unrestricted males			
786[1]	760–815	31	448	25.0 ± 0.5[2]	4.61 ± 0.12
864	797–979	27	345	24.1 ± 0.8	4.62 ± 0.03
		46%-Restricted males			
818[1]	814–821	38	275	22.4 ± 0.8	4.28 ± 0.09
926	832–994	10	225	21.7 ± 0.9	4.22 ± 0.09
1082[1]	1042–1143	16	257	22.2 ± 0.8	4.24 ± 0.09
		Unrestricted females			
795[1]	762–833	58	280	21.1 ± 0.6	4.00 ± 0.08
900	798–989	45	216	20.7 ± 0.7	4.01 ± 0.08
1063	997–1228	18	227	21.0 ± 0.7	4.03 ± 0.07
		46%-Restricted females			
821[1]	818–823	17	163	18.3 ± 0.3	3.77 ± 0.05
1150[1]	1036–1186	17	168	19.3 ± 0.5	3.84 ± 0.06

[1] Killed at this age while in good condition. Other rats were moribund or died.

[2] Standard deviations.

and linear plots gave points falling on the same straight line as the corresponding measurements of rats fed ad libitum This constant ratio was in accord with Huxley's principle of heterogony ('32).

Food intake and food utilization. The daily food intake of rats fed ad libitum was 19.5 gm and for the restricted males was reduced to 10.5 gm; that for females was reduced from 13.0 to 7.0 gm. At these levels of food restriction, the weight of males was the same as that of unrestricted females, and the weight of adult females was constant and equal to that of 56-day-old unrestricted females. Because the restricted males received 20% less food than unrestricted females and yet had an equal body weight and greater skeletal size, food utilization was more efficient in the males.

Organ weights. The ratio of heart or kidney weight to body weight was 25 to 30% higher in unrestricted rats over 1000 days of age than in 800-day-old animals; and in restricted rats, the ratio was 15 to 20% higher in the older age groups (table 2).

The ratio of pituitary weight to body weight did not change significantly with advancing age and was about the same for unrestricted and restricted animals. Pituitary weight in relation to body weight was higher in females but absolute pituitary weight was nearly the same in the two sexes.

The proximal tibial epiphysis. The appearance of the epiphyseal cartilage in restricted or unrestricted rats over 800 days old was the same as in 800-day-old rats. Newly formed trabeculae indicative of osteogenesis were absent and the plate was narrow and irregular as compared with the wide, even disc seen in growing bone (Berg, '60). Examination of the epiphyseal cartilage of 170-day-old restricted rats also revealed no signs of osteogenesis. Because new bone formation usually ceases at this age in unrestricted rats, it is reasonable to assume that full skeletal maturity was reached by restricted and unrestricted animals at about the same time, and that the smaller measurements of restricted rats were the result of slower growth rate during skeletal development.

Appearance and behavior. Restricted animals over 1000 days old resembled healthy young rats. Fur was glossy and clean, and the teeth were firm. As they were hungry, they were aggressive and climbed the doors of the cages in expectation of receiving food. The sleek appearance and liveliness of the restricted rats

TABLE 2

Organ weights of rats fed unrestricted and restricted diets

Mean age	No. of rats	Mean body weight	Mean heart weight	$\frac{\text{Heart weight}}{\text{body weight}} \times 10^4$	Mean kidney weight (both kidneys)	$\frac{\text{Kidney weight}}{\text{body weight}} \times 10^4$	Mean pituitary weight	$\frac{\text{Pituitary weight}}{\text{body weight}} \times 10^4$
days		*gm*	*gm*		*gm*		*gm*	
				Unrestricted males				
786[1]	31	448	1.73 ± 0.22[2]	39	4.55 ± 0.94	102	0.0113 ± 0.0016	0.25
864	27	345	1.92 ± 0.31	59	4.65 ± 0.69	141	0.0094 ± 0.0019	0.29
				46%-Restricted males				
818[1]	38	275	1.03 ± 0.06	38	2.14 ± 0.19	78	0.0074 ± 0.0015	0.27
926	10	225	0.96 ± 0.06	43	2.05 ± 0.21	91	0.0067 ± 0.0013	0.28
1082[1]	16	257	1.19 ± 0.18	47	2.47 ± 0.27	96	0.0071 ± 0.0012	0.28
				Unrestricted females				
795[1]	58	280	1.14 ± 0.19	41	2.51 ± 0.45	90	0.0112 ± 0.0023	0.40
900	45	216	1.19 ± 0.23	55	2.60 ± 0.61	120	0.0106 ± 0.0022	0.49
1063	18	227	1.24 ± 0.22	55	2.41 ± 0.37	106	0.0104 ± 0.0018	0.44
				46%-Restricted females				
821[1]	17	163	0.66 ± 0.03	40	1.34 ± 0.12	82	0.0054 ± 0.0007	0.34
1150[1]	17	168	0.82 ± 0.06	49	1.63 ± 0.13	97	0.0065 ± 0.0011	0.39

[1] Killed at this age while in good condition. Other rats were moribund or died.
[2] Standard deviations.

contrasted sharply with the obesity and sluggishness of the animals fed ad libitum. The latter had coarse, soiled fur, and dark brown pigment covered the nose and hair of the snout and around the eyes. The incisor teeth were long and brittle. A high percentage of unrestricted rats developed weakness or paralysis of the hind legs, loss of tail movement and marked kyphosis of the dorsal spine.

Mortality rates. Data for mortality in relation to age are shown in the table 3. The values of 100 P_t (the probability of death) are equal to the number of deaths in each 100-day age period, divided by the number of rats alive at the start of that age period. Among the restricted females there were so few deaths that the P_t values were computed over a longer age interval, but converted to the 100-day basis. These data permit us to calculate the life expectancy, in days, by use of the equation:

$$t = \frac{1}{k} \log \left[\frac{1.6k}{P_o} + 1 \right]$$

where P_o is the probability of death at birth and k is a constant equal to $1/t$ ($\log P_t - \log P_o$) (Simms, '46).

Males fed a restricted diet were observed to have a life expectancy of 1005 days, an increase of about 200 days over that of the unrestricted control males (802 days).

Data for the female rats showed an increased life expectancy of 364 days as a result of food restriction (1294 as compared with 930 days).

Life expectancy was greater for females than for males both unrestricted and restricted. The difference with ad libitum feeding was 128 days, and with restricted food intake, 289 days.

Incidence of major diseases excluding tumors

Observations of the separate incidence of each of the lesions of the 4 major diseases and of the combined incidence of these diseases showed a marked decrease

TABLE 3

Mortality and survival rates of rats fed unrestricted and restricted diets

Age range	Unrestricted				46%-Restricted			
	No. deaths	100 P_t = probability of death[1]	Survivors No.		No. deaths	100 P_t = probability of death[1]	Survivors No.	
days				%				%
			Males					
300–399	0	—	89	100	0	—	79	100
400–499	2	0.022	87	98	2	0.026	77	99
500–599	6	0.069	81	91	1	0.013	76	96
600–699	19	0.235	62	70	4	0.053	72	91
700–799	35	0.565	27	30	8	0.111	26[2]	80
800–899	20	0.741	13	8	4	0.154	22	69
900–999	7	1.000	0	0	6	0.273	16	50
1000–1099					10	0.625	6	19
1100–1199					6	1.000	0	0
			Females					
300–399	0	—	79	100	0	—	39	100
400–499	2	0.025	77	97	0	—	39	100
500–599	3	0.039	74	94	2 }	0.026	37	95
600–699	5	0.068	69	87	0 }		37	95
700–799	6	0.087	63	80	0 }	0.070	20[2]	95
800–899	22	0.349	41	52	2 }		18	87
900–999	23	0.561	18	23	1	0.061	17	84
1000–1099	14	0.778	4	5	2	0.118	15	77
1100–1199	4	1.000	0	0	1	0.068	14	73

[1] 100 P_t is the probability of death within a 100-day period among the rats alive at the beginning of the period.

[2] At about 800 days of age 38 male rats and 17 female restricted rats were killed while in good condition, reducing the number of survivors. The data for percentage surviving have been corrected accordingly.

in the number of lesions in restricted rats as compared with unrestricted ones (tables 4A and 4B). This decrease in number of lesions is interpreted as a delay in the onset of lesions. The observations in 800-day-old rats were reported previously (Berg and Simms, '60). The combined incidence of renal, vascular or myocardial lesions in restricted males (table 4A) increased with advancing age from 24% at 800 days of age to 60% at 900 days and to 75% at 1100 days, but the frequency of lesions in unrestricted males at 800 and 900 days was 100%.

For females, the combined incidence of renal, vascular or myocardial lesions in restricted rats was zero at 800 days and 24% at 1100 days as compared with 60 and 90% in unrestricted rats at corresponding ages (table 4B). Onset of disease was earlier, and incidence and severity of lesions were greater in males than in females, both restricted and unrestricted. These differences were reflected in the life expectancy and mortality rates of the two sexes (table 3). Data for three moribund restricted females from 800 to 1000 days old were not listed in table 4B, but included in table 3. All of these rats developed tumors but were free from other lesions.

In both males and females the lesions in the restricted rats were less severe than those in the ad libitum-fed animals of the same age (table 5). The former were early or moderate, whereas the latter were largely severe. However, if we compare the lesions in the restricted animals with those of ad libitum-fed animals having the same total incidence of lesions (data of 1957, Simms and Berg) we find that the distribution of severity is consistent with that previously noted during the early stages of the diseases. In other words, the incidence curves appear to have been shifted to a later age as a result of limited food intake.

Muscular dystrophy was treated separately in tables 4A and 4B because this condition did not develop before 800 days. In males, the incidence of skeletal muscle lesions at 900 days of age was 74% for unrestricted rats as compared with 30% for restricted animals. At 1100 days, the percentage for the latter was unchanged, but in another study on muscular dystrophy 100% of unrestricted males had lesions at 1000 days of age (Berg, '56). In females, the incidence was 88% for unrestricted rats at 1050 days as compared with 41% for restricted rats at 1100 days.

Incidence of tumors. The retarding effect of food restriction on the development of tumors is shown in table 6. At 800 days of age the total incidence of neoplasms in both sexes was lower in restricted animals than in unrestricted ones. For males the reduction was from 58 to 26% and for females from 41 to 12%. The P values for these differences were significant at the 1% level in males and below the 1% level in females. After 800 days of age, the number of animals with tumors increased in both unrestricted and restricted groups. In males the incidence was practically the same in ad libitum-fed and restricted rats. In females the incidence was 83% for unrestricted rats as compared with 53% for restricted ones, a difference that was not statistically significant. Retardation of tumorigenesis by food restriction was temporary for most neoplasms, but in restricted females up to 1200 days old the following tumors failed to develop: mammary fibroadenoma, islet adenoma of the pancreas and a mixed group of benign tumors. In 1948, Saxton et al., working in McCay's laboratory, noted a delay in onset of tumors in rats fed a severely restricted diet.

DISCUSSION

The favorable influence of dietary restriction on life expectancy and onset of three major diseases observed previously in rats up to 800 days of age extended to animals up to 1200 days old. Also, onset of skeletal muscle degeneration, a condition which rarely appeared before 800 days, was delayed. With advancing age, the incidence of the 4 major diseases increased in restricted rats, but severity of lesions, as well as total number, was less than in younger unrestricted animals. Owing to later onset of lesions, life expectancy was greater in females than in males, restricted and unrestricted.

Tumorigenesis, also, was delayed by food restriction, but the retardation was

TABLE 4A

Separate and combined incidence of microscopically observed lesions[1] of 4 major diseases in rats fed unrestricted and restricted diets (males)

Mean age	No. of rats	Glomerulonephritis: Total with lesions No.	Glomerulonephritis: %	Periarteritis: Total with lesions No.	Periarteritis: %	Myocardial degeneration: Total with lesions No.	Myocardial degeneration: %	Total no. of rats with renal, vascular or myocardial lesions	% with lesions	Muscular degeneration: Total with lesions No.	Muscular degeneration: %
days			%		%		%				%
Unrestricted											
786[2]	31	31	100	22	71	28	90	31	100	4	13
864	27	27	100	20	74	24	96	27	100	20	74
46%-Restricted											
818[2]	38	0	0	1	3	9	24	9	24	2	5
926	10	4	40	2	20	2	20	6	60	3	30
1082[2]	16	7	44	4	25	9	56	12	75	5	31

[1] All grades of severity.
[2] Killed at this age while in good condition. Other rats were moribund or died.

TABLE 4B

Separate and combined incidence of microscopically observed lesions[1] of 4 major diseases in rats fed unrestricted and restricted diets (females)

Mean age	No. of rats	Glomerulonephritis: Total with lesions No.	Glomerulonephritis: %	Periarteritis: Total with lesions No.	Periarteritis: %	Myocardial degeneration: Total with lesions No.	Myocardial degeneration: %	Total no. of rats with renal, vascular or myocardial lesions	% with lesions	Muscular degeneration: Total with lesions No.	Muscular degeneration: %
days			%		%		%				%
Unrestricted											
795[2]	58	33	57	16	28	14	24	34	60	1	2
900	45	37	82	15	33	29	64	42	93	17	38
1063	18	12	67	3	17	11	61	16	89	15	88
46%-Restricted											
821[2]	17	0	0	0	0	0	0	0	0	0	0
1150[2]	17	1	6	1	6	1	6	4	24	7	41

[1] All grades of severity.
[2] Killed at this age while in good condition. Other rats were moribund or died.

TABLE 5

Severity of microscopically observed lesions of 4 major diseases in rats fed unrestricted and restricted diets

Mean age	No. of rats	Glomerulonephritis				Periarteritis				Myocardial degeneration				Skeletal muscle degeneration			
		0[1]	E	M	S	0	E	M	S	0	E	M	S	0	E	M	S
days																	
						Unrestricted males											
786[2]	31	0	1	11	19	9	2	4	16	3	3	11	14	27	1	1	2
864	27	0	0	0	27	7	4	3	13	3	0	5	19	7	1	7	12
						46%-Restricted males											
818[2]	38	38	0	0	0	37	0	1	0	29	5	4	0	36	0	0	2
926	10	6	2	2	0	8	2	0	0	8	1	1	0	7	0	2	1
1082[2]	16	9	5	2	0	12	1	3	0	7	3	3	3	11	0	3	2
						Unrestricted females											
795[2]	58	25	9	9	15	42	0	3	13	44	4	5	5	57	0	0	1
900	45	8	6	15	16	30	5	2	8	16	4	12	13	28	3	8	6
1063	18	6	2	7	3	15	2	0	1	7	1	2	8	2	0	0	15
						46%-Restricted females											
821[2]	17	17	0	0	0	17	0	0	0	17	0	0	0	17	0	0	0
1150[2]	17	16	1	0	0	16	0	1	0	16	0	1	0	10	2	5	0

[1] Zero indicates no lesions; E, "early" (slight) lesions; M, moderate; S, severe.
[2] Killed at this age while in good condition. Other rats were moribund or died.

TABLE 6

Incidence of tumors occurring separately or in combination in rats fed unrestricted and restricted diets

Mean age	No. of rats	No. of rats with tumors: Single tumors, Benign	Single tumors, Malignant	Multiple tumors[1], Benign	Multiple tumors[1], Benign and malignant	Multiple tumors[1], Malignant	Total rats with tumors: Number, Benign	Number, Malignant	Number, Both	All types
days										%
Unrestricted males										
786[2]	31	15	0	3	0	0	18	0	18	58
864	27	11	1	9	1	0	21	2	22	82
46%-Restricted males										
818[2]	38	8	2	0	0	0	8	2	10	26
926	10	3	1	3	2	0	8	3	9	90
1082[2]	16	6	1	3	3	0	12	4	13	85
Unrestricted females										
795[2]	58	17	1	5	1	0	23	2	24	41
900	45	13	3	18	3	0	34	6	37	82
1063	18	3	1	9	2	0	14	3	15	83
46%-Restricted females										
821[2]	17	2	0	0	0	0	2	0	2	12
1150[2]	17	5	2	1	1	0	7	3	9	53

[1] "Benign" indicates number of animals with more than one benign tumor; "benign and malignant," the number having both benign and malignant tumors; and "malignant," the number having more than one primary malignant tumor.

[2] Killed at this age while in good condition. Other rats were moribund or died.

not as great as with other diseases. Certain tumors, such as mammary fibroadenoma and islet adenoma of the pancreas in females, appeared to be more susceptible than other types of neoplasms to nutritional influences. Pioneering work on the inhibitory effect of underfeeding, on spontaneous tumors was done by Tannenbaum ('40), who showed that the initiation and growth of breast and lung tumors in mice were delayed by restricting food intake.

The present experiments demonstrate that a single factor, namely food intake, can affect the onset of the major degenerative diseases in the rat. Though the nature of the metabolic mechanism involved in this phenomenon is obscure, it is evident that ad libitum feeding accelerates the development of lesions, whereas food restriction at a level that provides for good nutrition and prevents the storage of excess body fat has a delaying effect.

With due reservations regarding the significance of similar findings in two unrelated species, it is worthy of note that overweight and obesity predispose to shortened life expectancy in man (Armstrong et al., '51; Marks, '56; Society of Actuaries, '59), and that the diseases leading to death in man affect the same tissues as those in the rat (kidneys, arteries and heart), though the lesions differ in nature.

McCay and associates ('43) compared their severely underfed old rats with young animals and suggested that increased longevity of their restricted rats was related to immaturity. Our studies with levels of dietary restriction which was considerably less severe than those used by McCay, demonstrated that life expectancy could be extended and onset of lesions delayed without seriously retarding growth or suppressing sexual maturity by undernutrition (Berg, '60; Berg and Simms, '60). Observations of the tibial epiphyses of our restricted animals showed that further osteogenesis was highly improbable. The presence of Graafian follicles in all stages of development, including ovulation, in the ovaries of restricted

females, and a high rate of fertility (Berg, '60), were proof of sexual maturity. On the basis of this evidence, hormonal influences controlling growth and sexual development were largely excluded in our work, and the results obtained with dietary restriction could be attributed to nutritional factors.

SUMMARY

Observations on rats up to 1200 days old showed that food restriction to a level 46% below intake of rats fed ad libitum resulted in an extension of life expectancy and a delay in the onset of major diseases, including neoplasms. With this amount of dietary restriction little retardation of skeletal growth or sexual maturity was observed, and no storage of excess body fat developed as in the rats fed ad libitum.

In comparison with unrestricted rats, longevity of restricted animals was increased about 200 days in males and about 350 days in females. Females had greater longevity than males fed the same diet. Life expectancy depended upon the age of onset of lesions. With advancing age, the incidence of disease increased in both the restricted and the unrestricted rats, but number and severity of lesions remained lower in the restricted animals than in those fed ad libitum. Tumorigenesis was also delayed by dietary restriction but retardation was not as great as with other diseases.

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Magnesium Requirement of Young Women Receiving Controlled Intakes[1]

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The present status of our knowledge of magnesium requirement and its relation to other dietary constituents has been summarized recently by O'Dell ('60). Magnesium is an important constituent of cardiac and skeletal muscle, of nerve tissue and bone. It is involved in protein, fat, nucleic acid and coenzyme synthesis; in glucose utilization and in neuromuscular activity. It is also an essential activator for several enzymes, some that transfer phosphate, some that are involved with the decarboxylation of pyruvic acid and others concerned with reactions of the Krebs cycle.

Tibbetts and Aub ('37) noted that with intakes of 300 mg of magnesium daily, medical students regularly showed positive balances. Duckworth and Warnock ('42) suggested 220 mg as the magnesium requirement for women, but this amount is low in view of results reported since that time. Leichsenring et al. ('51) found a mean daily retention of 11.6 mg in 9 college women receiving a magnesium intake of 260 mg but 4 of the women were in negative balance for a part of the 21-day period at this intake. More recently Scoular et al. ('57) have reported the magnesium intake and output for 86 young women during a 5-day period when they used analyzed self-chosen diets. Of the 26 subjects with daily intakes between 100 mg and 200 mg of magnesium, 22 were in negative balance; of the 12 subjects with intakes between 220 mg and 300 mg, 10 were in negative balance; and of the 48 subjects with intakes above 300 mg, only 7 were in negative balance.

The purpose of this paper is to add to our knowledge of the quantitative requirements of human subjects for magnesium by presenting the results of metabolic studies of healthy young women receiving a controlled diet with variations in magnesium intake. These results are one phase of a project that measured the range in metabolic response to controlled intakes of several nutrients studied simultaneously.

PROCEDURE

Information on the locations and times of the studies, the young women who served as subjects, and the levels of magnesium intake is given in table 1.[4] The subjects were judged to be in good health on the basis of a thorough medical examination.

The length of each study was divided into consecutive periods of 5 days each and designated A, B, C, D and so on. During the first 5 days of the study, period A, the subjects ate their customary self-chosen diets. They recorded their food consumption in estimated household measures and the magnesium content of

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TABLE 1

General information

Location and initial date of study	Length of study	Subjects—women students				Controlled Mg intake[1]	
		Number	Range				
			Age	Height	Weight		
	days		*years*	*cm*	*kg*	*mg/day*	*days*
Oklahoma State University February, 1956	40	9	19–22	161–178	56–71	247	15
						183	10
						280	10
University of Minnesota January, 1957	35	7	18–21	158–173	47–62	320	15
						236	15
University of Nebraska February, 1956	40	8	18–24	159–176	47–73	278	35
University of Alabama November, 1955	20	6	20–23	159–175	52–65	252	15

[1] During the first 5 days the subjects consumed self-chosen diets.

the self-chosen diets was calculated from figures given by McCance and Widdowson ('36, '47), supplemented by those given by Sherman ('52).

Beginning with the 6th day, the first day of period B, and continuing until the end of the study, all of the subjects were fed the standardized diet which had been developed by the Human Nutrition Research Division of the Agricultural Research Service and is described in detail in USDA Technical Bulletin no. 1126 (Meyer et al., '55). The diet includes ordinary foods which provide palatable meals but only small amounts of most of the minerals and vitamins. Supplements of synthetic and purified products are added so that the total intake provides adequate amounts of essential nutrients.

The intake closely approximated 11 gm of nitrogen daily, 80 gm of fat, 750 mg of calcium, and 950 mg of phosphorus, 60 mg of ascorbic acid, 0.8 mg of thiamine, and 1.0 mg of riboflavin. The exact intakes of these nutrients by the subjects at each location were determined by analyses of food composites (Meyer et al., '55). The basal diet supplied 250 gm of carbohydrate but more sugar was added as needed for sufficient energy value to maintain each subject at a constant weight. By calculation the amounts of trace elements and vitamins provided daily were: (in milligrams) iron, 10; copper, 0.8; iodine, 0.105; manganese, 2; zinc, 4; niacin, 7; pantothenic acid,[5] 4; pyridoxine, 0.8; choline, 300; also folic acid, 100 µg; cobalamin, 5 µg, vitamin A, 4000 I.U. and vitamin D, 400 I.U.

The magnesium in the standardized diet was supplied about equally by the foods and by magnesium gluconate which was incorporated into yeast rolls. The amount of the gluconate was altered to accomplish changes in the total magnesium intake.

Complete collections of urine and feces were made beginning with the first day of period A and continuing throughout the study. Carmine was used to mark the feces at the beginning of each 5-day period.

Magnesium was determined by the method of Orange and Rhein ('51) on aliquots of 5-day composites of food, urine, and feces which had been dry-ashed. The intensity of the color developed was measured in a Beckman spectrophotometer exactly 10 minutes after the addition of the reagents.

RESULTS AND DISCUSSION

The mean daily intake and balance of magnesium for the subjects in each state and for all subjects during different periods are shown in table 2 together with

[5] In Nebraska, response to pantothenic acid intakes of 8 mg and 13 mg was studied also, but the magnesium balances were not affected by these variations in the level of pantothenic acid.

TABLE 2

Mean daily intake and balance of magnesium for subjects in each state and for all subjects during different periods[1]

State and period	Intake[2]	Balance	Range in balance	Subjects in negative balance
	mg	*mg*	*mg*	*no.*
Oklahoma, 9 subjects				
Period A	317 ± 76[3]	53 ± 72[3]	− 75 to 134	2
Period B	251	23 ± 34	− 21 to 16	3
Periods C, D	247 ± 2	− 5 ± 14	− 24 to 16	6
Periods E, F, 8 subjects	183 ± 6	−33 ± 25	− 3 to −80	8
Periods G, H, 8 subjects	280 ± 3	5 ± 10	− 2 to 22	3
Minnesota, 7 subjects				
Period A	263 ± 64	4 ± 70	− 71 to 147	4
Period B	292	−29 ± 34	− 98 to 2	6
Periods C, D	320	28 ± 23	2 to 68	0
Periods E, F	238	−22 ± 36	−101 to 4	6
Period G	233	−43 ± 22	− 77 to −15	7
Nebraska, 8 subjects				
Period A	275 ± 54	48 ± 85	− 69 to 129	2
Period B	260	14 ± 49	− 54 to 71	3
Periods C, D	274	9 ± 21	− 20 to 42	3
Periods E, F	275	16 ± 23	− 12 to 52	3
Periods G, H	286	0 ± 40	− 87 to 26	2
Alabama, 6 subjects				
Period A	244 ± 46	19 ± 39	− 11 to 96	2
Period B	252	16 ± 23	− 8 to 47	2
Periods C, D	252	−11 ± 14	− 28 to 10	4
All states				
Period A, 30 subjects	279 ± 65	34 ± 65	− 75 to 147	10

[1] Each letter represents a 5-day period; as periods C, D equal 10 days, and E, F equal 10 days.
[2] Intakes for period A were calculated rather than determined by analysis.
[3] Standard deviation of observations.

the ranges in balances and the number of subjects in negative balance. Period B, the first 5 days that the women received the standardized diet, was considered a transition or adjustment period and the intake and excretion values were not included in calculations of subsequent means or in the regression equation. Also the values for periods beyond the first two with the same intake, namely, period G in Minnesota and periods E through H in Nebraska, were omitted from the statistical treatment and from figure 1 in order to keep the length of time at each intake the same for all subjects.

The magnesium balance of each subject at each level of intake for which she was studied is charted in figure 1 for both the controlled and the self-chosen diets. No significant difference was found in the magnesium retention of the subjects in the 4 locations when analysis of covariance was employed to adjust for differences in intake. Variation in retention between subjects was not reduced when intake and retention were calculated on the basis of per kilogram of body weight.

Results of regression analyses are shown in table 3. The equation and the line for regression of intake on balance are given in figure 1 for the controlled intakes and for the calculated self-chosen intakes. Magnesium excretion and retention increased significantly as intake increased. Because only 20 to 22% of the variation in excretion is explained by differences in the controlled intake, intake cannot be considered a good predictor of excretion. It can be used to predict retention, however, inasmuch as 44% of the variation in retention values during periods of controlled intake is explained by differences in intake.

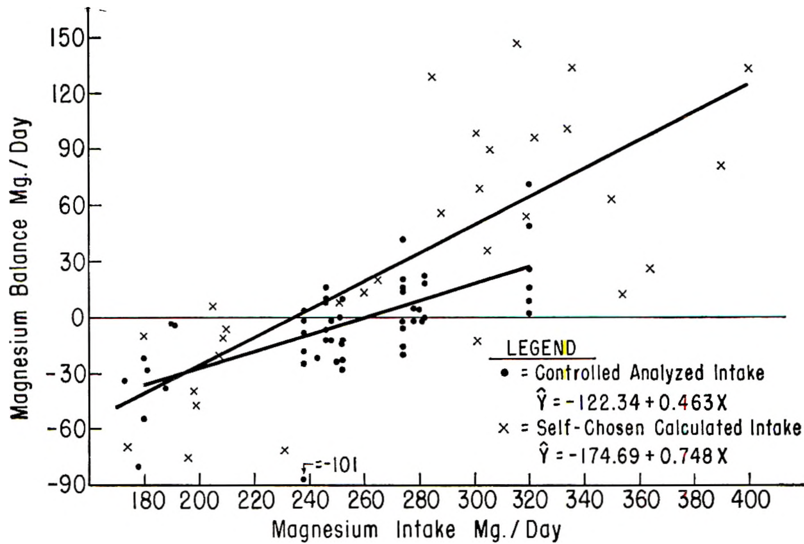

Fig. 1 Relation of intake to balance of magnesium with controlled and self-chosen diets.

TABLE 3

Regression of magnesium intake on excretion and balance

Regression	No.	b[1]	Proportion of variation explained by Mg intake[2]
Controlled magnesium intake (mg/day) on			
Urinary excretion, mg/day	53	0.22[3]	22
Fecal excretion, mg/day	53	0.32[3]	20
Retention, mg/day	53	0.46[3]	44
Self-chosen magnesium intake (mg/day) on			
Urinary excretion, mg/day	30	0.04	2
Fecal excretion, mg/day	30	0.21	11
Retention, mg/day	30	0.75[3]	57

[1] Change in excretion or retention for an increase of 1 mg/day intake of magnesium.
[2] Square of correlation coefficient.
[3] Significant at 1% level.

The regression values for period A must be interpreted cautiously because the magnesium intake was calculated from records of food intake estimated in terms of household measures rather than by quantitative analysis of actual intake. There was, however, a highly significant relationship between intake and retention, and 57% of the variation in retention in period A was explained by differences in intake. The calculated magnesium content of the self-chosen diets ranged from 174 mg to 400 mg daily. Ten subjects had intakes below 250 mg and 9 of these were in negative balance. Of the 20 subjects with calculated daily intakes above 250 mg, only one was in negative balance.

In table 4 the data have been grouped on the basis of the controlled levels of magnesium intake at which the women were studied. Both urinary and fecal excretion as well as retention increased directly with intake. Coefficients of variation for intake and excretion were stable for each of the groups of magnesium intakes. Coefficients of variation for retention were about 80% for both the low magnesium intake, 173 to 191 mg per day, and for the high intake, 320 mg per day, and were about 200% for the two intermediate intake groups. Thus there is little doubt about daily intakes from 173 to 191 mg being inadequate, or about an intake of 320 mg being adequate, for

TABLE 4

Means, standard deviations, and coefficients of variation for intake, excretion, and balance of magnesium for all subjects, grouped according to controlled magnesium intakes

Intake group	Statistic	Intake	Urine	Feces	Balance	Subjects in negative balance
mg/day		*mg/day*	*mg/day*	*mg/day*	*mg/day*	%
173–191(8)[1]	Mean	183	88	128	−33	100
	S.D.[2]	6	12	31	26	
	C.V., %[3]	3	14	24	77	
233–252(22)	Mean	245	99	158	−12	77
	S.D.	6	19	26	24	
	C.V., %	2	19	17	196	
274–282(16)	Mean	277	101	170	7	37
	S.D.	3	15	23	16	
	C.V., %	1	15	14	223	
320(7)	Mean	320	123	169	28	0
	S.D.	—	12	24	23	
	C.V., %	—	10	14	83	

[1] Figures in parentheses indicate number in group.
[2] Standard deviation of observations.
[3] Coefficient of variation.

maintaining magnesium equilibrium in these women. Six, or 37%, of the 16 women who had daily intakes between 274 and 282 mg of magnesium were excreting slightly more than they were taking in, but the extent of their negative balances was slight. Three of the 6 women had daily balances of −2 mg and the other three had balances of −6, −16, and −20 mg of magnesium daily.

In the 8 Nebraska subjects it was possible to observe the response to a constant magnesium intake for 30 days. The mean daily balances for the three consecutive 10-day periods CD, EF, and GH were 9 mg, 16 mg, and 0 mg, respectively, and were not significantly different. The mean intake for the 30 days was 278 mg and the mean balance was 8 mg with a standard deviation of 14 mg.

When Meyer et al. ('55) were developing and testing the standardized diet used in the present study, they determined magnesium balances for 6 young women for 35 consecutive days. At an intake of 181 mg of magnesium daily, the mean daily balance was −26 ± 18 mg and one of the 6 subjects was in equilibrium. Comparable results were obtained with the 8 Oklahoma subjects during only 10 days at an intake of 183 mg of magnesium daily. The mean balance was −33 ± 25 mg daily, all subjects were in negative balance, and the range in balances was from −3 to −80 mg daily.

The balances obtained in the present study during the 10-day periods of controlled intake suggest that a daily magnesium intake of more than 280 mg and perhaps as much as 320 mg would have been needed to insure equilibrium in all of these 30 subjects. On the basis of reports in the literature and the results of this study, a magnesium requirement of about 300 mg daily seems indicated for women.

A deficiency of magnesium has never been considered a dietary problem in the U.S.A. In its report of recommended dietary allowances, the Food and Nutrition Board ('58) states " . . . it is difficult to visualize a human diet deficient in this element (magnesium)." In general a diet that is adequate in other essential nutrients, especially protein of high quality, is considered to supply generous amounts of magnesium. When a magnesium deficiency occurs, it is usually associated with alcoholism or electrolyte imbalance, rather than an inadequate dietary intake. As the interrelationships among dietary essentials, including the role of magnesium in fat metabolism, are elucidated, the importance of magnesium intakes ade-

quate for maintaining equilibrium or retention will be established.

SUMMARY

Metabolism studies were conducted on 30 young women to determine their magnesium balances when they received a controlled standardized diet. The daily magnesium intakes ranged from a low of 173 mg, through intermediate amounts of 230 and 250 mg, to higher intakes of 280 and 320 mg. Each subject was studied at one, two or three levels of magnesium intake for at least 10 days following an adjustment period when the standardized diet was supplied.

At daily magnesium intakes of 173 to 191 mg the mean balance was −33 mg, and at intakes of 233 to 252 mg, the balance was −12 mg. With intakes of 274 to 282 mg mean retention was 7 mg, but 6 of the 16 subjects were in slight negative balance. Subjects receiving a daily intake of 320 mg had a mean daily retention of 28 mg. The relationship between the intake and the retention of magnesium for these women was significant at the 1% level. This was also true for a 5-day period when subjects consumed their self-chosen diets and the magnesium content of the estimated food intake was calculated from tables of food composition rather than by analysis.

The balances obtained during the periods of controlled intake suggest that a daily magnesium intake of more than 280 mg and perhaps as much as 320 mg would have been needed to insure equilibrium in all of the young women in the present study. On the basis of reports in the literature and the results of this study, a magnesium requirement of about 300 mg daily seems indicated for women.

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Effect of Thyroid Activity on the Absorption, Storage and Utilization of Orally Administered Vitamin A Aldehyde (Retinal) in Rats[1]

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In parallel experiments, Cama et al. ('57) studied the effect of hyper- and hypothyroidism on the conversion of β-carotene and retinal to vitamin A, assuming that the conversion of carotene to vitamin A *in vivo* takes place in two stages, (a) β-carotene → retinal (Glover and Redfearn, '54) and (b) retinal → vitamin A (Glover et al., '48). Cama et al., in their study, confirmed the observations of Johnson and Baumann ('47) by demonstrating that hyperthyroid rats stored more and that hypothyroid rats stored less vitamin A in the liver than their controls when β-carotene was administered orally; also that under identical experimental conditions, hyperthyroid rats fed retinal stored less liver vitamin A and hypothyroid rats fed retinal showed more vitamin A in the livers. It was further demonstrated that thyroid activity did not have any direct effect on the conversion of orally administered retinal to vitamin A. According to Johnson and Baumann ('48), the depletion of vitamin A from the liver, depended not only on the basal metabolic rate, being increased by thyroid and decreased by thiourea or thiouracil, but also on the rate of growth. The effect of restricted growth in retarding the depletion of vitamin A, however, was more marked than the effect of the thyroid in accelerating the depletion of vitamin A, when given at the same time.

In the present investigation, the effect of thyroid activity on the absorption of retinal from the intestines of rats fed retinal and on the utilization of liver vitamin A was studied, in an attempt to elucidate the cause for the differences in liver vitamin A levels of rats fed carotene and retinal under identical conditions. It will be shown that decreased liver storage of vitamin A in hyperthyroid and increased liver storage in hypothyroid rats fed retinal are the result of differences in the extent of utilization only.

MATERIALS AND METHODS

Oil solution of retinal for feeding. Pure retinal was prepared by the oxidation of vitamin A alcohol[2] with manganese dioxide[3] (Cama et al., '57). The product, after chromatography over 10% (v/w) water-treated Alumina,[4] had an $E^{1\%}_{1\,cm}$ of 1513 at 370 mμ (light petroleum ether).[5] It was approximately 90% pure on the basis of $E^{1\%}_{1\,cm}$ value of 1690 for crystalline retinal.

The method for preparing the oil solution of retinal was the same as that reported earlier (Cama et al., '57). The concentration of retinal in the oil solution was such that a daily dose of 0.1 ml of the oil contained 1 mg of retinal and 0.5 mg of α-tocopherol.[2]

Preparation of animals and plan of experiment. Four-week-old weanling albino rats of both sexes, weighing between 25 to 40 gm were used. Littermates of the same sex were kept in one set to facilitate comparison. From each set of

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[2] Supplied through the courtesy of Hoffmann-La Roche Ltd., Switzerland.

[3] B.D.H. Laboratory reagent.

[4] Alumina, specially prepared for chromatographic adsorptions, E. Merck, Germany.

[5] Light petroleum (b.p. 40 to 60°C) obtained from Burmah Shell is left over $KMnO_4$, washed, dried over $CaCl_2$ and twice distilled before use.

rats, an almost equal number were allotted to control, hyperthyroid and hypothyroid groups. The rats in the control group received only the vitamin A-free diet throughout the experimental period, whereas the animals in the hyper- and hypothyroid groups received 0.125% of iodinated casein[6] and 0.1% of thiourea[7] in the vitamin A-free diet, respectively, after they had been fed the vitamin A-free diet for the initial two weeks during depletion.

The vitamin A-free diet contained the following: (in per cent) casein (ether-extracted), 18; cornstarch, 63; sugar, 10; refined arachis oil, 5; salt mixture,[8] 4; also the following vitamins per kilogram of diet (in milligrams) menadione, 5; thiamine·HCl, 5; pyridoxine, 5; riboflavin, 5; niacinamide, 50; pantothenic acid, 50; biotin, 0.5; folic acid, 0.5; inositol, 100; (in micrograms) α-tocopherol, 100; calciferol, 100; and choline chloride, 1 gm.

In the first experiment, 0.1 ml of the oil solution of retinal was fed daily to control and experimental groups for three days. The feces were collected daily from the time of feeding the first dose of retinal until the end of the experiment—then stored at − 10°C until analyzed. On the third day, the animals were dissected two hours after the last dose and the blood was collected by cardiac puncture. The intestines were washed with 0.9% saline and the washings stored at 0°C for analysis of retinal and vitamin A. The washed intestines, colon, stomach, liver and kidney were stored at − 30°C until analyzed. The intestinal washings and the sera of rats were analyzed on the same day.

In the second experiment, the rats were fed the oil solution of retinal for three days as in the first experiment. After this period, dosing with retinal was discontinued and the animals were fed the respective diets for an additional 5 days. On the 6th day, animals were killed and the livers analyzed for vitamin A.

Preparation of tissue extracts. Petroleum ether extracts of sera from individual rats were prepared as follows: an equal volume of ethanol was added to a measured volume of the serum and the mixture extracted with light petroleum ether in a separatory funnel. The solvent was removed *in vacuo* and the residue dissolved in 0.5 ml of chloroform;[9] 0.4 ml of this solution was used for the estimation of vitamin A in serum.

The intestinal washings from each rat were triturated with an equal volume of ethanol and extracted three times with light petroleum ether. The extract was dried over anhydrous sodium sulphate,[10] reduced in volume and checked for vitamin A and retinal by spectrophotometry and chromatography over 5% (v/w) water-treated Alumina. In a mixture, vitamin A and retinal were estimated as described later.

All other tissues from each rat, such as colon, intestines, feces, liver and kidney, were ground separately with sand and sodium sulphate and extracted 4 times with light petroleum ether on a warm water bath. Extracts were then dried over anhydrous sodium sulphate, reduced in volume and analyzed for either vitamin A or retinal. Extracts of colon and feces were saponified and the unsaponifiable fractions analyzed for vitamin A and/or retinal.

Estimation of vitamin A. The serum and kidney vitamin A were estimated by the antimony trichloride color test at 620 mμ whereas vitamin A in liver and intestines was estimated by the three-point correction method of Cama et al. ('51). A few parallel estimations in liver and intestines were carried out by the antimony trichloride color test at 620 mμ and good agreement was observed between the values obtained by the two methods. All values were converted into international units to facilitate comparison.

Estimation of retinal. Retinal in stomach extracts was estimated by the antimony trichloride color test at 664 mμ and the values were expressed in international units (1 gm of all-*trans* retinal = 3.05×10^6 I.U., Ames et al., '55).

Estimation of vitamin A and retinal in a mixture. To estimate a mixture of vitamin A and retinal, as found in the intestinal washings from rats, the antimony trichloride color test was carried out at 620 and 664 mμ. From the known values

[6] Boots Drugs Company, United Kingdom.
[7] British Drug Houses Ltd., United Kingdom.
[8] Hawk and Oser ('31).
[9] B.D.H. Analar quality.
[10] E. Merck, Germany.

of E_{620}/E_{664} for vitamin A (13.6; Cama et al., '51) and E_{620}/E_{664} for retinal (determined in the present investigation as 0.5571), E_{620} corrected for retinal and E_{664} corrected for vitamin A could be calculated. The amounts of vitamin A and retinal were then computed from the corrected E values at 620 and 664 mμ, respectively, and expressed as total international units of vitamin A and retinal. The sum of the international units of retinal observed in stomach extract and those of vitamin A and retinal in the intestinal washings was used to denote the unabsorbed vitamin in the gastrointestinal tract.

Significances of the differences between the control and the treated groups was analyzed statistically by the "t" test of significance for paired values as given by Davies ('49).

RESULTS

The results of the tissue analysis for vitamin A and/or retinal are presented in tables 1 and 2.

The unsaponifiable fractions of the colon and feces, analyzed separately, showed neither specific absorption in the ultraviolet nor blue color with antimony trichloride, thereby indicating absence of vitamin A or retinal in the colon and feces.

The unabsorbed vitamin in the gastrointestinal tract of hyperthyroid rats was significantly less than that in the respective controls ($P < 0.01$), whereas no such difference was observed with hypothyroid rats (table 1). The hyperthyroid rats had more vitamin A in serum ($P < 0.05$) and in the intestine ($P < 0.1$) than controls but the differences in serum and intestinal vitamin A levels between control and hypothyroid animals lacked significance (table 1). No significant differences between the control and treated groups were observed in vitamin A levels of kidneys.

In the first experiment, in which the animals were sacrificed two hours after the last dose, rats in both hyperthyroid as well as hypothyroid groups had significantly higher liver vitamin A reserves than controls ($P < 0.005$ and $P < 0.001$, respectively, table 1). In the second experiment, in which 5 days were allotted after the last dose for the animals to use up liver reserves, hyperthyroid rats had less liver vitamin A than controls ($P < 0.05$), whereas the hypothyroid animals showed the same pattern as in the first experiment (table 2).

Vitamin A was always detected by its characteristic absorption at 328 mμ (light petroleum ether) in intestinal washings of control and treated groups. Retinal was also found in intestinal washings but could be detected only after chromatography over 5% (v/w) water-treated Alumina.

DISCUSSION

The liver vitamin A levels in rats, whether derived from carotene or retinal, should be a net result of the extent of three steps, namely, (1) conversion of the precursor to vitamin A in the intestines; (2) absorption of vitamin A from the intestines; and (3) utilization of the liver reserves. Cama et al. ('57) showed that thyroid hyperactivity lowered the liver vitamin A in retinal-fed rats but had no effect on the conversion of intravenously administered retinal to vitamin A. Thus, in the present study, the influence of the thyroid on absorption and utilization of vitamin A in retinal-fed rats was investigated.

In the first experiment, the animals were sacrificed two hours after the last dose. The unabsorbed vitamin A and retinal remaining in the gastrointestinal tract from the last dose of retinal, as well as serum, fecal and colon vitamin A were determined separately for each rat. Vitamin A was also estimated in liver, kidney and intestine.

The absence of vitamin A or retinal in the feces and colon suggested that the animals in the control and treated groups could absorb 1 mg of retinal daily when sufficient time was allowed for absorption. In this experiment, however, since the animals were sacrificed two hours after the last dose of retinal, some unabsorbed vitamin could be detected in gastrointestinal tract. This unabsorbed vitamin was significantly less in hyperthyroid animals than the controls (table 1), probably because of a faster rate of absorption in the hyperthyroid animals as compared with the controls. The higher levels of serum and intestinal vitamin A of hyperthyroid

TABLE 1

Effect of iodinated casein and thiourea on vitamin A and retinal in the gastrointestinal tract and on vitamin A in the serum, intestine, kidney and liver of rats fed 1 mg of retinal in arachis oil for each of three days (animals killed 2 hours after the last dose)

Material analyzed	No. of observations	Group	Total vitamin A and/or retinal	Mean Δ (experimental − control)	S.E. Δ[1]	P	Significance
			I.U.				
Gastrointestinal tract (stomach + intestinal washings)	8 8	Control Iodinated casein	1231 849	−382	±108.4	< 0.01	Significant
	8 8	Control Thiourea	1126 982	−144	±169.6	> 0.4	Not significant
Serum	7 7	Control Iodinated casein	37.37 58.04	+ 20.67	± 7.64	< 0.05	Significant
	8 8	Control Thiourea	45.21 69.67	+ 24.46	± 13.96	> 0.1	Not significant
Intestine	12 12	Control Iodinated casein	164.3 224.4	+ 60.1	± 27.45	< 0.1	Significant
	13 13	Control Thiourea	183.7 204.6	+ 20.9	± 26.82	> 0.4	Not significant
Kidney	11 11	Control Iodinated casein	10.0 11.8	+ 1.8	± 1.21	> 0.1	Not significant
	13 13	Control Thiourea	9.97 9.16	− 0.81	± 2.93	> 0.3	Not significant
Liver	12 12	Control Iodinated casein	2309 2924	+615	±170.5	< 0.005	Highly significant
	13 13	Control Thiourea	2298 2775	+477	± 96.63	< 0.001	Highly significant

[1] Standard error of the differences.

TABLE 2

Effect of iodinated casein and thiourea on vitamin A in the liver of rats fed 1 mg of retinal for each of three days (animals killed 5 days after the last dose)

No. of observations	Group	Total vitamin A	Mean Δ (experimental − control)	S.E. Δ[1]	P	Significance
		I.U.				
17	Control	2594	−315	±150.9	< 0.05	Significant
17	Iodinated casein	2279				
19	Control	2480	+507	±116.0	< 0.01	Significant
19	Thiourea	2987				

[1] Standard error of the differences.

rats two hours after the last dose also strongly supported such a probability. No such significant differences in the unabsorbed vitamin A and retinal, serum vitamin A or intestinal vitamin A could be observed between the control and the hypothyroid animals (table 1).

In the first experiment, rats in both the treated groups had higher liver vitamin A values compared with controls (table 1). This result was in agreement with that of Cama et al. ('57) for the thiourea-treated group but was contrary to that reported by Cama et al. ('57) for the iodinated casein-treated group.

Because of the results obtained in the first experiment, another experiment was carried out (table 2), in which the livers of rats were analyzed 5 days after the last dose of retinal had been fed, so as to give the animals enough time to utilize liver reserves. Here, observations for the liver were altered in hyperthyroid rats, which stored less vitamin A than controls, probably due to greater depletion. Liver values remained the same, however, in hypothyroid groups, which had more vitamin A than controls, as observed in the first experiment and in the earlier study (Cama et al., '57).

It appears, therefore, that in the first experiment, the faster rate of absorption of the last dose by hyperthyroid rats temporarily altered the liver values in these animals. In the hypothyroid animals, no significant differences from the controls could be observed for rate of absorption, and the liver vitamin A was consistently high, probably due to slow depletion of vitamin A reserves.

The results of the two experiments in the present study and those of Cama et al. ('57) suggest that, though the rate of absorption of retinal by hyperthyroid rats was faster than that of controls, resulting in temporary rise in liver vitamin A, the differences in the liver vitamin A levels were due only to differences in the extent of utilization by the control and treated groups.

Both vitamin A and retinal were present in the intestinal washings. This was not the experience of Glover et al. ('48) who observed only unchanged retinal in the intestinal washings after oral dosing with retinal. Possibly the vitamin A which is formed from retinal in the inner lining of the intestinal wall is partly released into the intestine and hence detected in the washings.

From the present study and that of Cama et al. ('57) the question arises of why thyroid activity has opposite effects on the liver vitamin A levels when β-carotene or retinal is fed under identical conditions. If the effect of thyroid were only on the utilization of vitamin A by the animals, then, under identical experimental conditions, feeding of β-carotene or retinal should give similar results. If the thyroid hormone increases the absorption of vitamin A formed from the precursor in the intestines, as suggested by Cama and Goodwin ('49), then a similar effect should occur whether vitamin A is derived from β-carotene or retinal, and hyperthyroid rats should store a greater quantity regardless of the source. This suggests that in carotene-fed rats, the thyroid has an influence on some factor other than absorption and utilization. If we assume retinal to be an intermediate in the two-stage conversion of β-carotene to vitamin A in the intestines, it may well be that the additional effect of thyroid possibly occurs in the first stage of conversion of caro-

tene to vitamin A. Further work is necessary to prove this hypothesis.

SUMMARY

The effect of iodinated casein and thiourea on absorption, storage and utilization of vitamin A in rats fed retinal orally has been investigated. Rats in both the control and the treated groups absorbed 1 mg of retinal per day when sufficient time was allowed. However, the rate of absorption of this dose, as indicated by unabsorbed vitamin and retinal remaining in the gastrointestinal tract, serum vitamin A and liver vitamin A levels two hours after the last dose of retinal, was faster in hyperthyroid rats, as compared with the controls. Thus, hyperthyroid rats were observed to have more vitamin A in livers if analyzed two hours after the last dose of retinal, whereas they stored less vitamin A if analyzed 5 days after the last dose, probably due to greater utilization. Conversely, the hypothyroid rats had consistently higher liver reserves than the controls whether analyzed two hours or 5 days after the last dose of retinal, probably due to poorer utilization of their liver reserves of vitamin A.

In control and treated rats, vitamin A was always detected in the intestinal washings analyzed two hours after the oral administration of the last dose of retinal.

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Effect of Dietary Protein and Zinc on the Absorption and Liver Deposition of Radioactive and Total Copper[1,2,3]

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The metabolism of any element such as copper, including the absorption from the intestinal tract, depends upon numerous factors in addition to the dietary concentration of the element. These other factors become especially important when one considers the effects of a toxic level of copper and the interactions of copper with other dietary nutrients.

Copper salts have always been regarded as toxic when ingested in more than trace amounts. Yet the specific level of dietary copper which may be considered toxic to a species has not been a matter of complete agreement. In early reports Boyden et al. ('38) found a 14-fold increase in the copper content of livers of rats fed a diet containing 500 ppm of added copper for one month. Cunningham ('31) and later Coulson et al. ('34) fed levels of 750 ppm of copper in the diets of rats, with no apparent ill effects. The results of more recent work by Mitchell ('53) and Barber et al. ('55) have indicated that the addition of relatively high concentrations of copper (150 and 250 ppm) to the diets of swine had beneficial effects. Preliminary work by Bass et al. ('56), in an attempt to confirm these findings, resulted in a loss of the animals owing to copper toxicity when 250 ppm of copper were added to the diets; although, later trials by these same investigators showed some beneficial effect from lower levels of added copper. These differences in results reported by the various investigators led to the suggestion that some factor in the diets may be responsible for the apparent differences in the absorption of the copper. It has been established that there are organic compounds in forages which will enhance the absorption of dietary copper by the rat (Mills, '54, '55).

In the investigations reported here, interactions of copper and protein with zinc and with each other have been considered in respect to liver accumulation of dietary copper and of radioactive copper, Cu^{64}.

EXPERIMENTAL

The diets were composed of casein and sucrose; mineral mix, Phillips and Hart ('35), 4%; vitamin fortification mixture,[4] 2%; purified cellulose, 3%; and vegetable oil, 8%. The levels of casein and sucrose were adjusted to give diets containing 10, 17.5 and 25% of protein. Copper phosphate, $Cu_3(PO_4)_2$, instead of copper sulfate was used in the diets which required the addition of high levels of copper, both because the copper phosphate eliminated the problem of rancidity and also because it did not affect the palatability of the feed at the levels used. The animals receiving the added copper consumed less feed than the others for the first few days after they started to receive the diet. After this time food consumption was about the same for

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[2] Data reported here were taken in part from a dissertation submitted by the senior author to the Graduate School of the University of Florida in partial fulfillment of the requirements for the degree of Doctor of Philosophy: Kulwich, R. 1951 Inter-relationships of molybdenum, copper and zinc in the simple-stomached animal.

[3] Florida Agricultural Experiment Station Journal Series, no. 1207.

[4] Mg/100 gm of ration: α-tocopherol, 11; ascorbic acid, 100; inositol, 11; choline chloride, 165; menadione, 5; p-aminobenzoic acid, 11; niacin, 10; riboflavin, 2.2; pyridoxine·HCl, 2.2; thiamine·HCl, 2.2; Ca pantothenate, 6.6; biotin, 0.044; folic acid, 0.2; and vitamin B_{12}, 0.003. Vitamins A and D were included at 1980 and 220 I.U. per 100 gm of diet.

all lots. Zinc carbonate, $ZnCO_3$, was used in the rations which required the addition of zinc. Piebald rats of the Long Evans strain raised in our laboratory were fed the rations ad libitum during the entire time of the trial up to the time of sacrifice.

The radioactive copper used in experiments 2, 3 and 4 was obtained from Oak Ridge as cupric nitrate, $Cu(NO_3)_2$, was converted to cupric chloride, $CuCl_2$ and administered orally to the animals by stomach tube. Approximately 24 to 30 hours after dosing, the rats were killed and the livers assayed for percentage of dose of the $copper^{64}$ and analyzed for total copper concentration. Total copper and zinc were determined by the method of McCall et al. ('58). For the $copper^{64}$ assay the livers were dried under infrared lamps, pre-ashed in beakers over Bunsen burners and ashed for 4 hours in a muffle furnace at 500°C. The livers were then broken up in the beakers with glass rods, transferred to glass beakers and radioactivity measured with a thin-window Geiger-Mueller tube.

In the first experiment, 8 male and 8 female rats, about 30 days old, were fed the ration indicated in table 1 for 52 days. At the end of this time the animals were killed and the livers taken for copper and zinc analyses.

In experiment 2, 54 weanling rats, 28 males and 26 females, were fed the diet indicated in table 2 for 15 days. After this time the animals received an oral dose of $copper^{64}$, and 36 hours later were killed and the livers taken for $copper^{64}$ and total copper analyses. The animals in this and the following experiments were fed the diets for the relatively short period of time in order that the amount of $copper^{64}$, found in the livers, would not be influenced by the amount of total copper present in the event there was a large build-up of total copper.

For the third experiment 24 weanling rats, 12 males and 12 females, the same age and approximately the same weight were fed the diets as indicated in table 2. After the animals had received these diets for 30 days, they were given an oral dose of $copper^{64}$ and approximately 30 hours later were killed and the livers taken for total copper and $copper^{64}$ analyses.

Two age groups of rats were used in experiment 4. Animals in the first group consisted of 18 males and 18 females about 8 weeks of age, with an average weight of 117 gm. In the second group there were 11 male and 12 female mature animals. Each group was divided into two lots and received the ration as indicated in table 2 for 15 days. At the end of this time all the animals received oral doses of labeled copper. Thirty-six hours after dosing, the animals were killed and the livers taken for total and radioactive copper analyses.

All data were analyzed by the split plot design as described by Cochran ('50).

RESULTS

No significant differences were observed between the results for males and females; hence the results for both sexes which received the same treatment were combined.

Based on the results of preliminary work it was expected that the addition of 1,000 ppm of copper to the diets would result in at least a 5- to 10-fold increase in liver copper. The data obtained from experiment 1 as shown in table 1 are unusual in that no significant differences were observed in the liver copper concentration between the animals receiving zero or 1,000 ppm of added copper.

The level of zinc added to the diets was only about one third that amount which may be considered toxic to rats and there is no indication that this addition of zinc had any effect on the concentration of copper in the livers at the mineral levels used in these diets. The level of protein used in the first experiment was higher than that normally used for studies with rats and this may have been the factor with the greatest influence of the concentration of copper in the livers.

In experiment 2 the length of time the animals were on experiment was reduced to 15 days as indicated in table 2. The same amounts of copper and zinc were added as in experiment 1 but the dietary level of protein was reduced to 17.5 and 10%. These levels were used because 17.5% as casein is the minimum amount that will completely fulfill the essential amino acid requirements of growing rats and the 10% level is definitely deficient.

TABLE 1

Concentration of copper and zinc in the livers of rats fed high levels of copper and zinc in 25% protein diets for 52 days

Intake		Mean and S.D. liver, copper and zinc	
Copper, added	Zinc, added	Copper	Zinc
ppm	*ppm*	*ppm*	*ppm*
0	0	23 ± 6	181 ± 26
1000	0	34 ± 14	146 ± 9
0	1000	27 ± 6	225 ± 6
1000	1000	25 ± 15	200 ± 24

TABLE 2

Effect of dietary protein level on the concentration of total and radioactive copper in the livers of rats fed high levels of copper

No. rats	Intake			Means and S.D. of liver copper	
	Protein (casein)	Copper added	Zinc added	Total copper	Cu^{64}
	%	*ppm*	*ppm*	*ppm*	*% of dose*
			Experiment 2[1]		
10	10	0	1000	26	0.4 ± 0.3
8	10	1000	0	444	2.0 ± 1.1
10	10	1000	1000	120	0.8 ± 1.1
9	17.5	0	1000	17	0.4 ± 0.4
8	17.5	1000	0	43	0.4 ± 0.2
9	17.5	1000	1000	15	0.3 ± 0.2
			Experiment 3[2]		
4	10	0	0	25 ± 2	0.7 ± 0.3
4	10	2500	0	918[3] ± 121	4.2[3] ± 4.0
4	17.5	0	0	21 ± 15	0.5 ± 0.1
4	17.5	2500	0	347[3] ± 312	3.6[3] ± 0.4
4	25	0	0	15 ± 2	0.4 ± 0.1
4	25	2500	0	36 ± 35	0.9 ± 0.0
			Experiment 4[4]		
18	25	2500	0	81 ± 32	0.8 ± 1.0
18	25	0	0	33 ± 11	0.6 ± 0.1
12	25	2500	0	90 ± 61	0.7 ± 0.3
11	25	0	0	44 ± 26	0.6 ± 0.1

[1] Rats fed diets 15 days.
[2] Rats fed diets 30 days.
[3] Significantly more than those receiving no added Cu ($P < 0.01$).
[4] Rats fed diets 15 days.

The average concentration of copper and percentage of dose of copper[64] observed in the livers of these animals is given in table 2.

The copper concentration in the livers of rats receiving the 10% protein diet with 1,000 ppm of added copper was more than 10 times that in the livers of the animals receiving the same level of copper in a 17.5% protein diet. The concentration was also more than 10 times that found in the livers of the animals in experiment 1 that received 1,000 ppm of added copper.

No standard deviation is listed in table 2, experiment 2, under "total copper" because the liver ashes from the animals in each treatment were inadvertently pooled for total copper analyses, and individual analyses for copper concentration were not available.

The results of experiment 1 indicated that the addition of 1,000 ppm of zinc had no effect on the accumulation of copper in the livers of the rats receiving a 25% protein diet with 1,000 ppm of added copper. The results of experiment 2 also show that

there was little effect when 17.5% of protein was fed; however, in the 10% protein level there was a definite indication that the addition of zinc to the diets containing the higher levels of copper decreases the concentration of copper in the livers.

Concentrations of 2.0% or more of the copper^{64} dose observed in the livers of some of the animals receiving the 10% protein diets with high copper should be interpreted as indicating an exchange rather than an accumulation of copper in the liver.

The apparent effect of increased dietary protein in limiting the liver storage of copper in the animals receiving the high-copper diet suggested the need for a study of the effects of protein on the accumulation of copper in the livers of the rats. This was the basis for the design of experiment 3. In a preliminary trial, 7 male and 8 female rats were fed a 25% protein diet containing 0.25% of copper for two months with no visible signs of toxicity in any of the animals; hence, in all subsequent trials the level of copper used was 0.25%. A summary of the data obtained in experiment 3 is given in table 2.

The results of experiment 3 show a highly significant effect of protein on the absorption of dietary copper. The livers of the animals receiving the 10% protein diet with 0.25% of added copper had approximately 35 times more copper than that found in the livers of the animals which received the same diet with no added copper. The concentration of copper in the livers of rats which received the 17.5% protein diet was about 15 times more for the animals fed 0.25% of copper than for those receiving no added copper. The liver content of the animals which received 25% of protein with 0.25% of added copper was only about twice that observed in rats consuming similar diets with no added copper.

The pattern of the differences in percentage of dose of labeled copper observed in the livers of these rats was about the same as that for the total copper but the magnitude was not nearly as great with respect to the radioactive copper. A large variation in the amount of radioactive copper was noted in the livers of animals fed the same diets, and considerable overlapping in the percentage of dose in the livers between animals receiving different diets. Except for a few animals there was good correlation between the percentage of dose and the total liver copper; however, as mentioned previously the high accumulation of copper^{64} probably represents an exchange rather than a deposition. These results and those from the two previous experiments indicate that a conclusion as to total liver copper accumulation based entirely on results with the labeled copper may be questionable.

The highly significant differences in the accumulation of liver copper observed with different levels of protein in the last experiment and the results obtained in experiment 1, showing no significant increase in accumulation of liver copper in rats fed 1000 ppm of copper and 25% protein, indicated a need for additional data regarding the accumulation of copper in the livers of rats fed high levels of dietary copper in a 25% protein diet. This was the reason for experiment 4 and the results are shown in table 2.

These results confirmed those found in experiments 1 and 3, at least with respect to the effect of the 25% protein level on the absorption of copper in a diet which contained 0.25% copper, although the total liver copper level for both lots is a little above that found in experiment 3. There were no significant differences in the concentration of liver copper between the animals fed the different levels of copper, and none of the animals were observed to have a copper concentration in the liver high enough to be considered toxic. Also, the higher total copper level was reflected in a higher percentage of dose of the labeled copper found in these livers.

DISCUSSION

Many investigators have pointed out that copper absorption, metabolism and excretion are markedly influenced by the presence of certain dietary factors. There is little understanding of how these factors affect copper absorption. Mills ('54), while conducting an investigation of the dietary components in forages that appeared to have an influence on the absorption of copper, noted that most of the copper in plant material is in the form of copper organic

complexes; although no specific evidence was presented, it it highly probable that these organic complexes are chelates. Results of further experiments by Mills ('55, '56) indicated that some organic compounds in forages enhance the absorption of dietary copper by rats; however, there was no mention of any compound in plants which would decrease the absorption of dietary copper by rats. Mills used copper[64] in his experiments and found 1.4 to 9.4% of the administered dose in the livers. These values are much higher than the 1.0 to 2.5% reported earlier by Comar et al. ('48). The rats used in the study by Mills, however, were in a state of extreme copper deficiency, whereas those used by other investigators were removed from full feed only for the 24-hour period just prior to dosage. The rats used in experiments reported here received full feed up to and after the time of dosage.

The results of the present study indicate that the dietary level of protein has a significant effect on the accumulation of copper in the livers of rats. An adequate or more than adequate level of dietary protein inhibited the accumulation of a toxic level of copper in the liver when large amounts of copper were ingested. The mechanism of this influence of protein is more than the inhibition of copper absorption through the formation of an unavailable copper protein compound as indicated by the failure of a high-protein diet to cause a reduction in the liver copper content on the low levels of copper intake. No indication of a copper deficiency was noted when normal levels of copper were fed with high levels of protein.

One possible mechanism by which protein or a degradation product of protein may prevent the accumulation of a toxic level of copper in the liver is through the formation of a copper-protein chelate which would be physiologically unavailable. Both copper and zinc form chelates with amino acids and peptides (Chaberek and Martell, '59). The formation of an unavailable copper or zinc chelate could explain the reason for a copper-zinc interrelationship similar to that reported by Van Reen ('53) which was not evident with rats receiving the high protein diet.

The results of the first experiment reported here indicate that zinc did not accumulate in the livers of rats fed 1,000 ppm of zinc, and results similar to these have been reported for rats and swine by Kulwich.[5] Available data suggest that with adequate protein in the diet, only a slight increase in zinc absorption occurs with increased levels of zinc, and under these conditions zinc would have less effect on preventing an accumulation of copper in the liver.

The lack of evidence of a copper-zinc interaction in the work being reported here when high levels of protein were included in the diet, would suggest that the effects observed by others when high levels of copper or zinc were added to the diets should be interpreted on the basis of the protein content of the diet.

It is probable that the physical and chemical characteristics of the different chelates formed by copper with protein or other dietary constitutents may affect their absorption from the intestinal tract. A copper-protein chelate formed with a low copper-to-protein ratio in the food may be one which is not rapidly absorbed. With a much higher ratio it is possible that other chelates or other types of chelates may be formed which are more rapidly absorbed. In the present study the rats fed diets with low copper-to-protein ratios had concentrations of copper in the livers that were within the normal range. Rats receiving higher copper-to-protein ratios had concentrations of copper in the livers of 10 times or more than normal.

The influence of protein on the accumulation of excessive copper in the livers of rats fed high-copper intakes may be the result of increased elimination of copper from the liver. This suggestion is supported by the relatively large amounts of radioactive copper compared with the relatively low amount of total copper found in the livers of the animals receiving diets containing the high levels of copper with high protein. Copper complexes formed and absorbed from diets with low copper-to-protein ratios may be more readily eliminated from the liver than those from diets with higher ratios.

[5] See footnote 2.

SUMMARY

Results of experiments reported in this study have shown that the accumulation of copper in the livers of rats fed high levels of copper may be influenced by such dietary factors as zinc and protein. This effect did not result from a simple interrelationship between copper and the other dietary factors but was a more complex interaction which was dependent upon the relative concentration of all the factors present.

The effect of the protein level in the diet was greater than the effect exerted by the levels of zinc, at least with respect to a toxic level of copper. At a dietary level of 25% of protein with the maximum level of zinc used in these experiments, any mutual or reciprocal influence between copper and zinc was apparently eliminated. The action of protein appeared to be both in the intestinal tract where a regulation of copper absorption occurred and in the liver where there was an increased elimination of copper as the concentration approached a toxic level.

Observations demonstrated that an accumulation of orally administered $copper^{64}$ in the liver might have indicated increased absorption but not necessarily increased availability of the element to an animal, and also, that a decreased liver storage of the element did not necessarily indicate a decrease in liver metabolism of the element.

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Effect of Source and Level of Dietary Protein on the Toxicity of Zinc to the Rat[1,2]

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The interactions of zinc with other dietary constituents are especially important in a study of zinc toxicity. It appears that no particular symptom may be specifically attributed to the zinc alone, but that the toxicity syndrome is observed as deficiency symptoms of several other nutrients.

Early studies with zinc by Sutton and Nelson ('38) and Smith and Larson ('46) indicated that the hypochromic microcytic anemia caused by feeding rats a relatively high concentration of zinc was actually the result of impaired utilization of iron for hemoglobin formation and this condition was corrected by the addition of copper salts to the diet. Later studies by Van Reen ('53) and Duncan et al. ('53) have extended this zinc-copper-iron interrelationship to include the iron-containing enzymes, catalase and cytochrome oxidase. These enzymes decrease in activity with high levels of dietary zinc and return to normal or even higher-than-normal values when copper salts are added above the required level.

Excessive dietary zinc causes a decrease in the concentration of copper in the liver of the rat and a recent study reported by Cox and Harris ('60) indicates that there is also a decrease in the concentration of iron in the livers of rats fed zinc at a high level. From these results it was suggested that it was this decrease in iron, and not copper, which is responsible for the anemia and reduction in activity of the iron containing enzymes. Grant-Frost and Underwood ('58) have stated that a high zinc intake may produce a deficiency of dietary components other than copper and iron necessary for normal hematopoiesis. Further support is added to this suggestion by the ability of animals to consume much larger concentrations of zinc than of many other metallic elements.

Still another dietary interaction involving zinc and a source of protein was reported by O'Dell and Savage ('60) and Kratzer and others ('59). They observed that chickens and turkeys fed isolated soybean protein as a source of protein showed a higher zinc requirement than those fed protein from casein or other sources. In the former work evidence was presented to show that the phytic acid in the soybean oil meal was responsible for this effect. In the work by Kratzer and others the zinc requirement was reduced by autoclaving the soybean protein or by the addition of EDTA (ethylenediaminetetraacetic acid) to the ration. This effect was explained on the basis that the zinc bound to the soybean protein and unavailable to the poults became available when the protein was denatured or when the zinc-EDTA chelate was formed.

The study reported here was undertaken to measure the effect of dietary source of protein, casein or soybean oil meal, and dietary level of protein, 20 or 30%, on rats fed zinc at a high level. Factors considered were final weights, hemoglobin values, liver accumulation of total copper, iron and zinc and liver deposition of copper^{64} and zinc^{65} from a single oral dose containing these radioactive elements.

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TABLE 1

Weight gains and hemoglobin values for rats fed normal and high levels of zinc with casein and soybean oil meal diets

Added Zn, ppm	20% protein, Casein 0	2500	5000	7000	5000[1]	5000[2]	20% protein, Soybean oil meal 0	2500	5000	30% protein, Casein 0	2500	5000	30% protein, Soybean oil meal 0	2500	5000
Av. weight, gm															
Males	289	282	180	148[3]	272	216	310	298	263[5]	320	313	213	281	290	283[5]
Females	198	200	161	114[4]	179	139	202	194	198[5]	250	204	185	203	186	198[5]
Av. Hb., gm/100 ml															
Males	13.4	11.5	7.3	5.7[3]	11.9	13.9	14.2	13.6	12.4[6]	14.5	13.6	6.0	14.2	14.1	13.3[6]
Females	13.6	12.3	7.7	5.7[3]	13.5	13.5	13.9	13.9	12.8[6]	14.4	13.7	10.0	14.0	13.9	14.1[6]

[1] 500 ppm Cu added as $Cu_3(PO_4)_2$.
[2] 500 ppm Cu + 1000 ppm Fe added as $Cu_3(PO_4)_2$ and $Fe_2(SO_4)_3$.
[3] Two died before experiment terminated.
[4] One died before experiment terminated.
[5] Significantly heavier than corresponding groups receiving casein, ($P < 0.01$).
[6] Significantly higher than corresponding groups receiving casein, ($P < 0.01$).

EXPERIMENTAL

The experimental design is shown in table 1. The basal diets were composed of casein or soybean oil meal (50% protein); sucrose; 4% of mineral mix (U.S.P. XIV, '55); 2.2% of vitamin mix;[4] and 4% of cottonseed oil.[5] The casein or soybean oil meal and the sucrose in each ration were varied to give the 20 or 30% protein level required. Three per cent of purified cellulose was added to the casein rations. Zinc was added as zinc carbonate, copper as cupric phosphate and iron as ferrous sulphate.

The radioactive copper was received from Oak Ridge as cupric nitrate. This was converted and administered to the animals as cupric chloride. The radioactive zinc received from Oak Ridge[6] as zinc chloride was used in this form.

One-hundred-eighty piebald rats of the Long Evans strain, raised in our laboratory, weighing about 50 gm each, were divided into 15 groups each containing 6 males and 6 females. These animals were fed the rations ad libitum during the time of the trial. After all the animals had received the diets for 8 weeks, two males and two females from each group were given an oral dose of about 1 ml containing 250 μc of copper^{64} and 2 μc of zinc^{65}. Twenty-four hours later the animals were weighed, killed, hemoglobin values measured, and the livers were taken for analysis of the radioactive and total copper and zinc and total iron. The remaining 120 rats (60 males and 60 females) were killed 5 days after the first group, body weights and hemoglobin measured and the livers taken for analysis of total zinc, iron and copper.

For the copper^{64} and zinc^{65} assay the livers were dried under infrared lamps, pre-ashed in beakers over Bunsen burners

[4] Vitamin mix included: (in mg/100 gm of ration) α-tocopherol, 11; ascorbic acid, 100; inositol, 11; choline chloride, 165; menadione, 5; p-aminobenzoic acid, 11; niacin, 10; riboflavin, 2.2; pyridoxine·HCl, 2.2; thiamine·HCl, 2.2; Ca pantothenate, 6.6; biotin, 0.044; folic acid, 0.2; vitamin B_{12}, 0.003; also (in I.U.) vitamin A, 1980 and vitamin D, 220.

[5] Wesson Oil, The Wesson Oil Company, New Orleans, Louisiana.

[6] Oak Ridge National Laboratory, Oak Ridge, Tennessee.

and then ashed for 4 hours in a muffle furnace at 500°C. After ashing, the livers were broken up in the beakers with glass stirring rods, transferred to 5-ml glass beakers and the radioactivity measured with a thin window Geiger-Mueller tube. The activity of both the copper^{64} and the zinc^{65} was measured within 12 hours after sacrifice, and the activity of the zinc^{65} alone was measured again a week later when all the copper^{64} had decayed to a point that there were no detectable counts in the standard. The activity of the copper^{64} was determined by difference. Total copper and zinc was determined by the method of McCall et al. ('58) and total iron by the phenanthroline method as described by Sandell ('50). Hemoglobin was determined by the acid hematin method. The analytical data were tested by analysis of variance as described by Snedecor ('59).

RESULTS AND DISCUSSION

The first visible symptom of zinc toxicity in the rat was a graying or fading of the dark areas of the haircoat. This was not a general overall fading but occurred in a definite, individual pattern for each animal and was more pronounced over the head and across the nose. At one stage there was a similarity in appearance between the faces of the affected rats and the familiar masks of the raccoon. As the severity of the toxicity continued the dark haircoats became silvery-brown in appearance and finally changed to the dull, yellowish-brown color so common in anemia. In advanced stages of zinc toxicity, alopecia was observed.

During the third week of treatment the first signs of a haircoat change occurred in the animals receiving 20% of casein with 5000 and 7000 ppm of added zinc. A similar fading of haircoat began to appear in those animals receiving 30% of casein with 5000 ppm of added zinc and those receiving the 20% casein diets with 2500 ppm of added zinc during the 4th and 5th weeks of the experiment. The first signs of fading occurred in the animals receiving 30% of casein with 2500 ppm of added zinc during the 6th week of the experiment. The last indication of a change in haircoat occurred during the 8th week of the experiment with those animals receiving 20% of soybean oil meal with 5000 ppm of added zinc. No change in haircoat was observed in animals receiving the 20% casein diet with 5000 ppm of added zinc plus the copper, or the added zinc plus copper and iron; and there was no sign in those animals receiving the 20% soybean diet with the zero or 2500 ppm of added zinc, or any of the animals receiving the 30% soybean diet.

The addition of 5000 ppm of zinc with or without the added copper and iron and the addition of 7000 ppm of zinc to the 20% casein diets restricted the rate of gain of the animals from the beginning of the experiment, although the animals that received added copper and added copper plus iron appeared normal in all other respects. Those animals receiving 30% of casein with 5000 ppm of added zinc grew normally for the first month and then lost weight until the end of the experiment. The average weights at time of sacrifice are shown in table 1.

The average hemoglobin values for the animals used in this study (table 1) followed the same general pattern as the weights for each treatment except for those groups receiving the added copper and iron. No definite numerical values were assigned to the appearance of the animals but in each instance appearance paralleled the hemoglobin values. Measurement of hemoglobin was made only at the time of sacrifice; however, the animals receiving 30% casein diets with 2500 ppm of added zinc and 20% of soybean with 5000 ppm of added zinc showed definite signs of achromotrichia with a normal hemoglobin range. This would indicate that the drop in hemoglobin value occurs sometime after the visible signs of zinc toxicity.

The average values for the percentage dose of copper^{64} and zinc^{65} observed in the livers of those rats receiving the radioactivity are shown in table 2. Little decrease occurred in the absorption of either copper^{64} or zinc^{65} under the conditions of this experiment. Interpretation of these data was difficult owing to the variability of the individual results and the small number of animals used for the radioactive study. It was not possible to use

TABLE 2

Liver deposition of an oral dose of Cu^{64} and Zn^{65} in rats fed normal and high levels of zinc with casein and soybean oil meal diets

	Protein source and level														
	20% Protein									30% Protein					
	Casein						Soybean oil meal			Casein			Soybean oil meal		
Added Zn, ppm	0	2500	5000	7000	5000[1]	5000[2]	0	2500	5000	0	2500	5000	0	2500	5000
% Dose CuC_4															
Males	2.6	1.1	3.1	7.0	0.4	0.1	1.3	0.2	0.4	1.4	5.9	0.1	3.9	1.2	1.2
Females	2.1	0.9	0.2	1.1	1.4	4.3	3.8	1.1	0.7	2.6	2.6	1.1	4.3	0.8	5.1
% Dose Zn^{65}															
Males	1.8	0.5	0.4	0.6	0.5	1.7	3.4	0.4	3.2	1.4	2.5	1.6	3.2	0.2	0.3
Females	3.4	1.1	0.4	2.9	4.4	5.1	1.6	0.4	2.2	1.4	0.7	2.3	2.1	0.7	0.4

[1] 500 ppm Cu, as $Cu_3(PO_4)_2$, added.
[2] 500 ppm Cu, as $Cu_3(PO_4)_2$, + 1000 ppm Fe, as $Fe_2(SO_4)_3$, added.

TABLE 3

Accumulation of total copper, zinc and iron in livers of rats fed normal or high levels of zinc with casein and soybean oil meal diets

	Protein source and level														
	20% Protein									30% Protein					
	Casein						Soybean oil meal			Casein			Soybean oil meal		
Added Zn, ppm	0	2500	5000	7000	5000[1]	5000[2]	0	2500	5000	0	2500	5000	0	2500	5000
Av. Cu[5]															
Males	33.0	14.0	17.0	12.5	16.0	24.0	27.0	24.5	16.0	22.5	22.5	15.5	28.5	22.5	17.5
Females	39.0	34.0	22.0	14.5	35.5	54.0	44.5	41.0	29.5	47.5	27.5	21.5	44.5	37.0	35.0
Av. zinc[5]															
Males	29.0	49.5	114.0	204	63.0	82.0	26.5	33.0[3]	75.0[3]	23.5	70.0	62.0	31.0	33.5[3]	57.0[3]
Females	22.0	89.0	164.0	255	165.0	196.0	29.5	31.5[3]	133.0[3]	27.0	87.5	129.0	37.0	34.0[3]	89.0[3]
Av. iron[5]															
Males	166	78[4]	82[4]	68[4]	47[4]	137[4]	112	106[4]	109[4]	126	75.0[4]	140[4]	115	105[4]	69[4]
Females	288	200[4]	134[4]	83[4]	87[4]	182[4]	237	161[4]	157[4]	245	121[4]	117[4]	219	190[4]	109[4]

[1] 500 ppm Cu, as $Cu_3(PO_4)_2$, added.
[2] 500 ppm Cu, as $Cu_3(PO_4)_2$, added, plus 1000 ppm Fe, as $Fe_2(SO_4)_3$, added.
[3] Significantly less than corresponding groups receiving casein, ($P < 0.05$).
[4] Fe in treated groups. Significantly less than control groups, ($P < 0.05$).
[5] Micrograms/gram of fresh weight of tissue.

large numbers because of the small amount of Cu^{64} available as a result of its short half-life, 13 hours.

Values showing the concentration of total copper, zinc and iron in the livers of the rats are given in table 3. Although a slight decrease was observed in the concentration of copper in the livers of the rats receiving the high-zinc diets this difference was not significant and probably did not produce the anemia which occurred in the animals. There is a decrease in liver copper concentration in all of the animals receiving high-zinc diets, even in those that received the soybean oil meal diets and showed no signs of zinc toxicity.

The concentration of zinc in the livers of both the males and the females increased as the dietary level of zinc increased, although significantly less zinc accumulated in the livers of those animals receiving the soybean oil meal rations. Both males and females receiving the 30% casein diet with 5000 ppm of added zinc had less accumulation of zinc in the liver than those receiving the 20% of casein with the 5000 ppm of zinc with added copper and iron. These results are unusual in that those animals receiving the 30% casein diets showed severe symptoms of zinc toxicity with low hemogloblin values, whereas those fed 20% of casein with the added copper and iron had normal hemoglobin levels and exhibited no particular signs of zinc toxicity.

A decrease was observed in the iron concentration in the livers of both the males and females for all groups corresponding to the increase in zinc ingested, including a slight decrease in that group receiving the added iron in the diet. The decrease in iron concentration in the animals receiving the soybean oil meal diets without any sign of zinc toxicity was equal to the decrease of iron in the animals receiving the casein diets, which had low hemoglobin values and other signs of severe zinc toxicity.

The results of zinc toxicity in the rat, including the hypochromic microcytic anemia, poor growth, reduction in liver catalase, and cytochrome oxidase, as well as the fading in haircoat color, have been explained previously on the basis that the increased level of zinc blocks the absorption or the utilization of copper, or of iron. In the reports, all of the symptoms of zinc toxicity were eliminated by the addition of copper to the diets, and in later work, Cox and Harris ('60) showed that the addition of iron to the diet had the same effect. In the present study the addition of either copper or copper and iron to the diets of the animals receiving the toxic level of zinc, increased the hemoglobin to the normal range and eliminated all other signs of zinc toxicity except the poor growth. It does not seem possible, however, that the small differences in the total copper, zinc, or iron content of the livers of these animals are enough to account for these changes.

The data obtained from this study suggest that the conditions arising from ingestion of a toxic level of zinc result from interactions with zinc and protein and which also involved other mineral elements. Zinc, copper and iron form stable chelates with amino acids and protein fragments (Chaberek and Martell, '59) and it is possible that the excess zinc could tie up enough of a required protein compound to impair the utilization of copper and iron. This would agree with the findings of Pal and Christensen ('59) that there is a strong possibility that metal chelates act as carrier or parts of a carrier for amino acid transport.

The limiting action of zinc under these conditions would have to be due to the combination of zinc with a specific compound within the protein supplement (soybean oil meal) and not just any chelate. The work of Kratzer and others ('59) showed that soybean protein increased the requirement of the chick for zinc and the addition of EDTA to this ration apparently made the zinc more available to the chick. Zinc and EDTA, in solutions at a physiological pH, form a very stable chelate. Perry and Perry ('59) have shown that the injection of EDTA changes the normal pathway of excretion of zinc increasing the urinary excretion of zinc by 10 times.

If the protein source makes this much difference on the availability of zinc, copper and iron, then it might be expected that the difference was effected by the

amino acid concentrations in the different proteins. A comparison of the amino acid content of soybean meal (Kuiken et al., '49) and that of casein (Almquist, '54) shows that, with the exception of arginine, these two protein sources have approximately the same amount of the essential amino acids. Gurd and Goodman ('52) have stated that zinc is tightly bound to the imidazole group of histidine which suggests the possibility that a histidine-zinc chelate may account for the differences. In soybean meal and casein, however, the histidine content is essentially the same. Van Etten and co-workers ('59) separated soybean protein into the soluble heat-coagulated and acid-precipitated fractions and found that the amino acid composition of these different fractions was essentially the same.

These considerations suggest the possibility that a structural feature in the protein is responsible for this difference in metabolic handling of metallic elements. The stability of a mineral-amino acid chelate depends to a large extent upon the relative position of the amino groups. From the data presented here it is proposed that specific protein compounds in soybean oil meal form stable chelates with zinc which decrease the accumulation of zinc in the livers when otherwise toxic levels are ingested and eliminate the adverse interaction with copper and iron. Utilization of copper and iron and possibly protein transportation within the body appear to be affected by this combination.

SUMMARY

Evidence was presented to show that the amount of zinc required to produce toxic symptoms in the rat and the severity of the symptoms depend upon the source and level of the dietary protein.

Rats fed excessive zinc in 20 or 30% soybean protein diets were significantly heavier, and showed higher hemoglobin levels and less total zinc accumulation in the livers than those animals receiving the same level of protein from casein.

The source of protein had no effect on the percentage of dose of radioactive copper or zinc or the total copper accumulation in the livers.

The addition of 500 ppm of copper and 500 ppm of copper plus 1000 ppm of iron to the diets of rats receiving 5000 ppm of zinc in a 20% casein protein diet increased the hemoglobin level and decreased the total accumulation of zinc in the liver but did not increase growth rate.

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Effects of Pyridoxine Deficiency upon Circulating Antibody Formation and Skin Hypersensitivity Reactions to Diphtheria Toxoid in Guinea Pigs[1]

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The deleterious effects of a pyridoxine deficiency upon circulating antibody formation in response to the stimuli of various antigens have been demonstrated repeatedly in the rat (Stoerk and Eisen, '46; Axelrod and Pruzansky, '55; Axelrod, '60). A requirement for this vitamin in the various phases of the anamnestic process has also been established in this species (Stoerk, '50; Axelrod, '58). The pyridoxine antagonist, deoxypyridoxine, has been used to advantage in these latter studies. Clearly, it is important to know whether these effects observed in the rat have general application to other species. Accordingly, in the present investigation, the effect of a pyridoxine deficiency upon circulating antibody formation has been studied in the guinea pig.

The possible effects of nutritional deficiency states upon hypersensitivity reactions represent a related area of general interest. Our observations in the pyridoxine-deficient guinea pig will be reported in this paper. The choice of the guinea pig as the experimental subject was dictated by the suitability of this species for hypersensitivity studies. In the course of these investigations, it became possible to assess the relative effectiveness of deoxypyridoxine as a pyridoxine antagonist in the rat and the guinea pig.

MATERIALS AND METHODS

Animals. Male, albino guinea pigs, 5 to 7 days old, of the Hampstead (Hartley) strain were kept in individual cages with wide-mesh screen bottoms and weighed weekly throughout the experiment.

Diets and supplements. A highly purified diet (no. 13) described by Reid and Briggs ('53) was used with the following modifications: powdered cellulose[3] was substituted for cellophane spangles[4] and the level of corn oil was reduced to 5% at the expense of sucrose. The control diet contained either 16 or 4 mg of pyridoxine·HCl per kg of diet, as indicated in the text of this paper. The pyridoxine-deficient diet was prepared by omitting pyridoxine from the control diet. In some instances a laboratory chow diet[5] was fed. All diets were fed ad libitum.

For injection, the requisite amounts of pyridoxine hydrochloride and deoxypyridoxine hydrochloride were dissolved in 1 ml of isotonic sodium chloride solution at pH 7.2 and administered intraperitoneally.

Serum antibody titrations. Anesthetized[6] guinea pigs were bled by cardiac puncture and serum antibody titres determined by the Stavitsky modification ('54) of the Boyden ('51) tanned erythrocyte technique. In addition, the classic Römer and Sames ('09) method was used in experiment 2 to measure diphtheria antitoxin levels of serum.

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[3] Solka Floc, obtained from Brown Company, New York.

[4] Obtained from Rayon Processing Company, Pawtucket, Rhode Island.

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

[6] By Nembutal, Abbott.

Skin tests. Skin reactivity following intradermal injections of 0.2 ml of a standard international formol diphtheria toxoid containing 40 Lf units determined the degree of hypersensitivity in immunized animals. Reactions were measured at the time intervals shown in each experiment. The technique for measuring the skin reactions provides repeated accurate readings on the same site even when the size of the lesions, or indeed their existence, cannot be detected visually. The loose skin of the flank is folded across the diameter of the inflamed area and the maximum thickness of the fold measured with calipers fitted with a magnifying dial. The utmost gentleness is used so as to interfere as little as possible with the natural development of the lesion. The results obtained are expressed as percentage change in thickness of this double layer of skin from its initial thickness before injection. This method has proved useful for analyzing immune and allergic reactions to diphtheria toxin and toxoid in guinea pigs (Long, '59).

EXPERIMENTAL AND RESULTS

Experiment 1. The animals were grouped as indicated in table 1. A pyridoxine-deficiency was induced by administering deoxypyridoxine to animals receiving a pyridoxine-deficient diet. An additional group, which received only the pyridoxine-deficient diet from the beginning of the experiment, is not included because of its high mortality. Thus, in a starting group of 10 animals, none survived the 8-week experimental period.

After 4 weeks on experiment, each guinea pig was immunized by the intraperitoneal injection of 0.15 ml of an alum-precipitated diphtheria toxoid preparation containing 50 Lf units per ml.[7] The animals were bled three weeks later and the primary response of circulating antibody determined. An identical dosage of the same diphtheria toxoid preparation was then administered as described and the animals bled a week later for determination of the secondary (booster) circulating antibody response. Skin tests were performed at this time.

The inhibitory effect of a pyridoxine-deficiency upon growth of guinea pigs is indicated in table 1. Other than an unkempt appearance of the fur. No obvious symptomatology was observed. Continuation of deoxypyridoxine administration for an additional 6-week period maintained the weight plateau but had no further deleterious effect upon appearance of the animals. In some instances, supplementation with excessive amounts of pyridoxine completely reversed growth inhibition.

The depressant effect of a pyridoxine deficiency upon circulating antibody formation to the antigenic stimulus of alum-precipitated diphtheria toxoid is shown in table 2. Impairment of the secondary response was most apparent. The immune response of guinea pigs fed the commercial chow diet did not differ from that of guinea pigs fed the control highly purified diet.

Skin hypersensitivity reactions of the early, Arthus-type, were considerably re-

[7] Kindly supplied by the Lederle Laboratories Division, American Cyanamide Company, Pearl River, New York.

TABLE 1

Inhibitory effect of pyridoxine deficiency upon growth (exp. 1)

Group	No. of animals	Average weight		
		Initial	At 4 weeks	At 8 weeks
		gm	*gm*	*gm*
Control[1]	5	120	366	563
Control[2]	8	125	287	453
Deoxypyridoxine-treated[3]	9	126	288	316

[1] Fed Purina Guinea Pig Chow for 8 weeks.
[2] Fed highly purified diet containing 16 mg of pyridoxine·HCl/kg for 8 weeks.
[3] Fed highly purified diet containing 16 mg of pyridoxine·HCl/kg of diet for 4 weeks and the pyridoxine-deficient diet for the remaining 4 weeks. Each animal received a daily intraperitoneal injection of 10 mg of deoxypyridoxine·HCl/100 gm of body weight during these last 4 weeks.

TABLE 2

Effect of pyridoxine-deficiency upon circulating antibody response to alum-precipitated diphtheria toxoid[1] *(exp. 1)*

Group	Primary response	Secondary response
Control (commercial chow)	1:400	1:40,000
	0	0
	1:200	1:80,000
	0	1:40,000
	1:20	1:20,000
Control (highly purified diet)	0	1:80,000
	1:400	1:40,000
	1:20	1:80,000
	0	1:2,000
	0	1:80,000
	1:400	1:320,000
	0	1:320,000
	0	1:200
Deoxypyridoxine-treated	0	0
	0	1:200
	1:20	1:2,000
	0	1:200
	0	1:2,000
	0	1:200
	0	1:2,000
	0	1:2,000
	1:400	1:20,000

[1] Agglutinating antibody titers of serum were determined by the tanned erythrocyte technique and values for individual animals presented in this table. In this method, determinations are made of final serum dilutions that are capable of agglutinating tanned erythrocytes upon which the antigen (diptheria toxoid) had been adsorbed. Thus, the magnitude of the titer (serum dilution) is a direct measure of the content of serum agglutinating antibodies.

duced in the pyridoxine-deficient animals (fig. 1).

Experiment 2. This experiment was conducted similarly to that of experiment 1 with the following differences: (1) the control highly purified diet contained 4 instead of 16 mg of pyridoxine·HCl per kg of diet. It was felt that the lower level of pyridoxine might aggravate the deficiency state produced by the subsequent administration of deoxypyridoxine; (2) animals fed the pyridoxine-deficient diet from the beginning of the experiment received three daily intraperitoneal injections of 1 mg of pyridoxine immediately upon their arrival at the laboratory in the hope that this treatment would lessen the high mortality noted in the similar group of experiment 1; and (3) dosage of the antigen was decreased and the antigen given by intramuscular instead of by intraperitoneal injection. This change was instituted in the belief that it would magnify

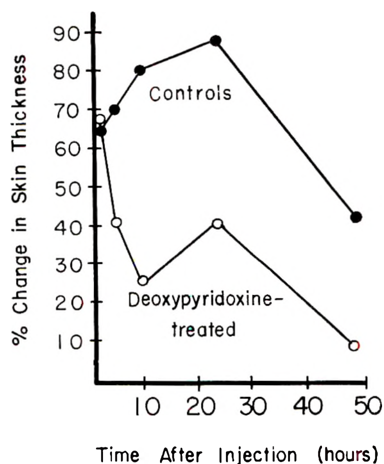

Fig. 1 Hypersensitivity in guinea pigs immunized with diphtheria toxoid as measured by skin reactivity to intradermally injected diphtheria toxoid (experiment 1). Intensity of the skin reaction was determined by the caliper method. Data of control animals fed commercial chow or the highly purified diet are combined since responses in these two groups were similar.

the differences in antibody response between control and deficient animals.

The control group (21 animals) received the highly purified diet containing 4 mg of pyridoxine·HCl per kg of diet throughout the experiment. The deoxypyridoxine-treated group (19 animals) received this control diet for 5 weeks and the pyridoxine-deficient diet for the remainder of the experiment. While the guinea pigs were receiving the pyridoxine-deficient diet, each animal was given a daily intraperitoneal injection of 10 mg of deoxypyridoxine·HCl per 100 gm of body weight. A dietary deficiency of pyridoxine was induced by feeding a third group of 15 animals the pyridoxine-deficient diet throughout the experiment. After 5 weeks, each animal was immunized by the injection of 1.0 ml of an alum-precipitated diphtheria toxoid preparation[8] containing 2.5 Lf units per ml into the adductor muscle of the hind legs. Three weeks later, the animals were bled for determination of the primary antibody response and an identical dosage of the same antigen administered intramuscularly. A final bleeding was performed 10 days later for evaluation of the secondary response. Skin tests were then performed.

Growth curves of the three groups in this experiment are given in figure 2. Growth of animals in the control group

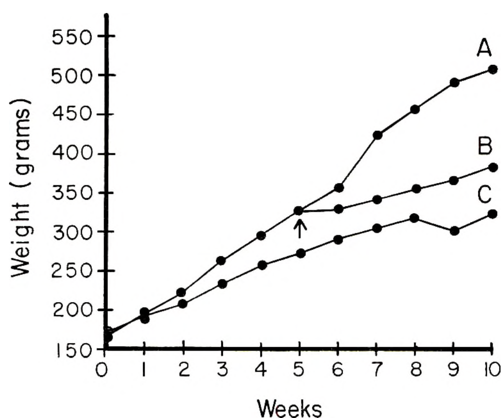

Fig. 2 Inhibitory effects of pyridoxine deficiency upon growth of guinea pigs in experiment 2. Group A, controls; group B, deoxypyridoxine-treated; group C, dietary-induced pyridoxine deficiency. Arrow indicates institution of deoxypyridoxine administration in group B as well as the beginning of immunization in all three groups.

and magnitude of the growth inhibitory effect of deoxypyridoxine were similar to those of the animals observed in experiment 1. As in the previous experiment, little effect of deoxypyridoxine treatment upon appearance of the animals was noted. Thus, decreasing the pyridoxine level of the diet from 16 to 4 mg per kg had no effect upon growth of the control animals or degree of the deficiency state produced by subsequent administration of deoxypyridoxine. Although growth of the guinea pigs fed the pyridoxine-deficient diet throughout the experiment was diminished, mortality in this group was not high. Thus, 11 of the 15 animals survived the 10-week period on this regimen. This result is in contrast with that observed in a comparable group of experiment 1 and demonstrated the efficacy of a brief pretreatment with pyridoxine in lessening mortality of guinea pigs fed the pyridoxine-deficient diet.

In agreement with observations made in experiment 1, agglutinating antibody formation as measured by the tanned erythrocyte technique was markedly depressed in guinea pigs treated with deoxypyridoxine (table 3). A similar depression in antibody response was seen in animals rendered pyridoxine-deficient by the omission of pyridoxine from the diet (table 3). Although no antibody formation could be detected with this sensitive method in a large proportion of the pyridoxine-deficient animals, every one of the controls possessed a demonstrable antibody titre. Diphtheria antitoxin production as measured by the Römer-Sames method ('09) was also reduced. Thus, 18 of the 21 control sera and only two of the 30 sera from pyridoxine-deficient animals contained one or more international units of diphtheria antitoxin per milliliter. Essentially, the Römer-Sames method measures the ability of an antiserum to neutralize diphtheria toxin. Toxin and antiserum are allowed to interact *in vitro* and the residual diphtheria toxin determined by the local toxicity of the toxin-antitoxin mixture upon intradermal injection into nonimmunized animals. The decreased antigenic stimulus used in experiment 2 produced a lower

[8] Wellcome Research Laboratories, London, England.

TABLE 3

Effect of pyridoxine deficiency upon circulating antibody repsonse to alum-precipitated diphtheria toxoid[1] (exp. 2)

Control	Deoxypyridoxine-treated	Dietary pyridoxine deficiency
1:2,000*	0	1:200
1:2,000*	0	1:2,000
1:2,000*	0	0
1:2,000*	0	0
1:40,000*	1:2,000	0*
1:2,000	0*	0
1:2,000*	0	1:200
1:80,000*	0	0
1:2,000*	0	1:200
1:2,000*	0	0
1:2,000*	0	0
1:2,000	0	
1:20,000*	1:200	
1:40,000*	0	
1:2,000	0	
1:2,000*	0	
1:2,000*	0	
1:2,000*	0	
1:2,000*	0	
1:20,000*		
1:2,000*		

[1] Agglutinating antibody titers of serum were determined by the tanned erythrocyte technique and values for individual animals presented in this table. Primary responses were negative in all groups and only secondary responses are given.

* Indicates sera containing one or more international units of diphtheria antitoxin per milliliter as determined by the Römer-Sames method. All other sera contained less than one international unit of diphtheria antitoxin per milliliter. All assays were performed on the same sera used for determination of circulating antibody by the tanned erythrocyte method.

content of agglutinating antibodies than that employed in experiment 1 (table 3). This difference was manifested in both control and deficient animals.

The degree of skin reactivity observed in experiment 2 (fig. 3) differed from that of experiment 1 (fig. 1). This change reflects the influence of variation in dosage and route of injection of the immunizing antigen. As in experiment 1, however, there was a diminution in the early, Arthus-type, skin reaction to diphtheria toxoid both in the deoxypyridoxine-treated animals and in those rendered deficient by dietary means alone (fig. 3).

DISCUSSION

In confirmation of results reported by Reid ('54), the present experiments have demonstrated that pyridoxine deficiency

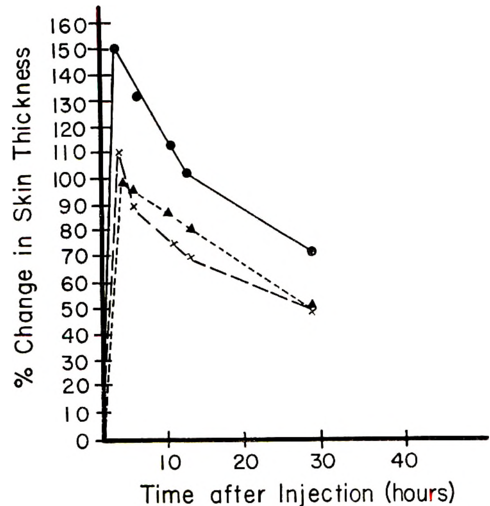

Fig. 3 Hypersensitivity in guinea pigs immunized with diphtheria toxoid as measured by skin reactivity to intradermally injected diphtheria toxoid (experiment 2). Intensity of the skin reaction was determined by the caliper method. ●—●, indicate control; ×- - -×, deoxypyridoxine-treated; ▲------▲, dietary-induced pyridoxine deficiency.

can be produced in young guinea pigs by omitting pyridoxine from the diet. In addition, a pyridoxine deficiency has been induced by transferring more mature guinea pigs from a control highly purified diet to one deficient in pyridoxine and administering deoxypyridoxine concomitantly. It is noteworthy that the guinea pig is more resistant to the pyridoxine-antagonistic action of deoxypyridoxine than the rat. Thus, the dosage of deoxypyridoxine used in the present experiments reduced growth but did not elicit any other obvious deficiency signs. Under comparable conditions, the same dosage of deoxypyridoxine in the rat induces a more profound deficiency state as defined by weight loss, mortality and skin pathology. With respect to antibody production, however, the rat and the guinea pig react similarly to a lack of pyridoxine; in both species, antibody production is markedly depressed. This inhibitory effect in the rat has been documented with a variety of antigenic stimuli. The present experiments have demonstrated the deleterious effect of a pyridoxine deficiency in the guinea pig upon the production of circulating antibodies to diphtheria toxoid. Serum antibodies were

determined by both the tanned erythrocyte method for agglutinating antibodies and the Römer-Sames method ('09) for diphtheria antitoxin. These effects of the deficiency cannot be attributed to the concomitant state of inanition since no inhibition of antibody formation has been observed in many experiments with inanition control guinea pigs.[9] Well-defined studies relating nutritional state to the process of antibody formation in the guinea pig are limited. Other than the present demonstration of the role of pyridoxine, only ascorbic acid has been implicated as a dietary factor required for antibody production in this species (Long, '50; Bersins, '55). In man, a pyridoxine deficiency induced by deoxypyridoxine did not affect antibody response to typhoid vaccine or the A and B blood group substances (Wayne et al., '58).

Since pyridoxine deficiency depressed the formation of circulating antibodies, it is not unexpected that it also depressed the early, Arthus-type, reaction which accompanies the development of immunity to diptheria toxoid (Ben-Efraim and Long, '57; Long, '59). Similar effects have been observed in mice by Hargis et al. ('60). These authors have demonstrated that circulating antibody formation and anaphylactic sensitivity to horse serum were markedly diminished in pyridoxine-deficient mice. Whether a delayed, tuberculin-type, reaction to diphtheria toxoid can be influenced by pyridoxine deficiency in the guinea pig is not known.

Studies on the relationship of specific nutritive factors to hypersensitivity phenomena are few. In this respect, the experiments of Schneider et al. ('57) are most pertinent. These authors reported that susceptibility of homozygous BSVS mice to acute disseminated encephalomyelitis (ADE) was nutritionally dependent, folic acid and vitamin B_{12} being the most effective nutritional factors studied. Supplementation of a highly purified diet with these vitamins restored in great measure the susceptibility of the mice to ADE. Biotin was less effective. These findings were considered within the framework of the possibility that ADE could be a manifestation of a hypersensitivity reaction and, in fact, were taken as evidence in support of such a mechanism. This reasoning was based on the recognition of the role of certain vitamins in the processes of antibody formation. A relationship between hypersensitivity reactions and nutritive state is further suggested by studies on the homograft rejection phenomenon which has been categorized as a delayed hypersensitivity reaction. In rats, Axelrod et al. ('58) and Fisher et al. ('58) have described the marked effect of a pyridoxine deficiency in prolonging the survival of skin homografts. Similar results have been reported in mice by Hargis et al. ('60). Parkes ('59) has demonstrated that treatment of recipient mice with deoxypyridoxine increased markedly the survival time of intrastrain as well as interstrain ovarian homografts. Of particular note were the striking effects obtained with heterografts of rat ovaries into deoxypyridoxine-treated mice. There is, therefore, a firm rationale for the suggestion that vitamin deficiency states, induced by dietary means or the application of vitamin antagonists, may be utilized to ameliorate the undesirable sequelae of hypersensitivity reactions.

SUMMARY

These studies were designed to investigate the effects of a pyridoxine deficiency in the guinea pig upon (1) circulating antibody response to the antigenic stimulus of diphtheria toxoid, and (2) skin hypersensitivity reactions to diphtheria toxoid injected intradermally into animals immunized previously with this antigen. Serum levels of circulating antibody were determined both by the tanned erythrocyte technique and by the Römer-Sames method for the estimation of diphtheria antitoxin.

Pyridoxine deficiency was produced in very young guinea pigs by feeding a highly purified diet lacking pyridoxine. The deficiency state was induced in more mature animals by administering the pyridoxine antagonist, deoxypyridoxine, to guinea pigs receiving the pyridoxine-deficient diet. Pyridoxine deficiency, produced by either of these procedures, depressed both circulating antibody formation and the degree of the early, Arthus-type skin hypersensitivity reaction to diphtheria toxoid.

[9] D. A. Long, unpublished observations.

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Effect of Food Restriction on Tissue Uptake and Urinary Excretion of Co^{60}-Labeled Cyanocobalamin by Various Animals[1,2]

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Recent studies have shown that rabbits subjected to total starvation accumulate greater amounts of native vitamin B_{12} in kidney tissue than normal well-fed animals, and during starvation urinary excretion of the vitamin also increases significantly (Rosenthal and Cravitz, '58). The accumulation of the vitamin in the kidney during starvation occurs with naturally occurring vitamin B_{12} or following an injection of cobalt-labeled cyanocobalamin (Rosenthal, '59). Since the accumulation of vitamin B_{12} following starvation is not altered from normal in liver, heart, skeletal muscle, spleen, and brain, a specific relationship between kidney tissue, starvation and vitamin B_{12} metabolism appears to exist. That the kidneys may play an important role in the metabolism of vitamin B_{12} other than an excretory function, has been suggested previously (Miller et al., '56).

It seemed important, therefore, to determine the effect of various degrees of inanition, and the length of time of starvation on the accumulation of vitamin B_{12} in the kidney; and also to determine whether the accumulation of vitamin B_{12} following starvation was increased in other laboratory animals such as the chicken, rat and guinea pig.

MATERIALS AND METHODS

Female New Zealand white rabbits weighing 2 to 3 kg, White Leghorn hens weighing 1.5 to 2.5 kg, female rats of the Wistar strain weighing 300 gm and female guinea pigs weighing 350 gm were placed in individual screen-bottom metabolism cages for determination of food consumption and the collection of urine. Food was withheld and after varying lengths of time of starvation, a 24-hour control urine collection was obtained. The animals were then injected with Co^{60}-labeled cyanocobalamin (specific activity approximately 1 mc per mg) intramuscularly in rabbits and chickens and intraperitoneally in rats and guinea pigs at a dose level of 0.05 μc per kg of body weight.[3] Starved animals were injected with the dosage based on their initial body weight. Control animals were treated the same, except that they were fed laboratory stock chow ad libitum. All guinea pig diets were supplemented with lettuce or carrots. Tap water was available ad libitum. Urine samples were collected in sulfuric acid for three days following the injection of radioactive vitamin. Animals were sacrificed by a blow on the head.

The livers and kidneys were weighed, dissolved in hot nitric acid and 5-ml aliquots of the digests and urine samples were assayed for radioactivity in a well-type scintillation counter, as previously described (Rosenthal, '59). The data shown in figure 1 indicate the days of starvation before injection of the vitamin plus the three days post injection.

RESULTS

In rabbits, the kidney accumulation of injected Co^{60}-cyanocobalamin was not altered significantly from control values of 0.35% of the injected dose per gm of

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tissue during the first few days of starvation (fig. 1). After 5 days of starvation, kidney radioactivity was markedly increased, and after 8 days of starvation reached a value of 2.8% of the dose per gm of tissue. The radioactivity in the liver increased slightly after two to 5 days of starvation, but tended to approach control levels when starvation was continued for 8 days. The changes in liver radioactivity are of doubtful significance. During the first few days of starvation, values for urine radioactivity were lower than control values but increased as starvation continued for 8 days.

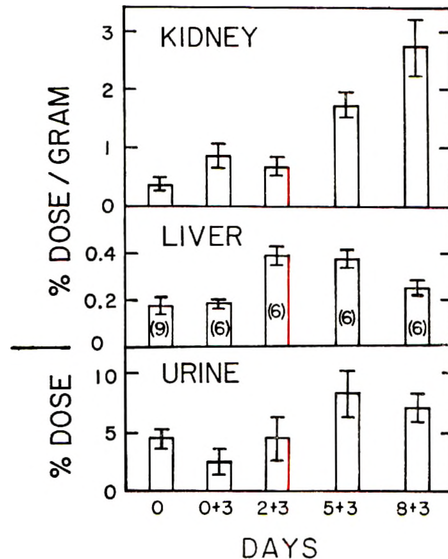

Fig. 1 The effect of duration of starvation on tissue uptake and urinary excretion of injected Co^{60}-labeled vitamin B_{12} in female rabbits. The vertical bars represent standard errors of the mean and the number of animals studied are shown in parentheses. The 0 indicates control animals fed for three days after injection and before sacrifice; 0 + 3 indicates animals fed until day of injection, then starved for three days; similarly, 2 + 3, 5 + 3 and 8 + 3 indicate two, 5 or 8 days of starvation prior to injection followed by three additional days of starvation after injection of the vitamin.

In rabbits subjected to partial food restriction, progressively increasing kidney accumulation and urine excretion of injected Co^{60}-cyanocobalamin was observed (table 1). In order to establish base line values for food consumption, all rabbits were fed ad libitum for 5 to 10 days until food intake became stabilized. When the animals were fed 75% of their control food consumption for 35 days, they gained weight and the radioactivity in the kidney increased slightly from 0.35 to 0.66% of the dose per gm of wet tissue, while radioactivity in liver tissue decreased. These changes are not significantly different from those in control animals. However, when the rabbits were fed 50% of their control food consumption for 18 days and 36 days or 25% of the control intake for 9 days and 29 days, respectively, or during complete starvation, the accumulation of radioactivity in kidney tissue was significantly elevated. The radioactivity in liver tissue was not altered when the 50% diets were fed but increased slightly (P =

TABLE 1

Effect of food restriction on tissue uptake and urinary excretion of injected Co^{60}-labeled vitamin B_{12} in female rabbits

Food intake	Days[1]	Weight change	Kidney	Liver	Urine
%		%	*% dose/gm*	*% dose/gm*	*% dose ± S.E.*
100(9)[2]	0+3	+9.5	0.35 ± 0.09[3]	0.15 ± 0.03	4.41 ± 0.70
75(5)	35+3	+22.6	0.66 ± 0.15	0.07 ± 0.02	5.04 ± 1.31
50(6)	18+3	−5.9	1.33 ± 0.39[4]	0.15 ± 0.01	7.67 ± 2.87
50(6)	36+3	+12.1	1.16 ± 0.29[4]	0.15 ± 0.02	6.30 ± 1.24
25(5)	9+3	−10.3	1.18 ± 0.24[4]	0.17 ± 0.01	6.32 ± 0.76
25(6)	29+3	−13.0	1.64 ± 0.35[4]	0.24 ± 0.02[5]	6.21 ± 1.53
0(6)	8+3	−26.7	2.77 ± 0.45[4]	0.25 ± 0.03[5]	7.08 ± 1.17[5]

[1] Figures represent the days maintained with the diets plus the same diet for three additional days following injection of Co^{60}-labeled vitamin B_{12}.
[2] Figures in parentheses indicate number of animals.
[3] Standard error of the mean.
[4] Significantly different from 100% food intake at 1% level.
[5] Significantly different from 100% food intake at 5% level.

TABLE 2

Effect of starvation on tissue uptake and urinary excretion of injected Co^{60}-labeled vitamin B_{12} in various animals

Species	Diet		Days[1]	Body weight		Kidneys (2)			Liver			Urine
				Initial	Final							
				kg	*kg*	*gm/100 gm body wt.*	*% dose/organ*	*% dose/gm*	*gm/100 gm body wt.*	*% dose/organ*	*% dose/gm*	*% dose*
Rabbit	Fed	(9)[2]	8+3	2.06	2.22	0.604	4.47 ± 1.05[3]	0.35 ± 0.09	3.75	11.72 ± 1.58	0.15 ± 0.03	4.41 ± 0.70
	Starved	(6)	8+3	2.43	1.63	0.573	27.57 ± 3.94	2.77 ± 0.45	2.69	11.58 ± 1.39	0.25 ± 0.03	7.08 ± 1.17
	P value						< 0.01	< 0.01			0.05	0.05
Chicken	Fed	(3)	8+3	1.65	1.64	0.418	6.53 ± 0.39	0.97 ± 0.11	2.18	15.29 ± 2.04	0.44 ± 0.04	
	Starved	(3)	8+3	2.14	1.74	0.389	12.73 ± 2.34	1.90 ± 0.30	1.05	4.79 ± 0.17	0.27 ± 0.01	
	P value						0.05	0.05		< 0.01	< 0.02	
				gm	*gm*							
Guinea pig	Fed	(6)	2+3	323	349	0.877	1.86 ± 0.13	0.62 ± 0.06	4.31	36.23 ± 1.30	2.53 ± 0.25	2.94 ± 0.31
	Starved	(6)	2+3	360	257	1.074	0.94 ± 0.15	0.35 ± 0.07	3.43	46.80 ± 5.95	5.57 ± 1.04	3.55 ± 0.49
	P value						0.01	0.01			0.02	
Rat	Fed	(6)	4+3	328	322	0.782	32.38 ± 2.59	12.85 ± 1.39	4.18	5.14 ± 0.32	0.38 ± 0.02	1.91 ± 0.58
	Starved	(6)	4+3	311	221	0.794	27.50 ± 1.59	15.83 ± 0.98	3.71	6.50 ± 0.28	0.82 ± 0.07	1.20 ± 0.49
	P value									< 0.01	< 0.01	
	Fed	(7)	8+3	303	305	0.698	20.74 ± 2.71	10.09 ± 1.46	3.19	5.87 ± 0.40	0.60 ± 0.09	2.39 ± 0.57
	Starved	(6)	8+3	291	187	0.813	25.99 ± 3.86	17.18 ± 2.63	2.37	7.75 ± 0.34	1.78 ± 0.20	2.59 ± 0.62
	P value									< 0.01	< 0.01	

[1] The figures represent the days fed ad libitum or subjected to complete starvation plus the same regimen for 3 additional days following the injection of Co^{60}-labeled vitamin B_{12}.

[2] Figures in parentheses indicate number of animals.

[3] Standard error of the mean.

0.05) with the 25% diets or after complete starvation. Although the urinary excretion of the injected dose was greater than that observed in the control animals for all conditions studied, only complete starvation resulted in a significant urinary excretion.

Since starved animals lose weight with concomitant changes in the size of various organs, the data were recalculated as shown in table 2. In 6 female rabbits starved for 11 days, a body weight loss of 33% occurred. The kidneys lost weight at the same rate as the body, as shown by the similar organ weight per 100 gm of body weight between normal (0.604) and starved (0.573) animals. The accumulation of the injected dose of radioactivity in the kidneys, when calculated as the percentage of the dose for either the total organ or per gm of tissue, was 6- to 8-fold higher in the kidneys from starved animals. Similar calculations for the liver demonstrate no alteration in accumulation of radioactivity for the total organ.

Similar experiments (table 2) were performed with rats starved for 7 days in the first experiment and 11 days in a second experiment, in hens starved for 11 days and in guinea pigs starved for 5 days. In rats starved for either 4 or 8 days prior to the injection of Co^{60}-cyanocobalamin, kidney accumulation was not significantly different between fed and starved animals when calculated either as percentage of dose per gm of tissue or percentage of dose in the total organ. Radioactivity in the liver, however, was significantly elevated ($P < 0.01$) in starved animals for both experiments and on either basis of calculation. The urinary excretion of the injected vitamin was the same for normal and starved animals in both experiments.

In starved hens, the radioactivity in the kidney of starved animals was slightly elevated ($P = 0.05$) but was markedly decreased in the liver of the animals. In female guinea pigs, kidney radioactivity was significantly decreased in starved animals but the increased liver radioactivity for starved animals is of doubtful significance. The urinary excretion in guinea pigs was not altered from normal values.

DISCUSSION

Results of these studies show that animals of different species vary considerably with respect to the accumulation of Co^{60}-cyanocobalamin in various organs as Miller et al. ('56) have observed previously. In rats, the kidneys appear to be the major site of vitamin B_{12} storage, whereas in other animals, most of the vitamin was stored in the liver. Marked variations in native vitamin B_{12} content in various tissues and whole blood and serum from various animals have also been observed by Scheid and Schweigert ('54), Couch et al. ('50) and Rosenthal and Brown ('54).

The studies reported here indicate that animals may be divided into three major categories based on the kidney: liver ratio of accumulated radioactivity in well-fed animals following an injection of Co^{60}-cyanocobalamin. The ratios range from 0.05 in guinea pigs to 5.0 for rats, whereas the ratios for rabbits and chickens are intermediate and range around 0.4. These ratios vary by a factor of 10, although it is difficult to explain the reason for this variation.

More significant was the response of various animals to starvation with respect to the accumulation of injected Co^{60}-cyanocobalamin in kidney and liver tissue. In rabbits subjected to starvation, a rapid increase in the accumulation of radioactivity in the kidneys occurred after a short lag period. This lag period suggests that labile caloric reserves may be sufficiently adequate to maintain normal function for a minimal period of time. After the labile reserves are depleted, kidney vitamin B_{12} accumulation increases steadily to high levels.

The relationship between starvation and kidney vitamin B_{12} accumulation also occurred in chickens and rats but to a lesser extent than in rabbits. Guinea pigs, which can withstand starvation for only short periods of time, failed to respond in the same way as rabbits, chickens and rats. In fact, the kidneys of starved guinea pigs accumulated smaller amounts of the injected vitamin than control animals fed ad libitum. On the other hand, liver tissue from starved guinea pigs and rats incorporated larger amounts of the injected

dose than ad libitum-fed control animals, but in the liver tissue from starved hens, the incorporation of radioactivity was markedly depressed.

Because restriction of food consumption in rabbits for 18 or 36 days to 50% of the ad libitum control values (a food intake adequate for maintaining body weight), resulted in elevated kidney accumulation strongly suggests the possibility that a dietary nutrient may be the limiting factor. Observations that the kidneys from other animals such as the rat and chicken also accumulate more cyanocobalamin under conditions of food restriction tend to support this view. Guinea pigs, however, responded in a manner opposite to the other animals. Although it is conceivable that some dietary substance is a limiting factor necessary for the incorporation of injected radioactive cyanocobalamin into rabbit tissues, the complex nature of metabolism during starvation makes other explanations tenable and must await further study.

SUMMARY

Adult female rabbits, subjected to total starvation, accumulated a higher percentage of injected Co^{60}-cyanocobalamin in kidneys and eliminated more of the dose in urine than normal, well-fed animals. Accumulation of the vitamin in liver was not altered under similar conditions. Chickens and rats responded to starvation to a lesser extent than rabbits, but guinea pigs failed to respond. Partial food restriction in rabbits resulted in progressive increases in kidney accumulation and urinary excretion of the injected vitamin as food intake decreases. It is suggested that some unknown factor necessary for the normal metabolism of vitamin B_{12} by the kidneys becomes limiting under conditions of total and partial food restriction.

ACKNOWLEDGMENT

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Nutritive Value of Maine Sardines

II. ANIMAL FEEDING TESTS[1,2]

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In an earlier report, Maine sardines were characterized with respect to a number of essential nutrients (Proctor et al., '61). The results of that investigation indicated that this processed food may be a good, inexpensive source of a number of nutrients.

Although the analytical techniques used in these tests demonstrate the presence of nutrients, they give no information concerning their availability. Moreover, the extent to which a particular foodstuff can act as an important food source depends ultimately upon its ability to support the growth of young animals and its utilization for maintenance of adult animals. Therefore, animal feeding studies, designed to test the ability of sardines to support the growth of young animals, were undertaken.

Since more than one process is used by the sardine industry, it was felt that some measurement should also be made of the effect of various processing methods on the availability of nutrients. For this reason, sardines processed in two ways were included in this study.

It was recognized at the outset of these tests that an investigation of the availability of all the essential nutrients of a specific food product in a single test would be unwieldy and impractical. Therefore, these investigations were designed to study only the utilization of the protein and fat of sardines in terms of their ability to support growth and development of the rat.

Because sardine oils contain relatively large amounts of unsaturated fatty acids which have been shown to be effective in alleviating hypercholesterolemia, measurements of serum cholesterol were made to determine the influence of whole sardine diets on this metabolite.

MATERIALS AND METHODS

Ninety male Sprague-Dawley rats (Charles River Laboratories) of about the same body weight were divided into three groups of 30 rats each. Rats in each group averaged about 70 gm, ranging from 64 to 76 gm. The animals were housed in individual screen-bottom cages in temperature- and humidity-controlled quarters. Food and water were supplied ad libitum. Food intake was measured daily, and the animals were weighed twice weekly.

The diets were prepared as agar gels. This type of ration has been found to facilitate the measurement of food intake since it reduces scattering to a minimum. The diets are presented in table 1 and were prepared according to the method of Miller and Allison ('58). In the composite sardine diets, protein and fat were supplied by a combination of 4 brands of Maine sardines, which had been steam-cooked prior to canning. The sardines for this composite were prepared by opening and draining equal numbers of cans of the 4 brands and then grinding the fish in a Hobart meat grinder. The ground fish were then thoroughly mixed before inclusion in the ration.

Preparation of the brand "E" sardine diets was essentially the same except that the fish were from a lot that had been

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[2] Contribution no. 409, from the Department of Nutrition, Food Science and Technology.

TABLE 1
Composition of diets

	Brand "E" sardine diet	Composite sardine diet	Control diet
	gm	*gm*	*gm*
Brand "E" sardines	500	—	—
Sardine composite	—	500	—
Casein[1]	—	—	120
Sucrose	400	400	400
Lard	—	—	60
Salts[2]	40	40	40
Cod liver oil	20	20	20
Agar	30	30	30
Water	100	100	420
Vitamins[3]	2	2	2

[1] Casein is approximately 90% protein and therefore supplies to the ration 108 gm of protein.
[2] Wesson modification of the Osborne and Mendel ('32) salt mixture.
[3] The vitamin mix supplies per kg of diet: (in milligrams) thiamine·HCl, 10; riboflavin, 10; pyridoxine, 10; Ca pantothenate, 30; niacin, 25; menadione, 4; inositol, 500; biotin, 0.5; choline, 1000; and α-tocopherol, 40 I. U.

fried rather than steam-cooked prior to canning.

Upon analysis, the composite of sardines was shown to contain 62.8% of water, 22.6% of protein and 11.5% of fat, whereas the brand "E" sardines contained 58.3% of water, 24.1% of protein and 15.4% of fat. Details concerning amino acids, vitamins and other nutrients are given in the previous paper of this series (Proctor et al., '60).

Casein and lard were the sources of protein and fat in the control ration. The levels of casein and lard in the diets fed the control animals were designed to give protein and fat levels approximating those of the sardine ration. Since only protein and fat utilization were to be studied, adequate amounts of vitamins and minerals were added to each ration.

Food intake was measured daily and was calculated on a wet-weight basis since previous experience by one of the authors with these rations indicated that the loss in water by evaporation after being allowed to stand overnight in a cage was less than 2%.

Blood was taken from 10 animals of each group by direct cardiac puncture at zero, 4, 12 and 24 weeks. The serum was obtained by allowing the blood to clot, centrifuging and collecting the supernatant. Total cholesterol was then measured using Bloor's method ('16).

At the completion of the study, the animals were sacrificed using ether. Ten animals of each group that had not been used for cardiac punctures were autopsied and the tissues examined histopathologically. Ten animals were selected randomly from the remaining 20 in each group for carcass analysis.

The carcasses were prepared for assay by first freezing them and the bones were then crushed with a mallet. The sample was ground three times in a Hobart meat grinder. Aliquots of this sample were taken and analyzed for protein (A.O.A.C., '55b), fat (Jacobs, '51) and moisture (A.O.A.C., '55a).

Data were analyzed for standard deviation of the mean and significance determined by the "t" test (Richardson, '44).

RESULTS AND DISCUSSION

Chemical characterization of sardines (Proctor et al., '61) had indicated that this food product was a good source of protein and fat. The results of these animal feeding tests support these contentions. Animals fed a ration in which sardines supplied all of the protein and fat grew at rates similar to those fed a synthetic ration which, in this laboratory, had proven adequate for growth (fig. 1). Animals fed the composite sardine diet gained approximately 31.5 gm per week for the initial growing period, whereas animals fed the casein-lard control diet gained approximately 32.5 gm per week for the same interval. The greatest growth rate was observed in rats fed the brand

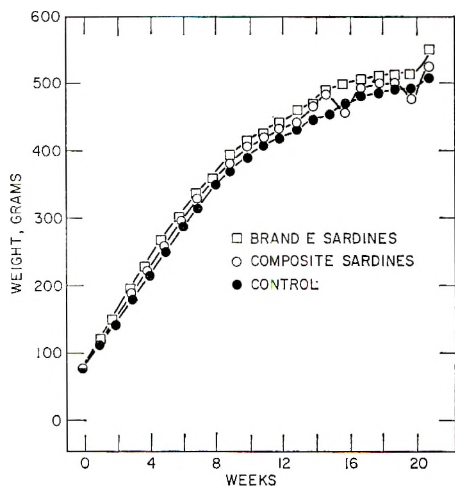

Fig. 1 Growth of animals fed sardines.

"E" sardine diets, when weight gain averaged about 35 gm per week for this growth period. At the conclusion of the test, the mean weights of the three groups were essentially the same.

More specific evidence for the value of sardines as a food, however, is indicated in the measurement of food efficiency (fig. 2). Animals fed the composite and brand "E" diets utilized their rations for weight gain to an extent at least equal to animals fed the control rations. When the entire experimental period is considered, both groups of sardine-fed rats exhibited the same food efficiencies as the animals fed the casein-lard control diets (table 2).

When protein efficiencies are considered, similar results are obtained. As illustrated in table 2, both groups of sardine-fed rats tended to utilize the protein of their rations during the first 4 weeks of the study to the same extent as the animals fed the control diets. Although protein utilization appeared to be somewhat greater in animals fed sardines, these differences were not statistically significant. The apparently low protein efficiency ratio of the controls during the first 4 weeks may have been a function of the age of the animals at the initiation of the test. Protein efficiency measurements are generally performed on weanling animals with an initial weight of about 50 gm, whereas 70-gm animals were used in this test.

The results of the carcass assays also appear to indicate greater protein utilization by the sardine-fed rats (table 3). Again, however, this tendency is not statistically valid. Animals fed sardines, both as a composite and as a single brand, as a source of protein and fat appeared to deposit somewhat less fat and more protein than animals fed the control diets.

The apparent advantage in the utilization of sardine protein as compared with casein is not considered significant and is probably a function of the biological variability inherent in any animal investigation.

As indicated in figure 3, essentially no significant effect on serum total cholesterol was noted under the conditions of this test when drained canned sardines were the sole source of protein and fat in the diet. Serum cholesterol levels in both control and experimental groups were similar during the 6-month feeding period. In addition, histopathological examination revealed no evidence of any pathological

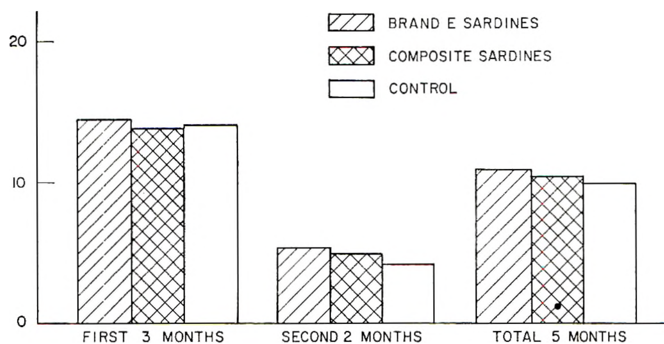

Fig. 2 Summaries of food efficiencies of animals fed sardines.

TABLE 2

Food and protein efficiencies of animals fed sardine and control diets

	Δ Body weight	Food intake	Protein intake	Food efficiency ratio	Protein efficiency ratio
	gm	*gm*	*gm*		
0 to 28 Days					
Brand "E"	171 ± 5[1]	704.4 ± 66.9	87.6 ± 8.2	0.24 ± 0.03	1.95 ± 0.19
Composite	159 ± 5	712.0 ± 65.6	89.7 ± 8.3	0.22 ± 0.03	1.77 ± 0.18
Control	152 ± 5	707.5 ± 67.3	93.3 ± 8.9	0.21 ± 0.02	1.63 ± 0.18
0 to 84 Days					
Brand "E"	357 ± 5	2448.7 ± 210.0	301.2 ± 25.8	0.15 ± 0.01	1.19 ± 0.11
Composite	340 ± 4	2473.1 ± 201.1	311.6 ± 25.3	0.14 ± 0.01	1.09 ± 0.10
Control	337 ± 4	2402.6 ± 204.5	317.1 ± 26.9	0.14 ± 0.01	1.07 ± 0.10
0 to 140 Days					
Brand "E"	452 ± 3	4163.0 ± 387.3	512.0 ± 47.6	0.11 ± 0.01	0.88 ± 0.08
Composite	423 ± 3	4098.0 ± 397.1	516.3 ± 49.0	0.10 ± 0.02	0.81 ± 0.14
Control	409 ± 3	4112.1 ± 369.5	542.8 ± 48.7	0.10 ± 0.01	0.75 ± 0.10

[1] Standard deviation of the mean.

TABLE 3

Carcass analysis of rats fed sardine and control diets

	Moisture	Protein		Fat		Protein fat ratio
		Dry	Wet	Dry	Wet	
	%	%	%	%	%	
Brand "E" sardines	54.04 ± 4.31[1]	39.70 ± 7.73	17.89	54.20 ± 9.28	25.35	0.732 ± 0.192
Sardine composite	55.64 ± 3.82	41.49 ± 6.58	18.22	53.63 ± 6.08	24.85	0.774 ± 0.151
Control	52.52 ± 3.07	36.62 ± 4.49	17.29	54.20 ± 10.15	26.71	0.676 ± 0.154

[1] Standard deviation of the mean.

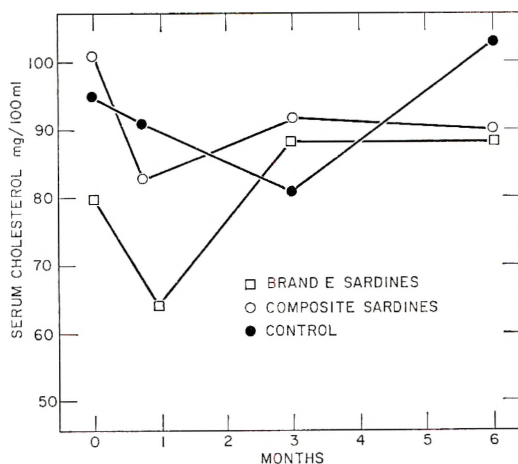

Fig. 3 Serum cholesterol levels of animals fed sardines.

lesions in any tissue examined, including those of the circulatory system.

Therefore, it may be concluded that sardines compare well with other good-quality foods in terms of their ability to support growth in young rats. These results indicate that the protein of sardines is sufficiently available for utilization by the rat and has an adequate pattern of amino acids to support growth. Also, under the conditions of this experiment, sardine oils, generally characterized as "liquid" because of greater concentrations of unsaturated fatty acids, are utilized at least as well as lard, a solid fat. Moreover, the various methods of preparing the fish, namely, by steaming and frying, do not differ greatly in their effect on the availability of protein and fat to the growing rat.

SUMMARY

Since an earlier investigation had indicated that sardines were a good source of a number of nutrients, a study was initiated to determine the availability of sardine protein and fat for growth and maintenance of rats. In addition, the effects

of two processing methods, steam-cooking and frying, on the availability and utilization of sardine protein and fat were determined.

No significant differences in growth, food and protein efficiency, or carcass composition were observed between rats fed a casein-lard control ration and animals fed either steam-cooked or fried sardines as a source of protein and fat.

It was concluded that the protein and fat of sardines was sufficiently utilized by the rat for growth and maintenance and that steam-cooking or frying prior to canning had no significant effect on the utilization of these nutrients.

ACKNOWLEDGMENT

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Nutritionally Induced Smooth Muscle Lesions in the Rat[1]

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It has been reported that whole cow's milk need be supplemented only with trace minerals to promote good growth when fed as the sole diet of the weanling rat (Kemmerer et al., '32). In the course of a series of studies in these laboratories, designed to determine the effect of nonfat cow's milk on rat growth and longevity, a striking number of lesions involving the smooth muscle component of the aorta and stomach has been noted in animals restricted to the experimental diet or modifications thereof. The specific and selective appearance of these lesions in rats following prolonged ingestion of nonfat cow's milk forms the basis of the present report.

MATERIALS AND METHODS

One-hundred-and-twenty Sprague-Dawley albino male rats were divided into 4 groups of 30 animals each and given the following diets and tap water ad libitum beginning at 6 weeks of age: group 1 received nonfat dry cow's milk of the nutrient composition determined by direct chemical and microbiological analysis shown in table 1; group 2, nonfat dry cow's milk plus 55 I.U. of vitamin A per rat per day administered by oral instillation from an aqueous suspension of the palmitate salt; group 3, nonfat dry cow's milk containing 1.5% of ammonium chloride plus 55 I.U. of vitamin A per rat per day administered as above; group 4, stock diet.[2] Vitamin A and ammonium chloride were included in the diets of groups 2 and 3 because previous studies had demonstrated that the addition of these compounds to a deficient diet comprised of wheat flour (28%), degerminated corn meal (35%), lard (18%) and sugar (19%) enriched with 39% of nonfat dry cow's milk materially enhanced rat growth and longevity (Dreizen et al., '60).

The rats were housed in individual cages with screen bottoms and kept in an air-conditioned room maintained at approximately 23°C. They were weighed every week and complete blood counts were performed on tail blood every 4 months. The animals were kept on the experimental regimen until they expired. At necropsy, the kidneys were removed and x-rayed for the presence of renal stones before fixation. Histologic sections of the cervical, thoracic abdominal and pelvic organs, fixed in 10% buffered formalin, were prepared and stained with eosin and hematoxylin for microscopic examination.

OBSERVATIONS

Growth rate and longevity data of the rats fed the various test diets are presented in figure 1. Essentially little difference was observed between the growth curves of rats receiving nonfat dry cow's milk either alone or supplemented with vitamin A or with vitamin A and ammonium chloride. Weight levels attained by the animals in groups 1, 2 and 3 were considerably lower than those of the stock diet controls (group 4). Whereas inclusion of vitamin A in the diet of nonfat dry cow's milk failed to influence longevity, addition of 1.5% of ammonium chloride significantly extended the mean age at death from 36.2 weeks for group 1 and 37.5 weeks for group 2, to 46.8 weeks for group 3 ($P < 0.05$); these, however, were all substantially below the mean value of 71.1 weeks for the rats in group 4 fed the stock diet ($P < 0.01$).

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[1] These studies were supported by grants from the Charles F. Kettering Foundation and the Carnation Company.

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

TABLE 1

Nutrient value of nonfat dry milk as determined by direct analysis[1]

Amino acids		Minerals		Vitamins and lipids	
	gm/100 gm		*mg/100 gm*		*U.S.P. units/100 gm*
Alanine	1.20	Calcium	1,530.0	Vitamin A	243
Arginine	1.66	Cobalt	0.002	Vitamin D	50
Aspartic acid	1.73	Copper	0.11		*mg/100 gm*
Cystine	0.38	Iodine	0.46	Thiamine	0.432
Glutamic acid	4.57	Iron	0.74	Riboflavin	1.90
Glycine	0.62	Magnesium	100.0	Niacin	1.73
Histidine	0.96	Manganese	0.06	Vitamin B_6	0.38
Isoleucine	2.55	Phosphorus	1,120.0	Pantothenic acid	2.58
Leucine	3.63	Potassium	1,530.0	Vitamin B_{12}	0.00106
Lysine	3.20	Sodium	514.0	Folic acid	0.0031
Methionine	1.34	Zinc	1.5	Choline	19.7
Phenylalanine	1.67			Biotin	0.012
Proline	3.07			Inositol	80.3
Serine	2.26			*p*-Aminobenzoic acid	0.01
Threonine	1.51			Vitamin C	7.58
Tyrosine	1.67			Vitamin E	0.0079
Tryptophan	0.42			Linoleic acid	340.6
Valine	2.73			Linolenic acid	4.42
				Arachidonic acid	0.325
				Cholesterol	1.9

[1] Carbohydrate, 51.40%; protein, 34.20%; ash, 7.58%; moisture, 5.91%; fat, 0.65%; fiber, 0.26%.

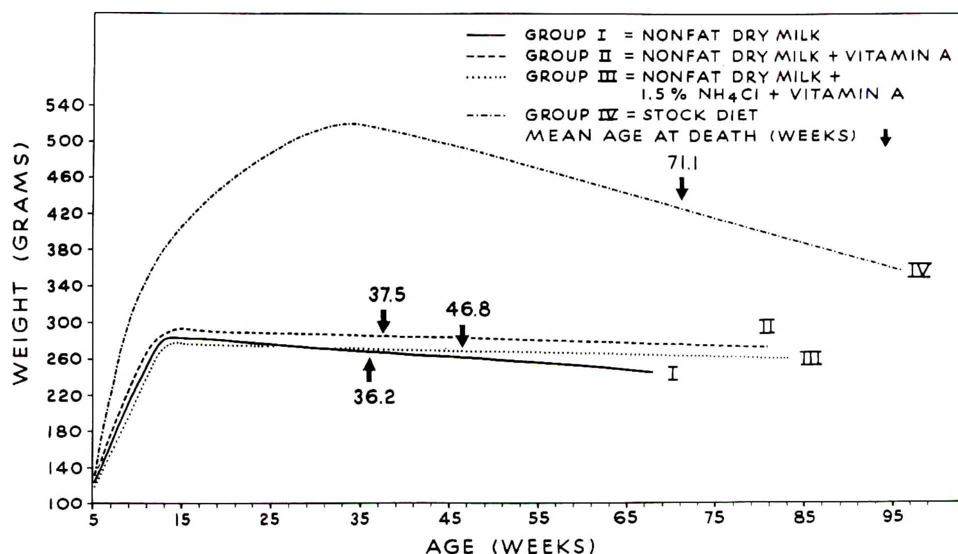

Fig. 1 Comparison of growth and longevity of rats fed experimental diets.

The rats restricted to the nonfat milk diet developed severe microcytic hypochromic anemia which was not alleviated by the addition of vitamin A and ammonium chloride. Smears from tail blood revealed a pronounced degree of anisocytosis, poikilocytosis and polychromatophilia. The mean blood values for each group at the end of the first 4 months of study are shown in table 2. Levels obtained in the succeeding 4-month intervals were similar. Blood counts for the animals maintained with the stock diet compared favorably with reported normal values for rats of this age, sex and strain (table 2).

The distribution of histologically confirmed smooth muscle lesions in the aorta and stomach of rats restricted to the test

diets is presented in table 3. Irrespective of the severity of smooth muscle involvement, no histologic evidence of concomitant degenerative changes in the cardiac and skeletal muscle was observed in any of these animals. The frequency of occurrence of smooth muscle lesions ranged from 50.0% for group 1 and 67.7% for group 2, to 73.3% for group 3. In each group there was a slightly greater incidence of lesions in the stomach than in the aorta. In general, the frequency of occurrence of the smooth muscle pathology increased with increasing age. The earliest age at which these lesions were detectable either grossly or microscopically was 175 days (25 weeks). None of the animals fed the stock diet (group 4) showed comparable changes in the aorta and stomach regardless of age at expiration.

Representative of the characteristic gross appearance of the aortic lesions is the specimen shown in figure 2. Involvement was most pronounced in the arch and in the thoracic segment. Lesions presented as irregular, bulging, inelastic plaques, streaks or annular bands. They were gray-white, and gritty and rigid in consistency. The annular bands were most prominent at the level of the intervertebral discs. When numerous, they gave a bamboo-stick impression to the aorta.

Microscopically, the most striking changes were noted in the tunica media. The usual pattern of parallel elastic lamellae separated by layers of smooth muscle was replaced by patches of degeneration and necrosis involving both tissue elements. The elastic lamellae were split and fragmented and the intervening smooth muscle converted into necrotic debris containing in some areas connective tissue ground substance, proliferations of fibroblasts, collagen scars and deposits of calcium salts. The intimal changes were confined to an increase in the number of endothelial cells and a slight increase in the amount of connective tissue ground substance. Examples of aortic lesions varying in severity and character observed by microscopic examination are presented in figure 3.

The specimen shown in figure 4 illustrates the characteristic gross pathologic changes which developed in the stomach of a significantly high proportion of rats fed the nonfat milk diets (groups 1, 2 and 3). The smooth glistening external surface of the stomach was superceded by dull white coarse plaques arranged as discrete or coalesced papular thickenings or as

TABLE 2

Mean blood values of rats fed experimental diets, at 4 months of age

Group	Diet	Leucocytes	Erythrocytes	Hemoglobin
		no./mm³	*million/mm³*	*gm/100 ml*
1	Nonfat cow's milk	14,825	6.08	6.02
2	Nonfat milk + vitamin A	13,820	6.13	5.65
3	Nonfat milk + vitamin A + 1.5% NH_4Cl	14,764	8.02	8.52
4	Stock	16,520	9.39	15.22
Normal for Sprague-Dawley male rats[1]		17,826	8.88	14.8 to 15.7

[1] Gardner ('47).

TABLE 3

Distribution of smooth muscle lesions in aorta and stomach of rats fed the experimental diets

Group	No. of animals	Diet	Aorta only		Stomach only		Aorta and stomach		Total	
			No.	%	No.	%	No.	%	No.	%
1	30	Nonfat milk	1	3.33	4	13.33	10	33.33	15	50.00
2	30	Nonfat milk + vitamin A	3	10.00	7	23.33	10	33.33	20	66.67
3	30	Nonfat milk + vitamin A + 1.5% NH_4Cl	3	10.00	7	23.33	12	40.00	22	73.33
4	30	Stock	0	0.00	0	0.00	0	0.00	0	0.00

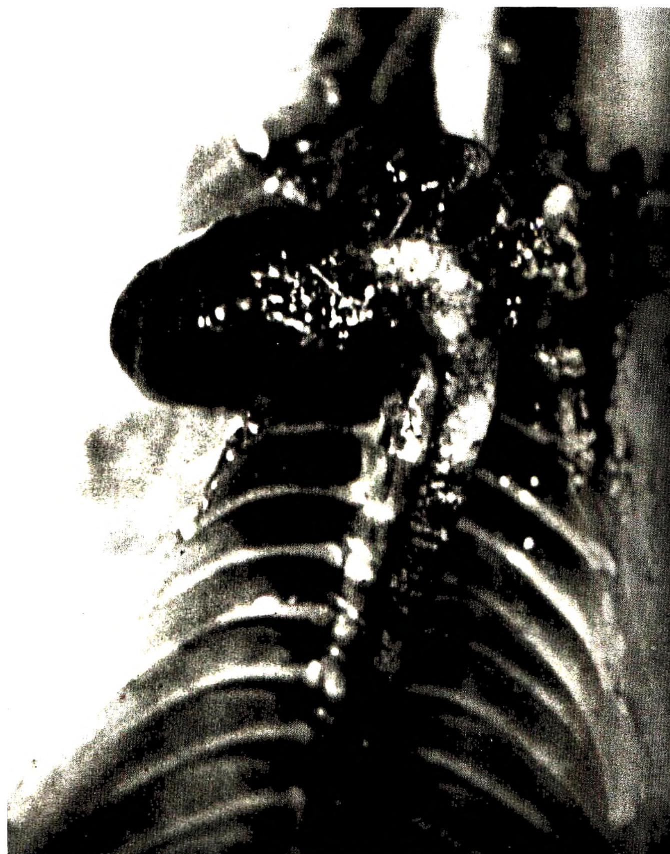

Fig. 2 Gross appearance of medial degeneration and necrosis in arch and thoracic aorta of rat fed nonfat dry cow's milk.

bands encircling the entire circumference from the lesser to the greater curvatures. In the most severe instances, additional striations followed the contour of the greater curvature and were most apparent at the point of attachment of the greater omentum. When involvement was slight-to-moderate, the lesions were localized to the pyloric region adjacent to the lesser curvature; when severe, the entire stomach was affected.

The microscopic appearance of the gastric lesions is shown in figure 5. Except for the deposition of calcium salts in the mucosal lining in a few animals, changes were confined exclusively to the circular and longitudinal muscle masses of the deep muscular layer, the muscularis mucosa being spared. Extensive degeneration and necrosis of the smooth muscle fibers with superimposed accumulations of calcium salts in the necrotic areas typified the microscopic findings. Irrespective of the degree of muscular degeneration, an inflammatory response represented by infiltration of polymorphonuclear leucocytes and mononuclear cells was minimal or absent.

Each of the animals that received the nonfat milk diets with and without 1.5% of ammonium chloride developed roentgenographically detectable renal stones ranging from flecks in the pelvis and calyces to the stag horn configuration apparent in figure 6. No stones were found in any of the rats receiving the stock diet (group 4). The parenchymatous microscopic changes in the kidney consisted of obstruction of many of the collecting tubules mainly by clear hyaline casts and occasionally by intraluminal collections of

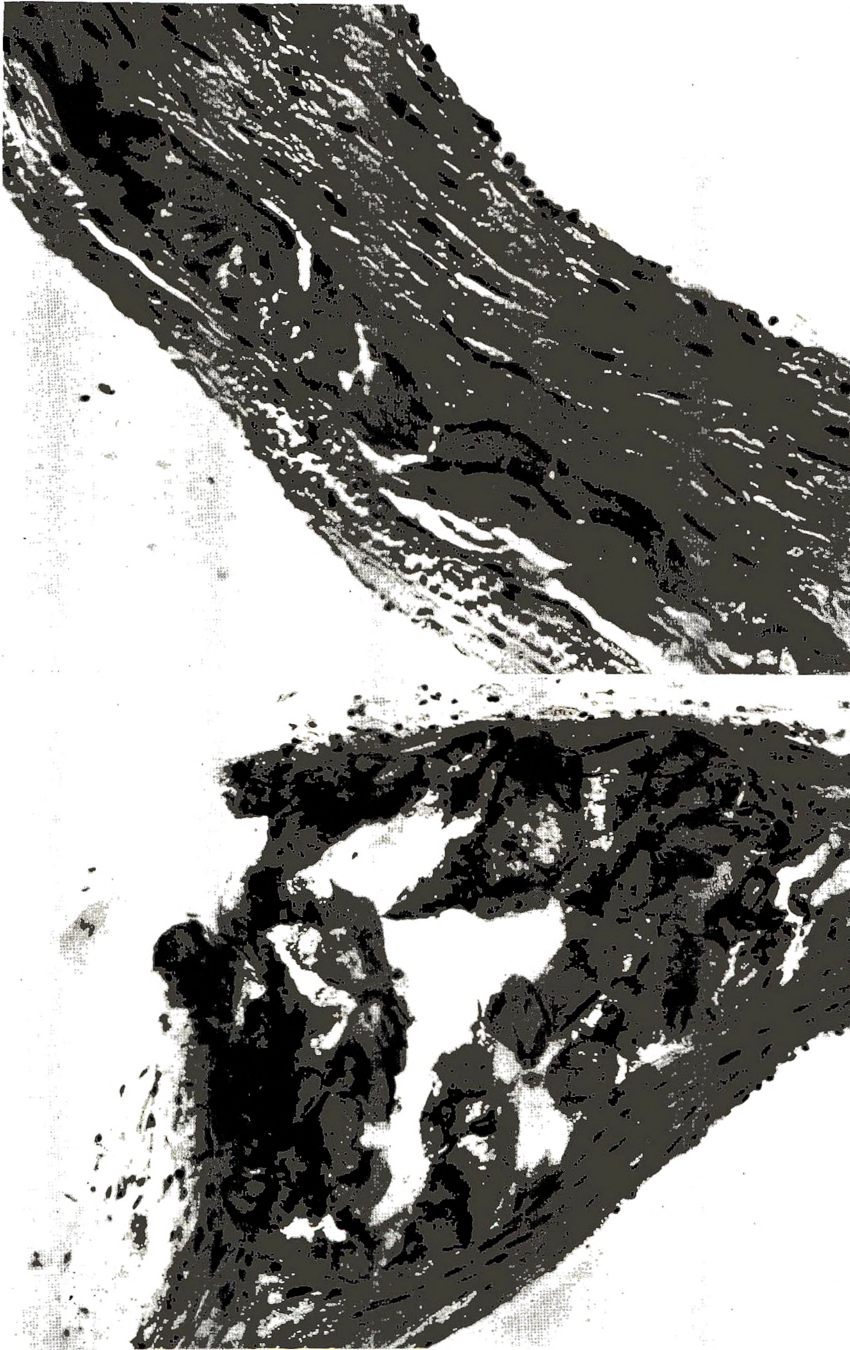

Fig. 3 High power photomicrographs of degeneration and necrosis in tunica media of aorta of rats fed nonfat dry cow's milk.

Fig. 4 Gross appearance of smooth muscle lesions in stomach wall of rat fed nonfat dry cow's milk.

calcium salts. Tubules so affected showed a pre-obstructive dilatation with flattened lining epithelial cells which frequently extended up into the cortex (fig. 7). Evidence of parenchymatous degeneration was exhibited by the proximal and distal convoluted tubules draining into the obstructed collecting tubules. The glomerular segments of the involved nephrons showed changes ranging from shrinkage of the capillary loops and widening of Bowman's space to hyalinization and complete obliteration of the glomerular apparatus. The pelvic epithelium demonstrated an inflammatory reaction which varied in intensity with the degree of stone formation, stasis and infection.

The most common causes of death of the animals on the various dietary regimens were severe anemia and chronic renal disease with urinary retention in the groups fed nonfat milk (groups 1, 2 and 3) and acute and chronic pneumonitis in the rats given the stock diet (group 4). Evidence of pneumonia was rarely found at necropsy in the groups restricted to the nonfat milk diets regardless of the age at death.

DISCUSSION

Arteriopathies characterized by necrosis of the tunica media of the aorta, resembling in detail Monckeberg's medial sclerosis in the human, have been produced experimentally in rats by severe choline deficiency (Hartroft et al., '52; Wilgram et al., '54; Wilgram and Hartroft, '55), hypervitaminosis D (Duguid, '30; Ham, '32; Kreitmaier and Moll, '38), intraperitoneal injection of poorly soluble sulfonamides (Lehr and Churg, '52), parathyroid excess (Lehr and Martin, '56), injection of anti-rat-kidney serum (Wissler et al., '54) and reduction or total interruption of the renal blood flow (Loomis, '46). Histologically identical lesions have been found to occur spontaneously in female breeder rats (Wilgram and Ingle, '59; Kittinger et al., '60). Male rats of the same strain and age show relatively little spontaneous cardiovascular disease (Wilgram and Ingle, '59). A common denominator in each of the aforementioned studies inductive of aortic medial necrosis was the development of nephropathies leading to renal malfunction.

Only Lehr ('59) has reported, heretofore, on the simultaneous appearance of smooth muscle lesions in the aorta and stomach wall of rats. Rats subjected to acute renal injury by the intraperitoneal injection of a single excessive dose of sodium acetylsulfathiazole demonstrated a predilection for smooth muscle degeneration in the aortic media and in the thick muscular wall of the pyloric half of the stomach. These lesions were ascribed to an overproduction of parathyroid hormone consequent to renal impairment. Similar lesions also attributed to an overproduction of parathyroid hormone have been produced by Lehr ('59) in bilaterally nephrectomized animals. In both instances, the development of disseminated smooth muscle necrosis was prevented by the prior removal of the parathyroid glands.

In this study, chronic renal injury and severe hypochromic microcytic anemia accompanied the appearance of smooth muscle necrosis in the aorta and stomach in a large number of rats fed nonfat dry cow's

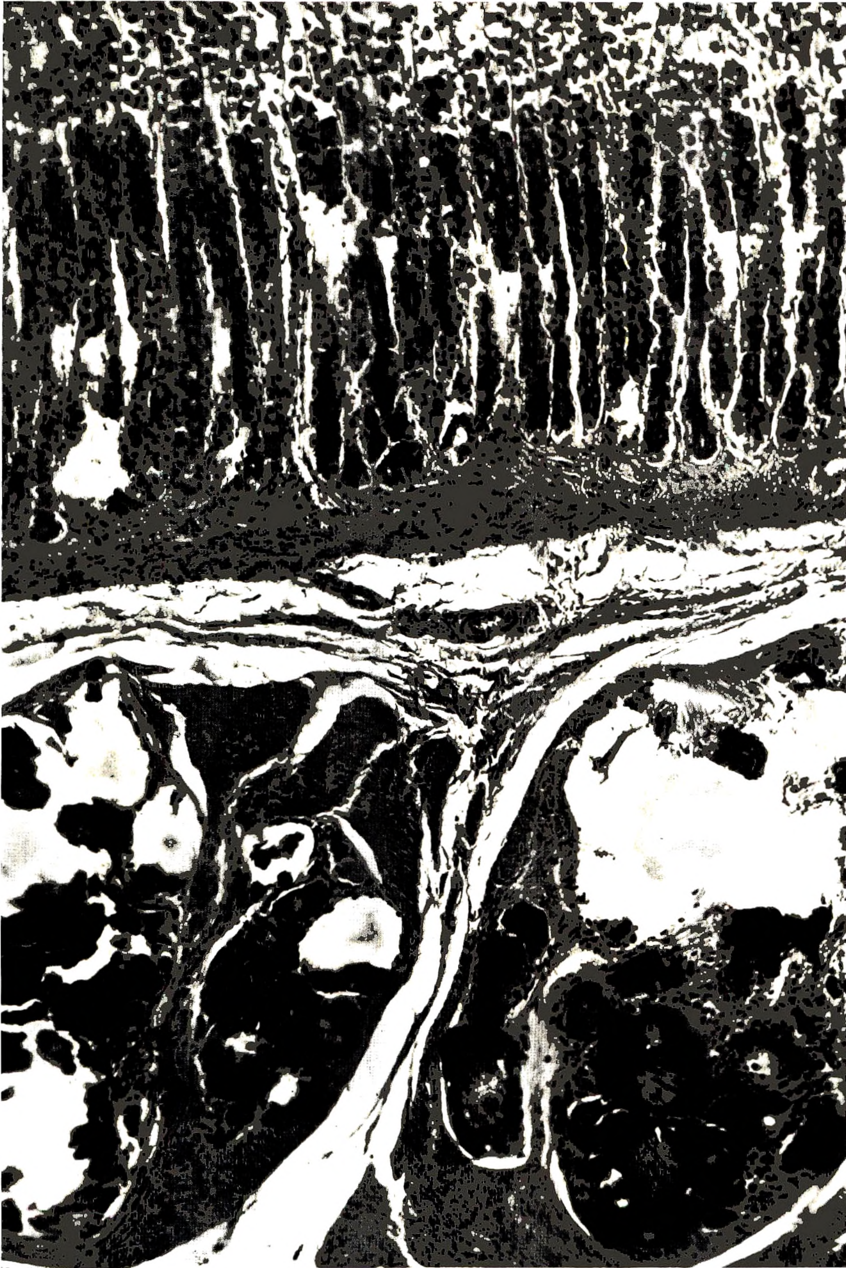

Fig. 5 High power photomicrograph of stomach wall of rat fed nonfat dry cow's milk showing degeneration and necrosis of the deep muscle masses and sparing of the muscularis mucosa.

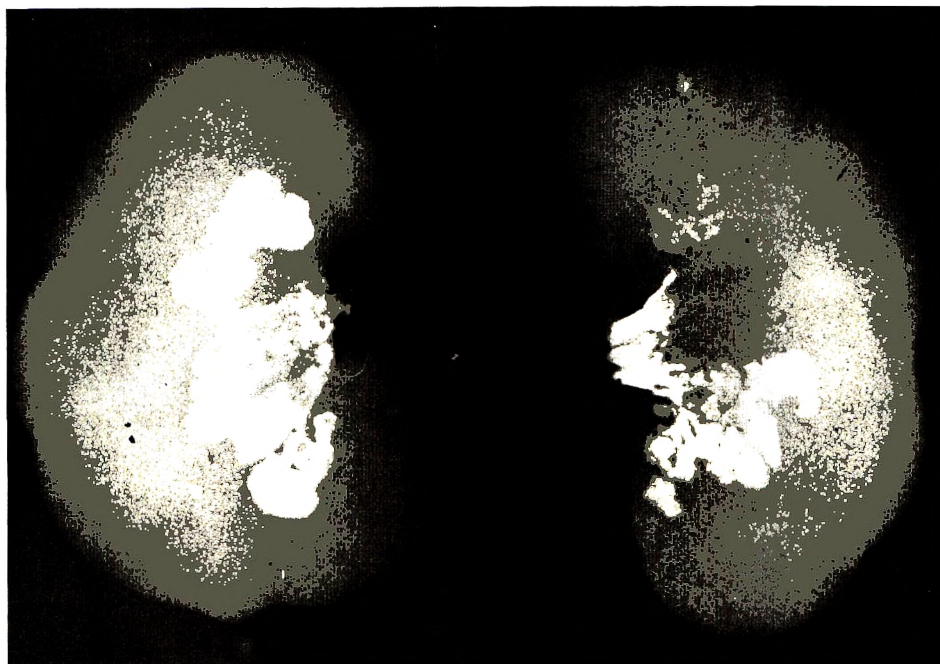

Fig. 6 Roentgenograms of kidneys of rat fed nonfat dry cow's milk showing extensive renal stone formation.

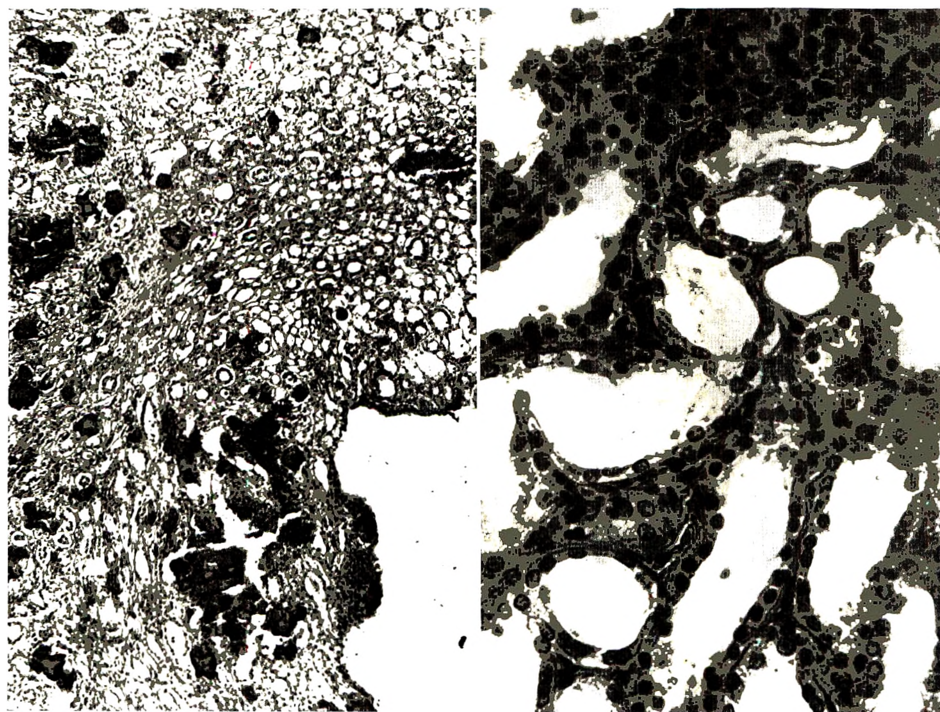

Fig. 7 High power photomicrographs showing obstructive nephropathy in medulla of kidney of rat fed nonfat dry cow's milk and dilatation of obstructed tubules with flattening of the lining epithelium.

milk with and without added vitamin A and ammonium chloride. Although ammonium chloride is highly effective in preventing nephrosclerosis in rats given overdoses of hydroxycortisone acetate (Selye et al., '45), addition of this salt to the nonfat milk increased longevity but failed to protect against renal, gastric and aortic pathology. The present findings cannot be attributed to a deficiency of choline or to an excess of vitamin D, each of which reputedly precipitates aortic medial necrosis and sclerosis in the rat. Rats maintained entirely with whole cow's milk supplemented with traces of iodine, manganese, copper, iron and cod liver oil throughout their life span do not develop aortic and gastric lesions of the type noted herein (McCay et al., '52). The presence of smooth muscle lesions in rats fed nonfat cow's milk and their absence in rats fed whole cow's milk and trace minerals suggests that specific nutrients in butter fat and/or trace minerals may act to preserve the integrity of rat smooth muscle. The identity of these factors and their possible influence on the parathyroid gland remains to be determined.

SUMMARY AND CONCLUSIONS

Smooth muscle degeneration and necrosis in the aortic media and in the thick muscular wall of the stomach have been produced in a high proportion of young albino male rats restricted to a diet of nonfat dry cow's milk and tap water beginning at 6 weeks of age. The disseminated muscular necrosis was accompanied by the development of severe anemia and renal disease. Addition of vitamin A and 1.5% of ammonium chloride to the experimental diet increased the incidence of smooth muscle lesions presumably by increasing the life span of the animals so treated.

The absence of smooth muscle pathology in reported studies of rats reared completely with a diet of whole cow's milk fortified with trace minerals suggests that unidentified nutrients in butter fat and/or specific trace minerals are essential for the maintenance of the integrity of smooth muscle in the rat.

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Effect of Various Tissues on the Growth of Sulfaguanidine-Fed Rats[1]

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Mackenzie and Mackenzie ('43) have demonstrated that the feeding of sulfaguanidine to rats results in goiter and growth inhibition. This and other evidence (Pitt-Rivers, '48) indicated that sulfaguanidine prevented the synthesis of thyroid hormones resulting in symptoms of thyroid deficiency. Ackerman ('57) reported that the inclusion of 5% of meat meal in the diet of rats fed 1% of sulfaguanidine could prevent or reverse the growth inhibition of the goitrogen. This same investigator reported later ('59) that meat meal was only partially effective in preventing the enlargement of the thyroid gland in rats fed sulfaguanidine or certain other goitrogens; also that certain animal tissues were found to stimulate the growth of sulfaguanidine-fed rats. Further work suggested that an unknown growth-promoting principle may be responsible for the growth-stimulating effect of hog duodenum.[2] Because it still seemed possible that thyroid hormone, present in low amounts in such tissues, might be the active principle, we initiated a study to determine the distribution and nature of tissues which stimulate the growth of sulfaguanidine-fed rats.

EXPERIMENTAL

Dried, powdered tissues were obtained from biochemical supply houses or meat packing plants. These were assayed by the curative-type growth assay described previously (Ackerman, '59). The tissue to be assayed was mixed into the sulfaguanidine-containing diet and fed for two weeks. Three rats (one male with two females) or two rats (one male with one female) were used for each test. This procedure has been found to be reliable since growth cessation is established for each rat before the animal is used for assay purposes, and subsequent growth is a response to the dietary supplement.

RESULTS AND DISCUSSION

Those tissues which have been observed to stimulate the growth of sulfaguanidine-fed rats are listed in table 1; and those found to be inactive in promoting growth are shown in table 2. For completeness, data which have been reported previously (Ackerman, '59) are summarized as a note under each table. The weight gain of rats used as controls at different times for these assays is summarized as one group in table 1. These rats gained 3 to 9 gm during the experimental period. A rigid comparison of the results of each assay in table 1 cannot be made since the tests were not all conducted at the same time, but those substances which resulted in a gain of 15 to 17 gm may be considered as having little growth-promoting activity. These have been arbitrarily classified depending on whether other preparations of the same tissue were active or inactive.

Consideration of table 1 suggests that tissues associated with digestion, the lymphatic system, and tissues associated with early growth, stimulate the growth of sulfaguanidine-fed rats. Data in table 2 show, however, that some samples of these tissues were inactive. Duodenal preparations have been consistently active.

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[2] Ackerman, C. J., and V. Tsou. Abstracts, Fifth International Congress on Nutrition, 1960, Washington, D. C., p. 27.
[3] Lot 5043, Nutritional Biochemicals Corporation, Cleveland.

TABLE 1

Tissues that stimulated growth of sulfaguanidine-fed rats[1]

Tissue[2]	Source[3]	Lot or control no.	No. of rats	Average 2-week gain
				gm
None	—	—	15	5
Duodenum	Cudahy	1566	3	51
Duodenum, hog	Cudahy	709	3	63
Duodenum	NBC	5043	3	17
Stomach, hog	Cudahy	697	3	41
Stomach lining	NBC	8487	3	22
Pancreas, hog	Cudahy	311	3	17
Thymus, beef	Cudahy	627	3	49
Thymus (2.5%)	NBC	6396	3	34
Thymus	NBC	—	3	38
Lymphatic powder	NBC	7372	2	41
Lymphatic powder	NBC	—	3	45
Lung	NBC	7517	2	47
Submaxillary	NBC	8068	2	39
Tonsil	NBC	7528	3	15
Ovaries, beef	Cudahy	699	3	45
Ovaries	NBC	4125	3	22
Placenta, beef	Cudahy	26	3	24
Mammary, beef	Cudahy	121	3	51

[1] Number of samples of active tissue reported previously (Ackerman, '59): duodenum, 2; stomach, 3; intestinal extract, 1; pancreas, 2; spleen, 1; mammary, 1; fish meal, 1; fish flour, 1, and tissue B, 1.

[2] Mixed in the diet at a level of 5% unless indicated otherwise by numbers in parentheses.

[3] Cudahy Laboratories, Omaha, Nebraska; Nutritional Biochemicals Corporation, Cleveland.

TABLE 2

Tissues that did not stimulate growth of sulfaguanidine-fed rats[1]

Tissue[2]	Source[3]	Lot or control no.	No. of rats	Average 2-week gain
				gm
Liver, hog	Cudahy	639	3	5
Liver, beef	Cudahy	740	3	3
Liver fraction "2"	NBC	8522	3	3
Liver extract	NBC	—	3	1
Blood powder	NBC	7412	3	15
Blood, dried soluble	NBC	1492	3	11
Blood, whole	NBC	7512	3	1
Blood, whole	Southeastern[4]	—	2	6
Heart, hog	Cudahy	376	3	2
Spleen	NBC	1995	3	3
Bone marrow, red	NBC	1789	3	6
Pineal powder, (3.5%)	NBC	9392	3	3
Egg yolk	NBC	7701	3	5
Enteric growth factor	Cudahy	488	3	14
Milk, dry, whole (10%)	Borden	—	3	2
Thymus	Southeastern[4]	—	2	6
Lung	NBC	—	3	8
Tonsil	NBC	—	3	0
Stomach lining	NBC	—	3	4
Placenta	NBC	—	3	8
Gastric mucin	NBC	—	3	1
Mammary	NBC	—	3	0
Orchic powder	NBC	—	3	9
Yellow corn (10%)	—	—	3	2

[1] Number of samples of inactive tissues reported previously (Ackerman, '59): hog stomach, 2; hog pyloric section, 1; extract of hog pylorus, 1; liver, 2; kidney, 1; brain, 1; fish meal, 3; fish flour, 1.

[2] See table 1, footnote 2.

[3] See table 1, footnote 3.

[4] Southeastern Biochemicals, Augusta, Georgia.

One sample[3] was weakly active and this preparation resulted in a 20-gm gain when the assay was repeated. Liver has been consistently inactive, and two out of 4 samples of blood were inactive. Two other samples of blood were weakly active, but these were arbitrarily included in table 2, as was enteric growth factor.

It is possible that the growth-promoting property of the tissues listed in table 1 is due to the presence of thyroid hormones, but the concentration of thyroid hormones in tissues of larger animals has not been determined. The distribution and relative concentration of radiolabeled thyroid hormones has been studied in rats (Van Arsdel et al., '54; Gross and Leblond, '47; Hatfield et al., '60; Tapley et al., '59) and guinea pigs (Ford et al., '57). Such studies show that all tissues exhibit an uptake of the administered dose of radiolabeled thyroid hormone, and the relative concentration of the radiolabeled hormones is highest in the liver, kidney and blood. If it is assumed that the distribution of thyroid hormone in larger animals is similar to that of laboratory animals and that the results reflect the actual concentration of thyroid hormones in tissues, then there is little correlation between the growth-promoting activity of the tissues listed in table 1 and their possible thyroid hormone concentration. Six liver preparations, one kidney and two blood samples were inactive. Thymus gland exhibits a low uptake of radiolabeled thyroid hormones (Gross and Leblond, '47; Van Arsdel et al., '54) but three of the 4 thymus samples assayed stimulated growth of sulfaguanidine-fed rats (table 1). Deiodinases present in liver and other tissues (Becker and Brudden, '59; Flock et al., '56, '57; Kalant et al., '55), however, could destroy an appreciable amount of the thyroid hormones during the commercial preparation of these tissues.

Other observations made in this laboratory indicate that further investigation of the nature of the growth-promoting principle in these tissues is warranted. Certain fractions prepared from hog duodenum were found to be devoid of iodine and were capable of stimulating the growth of sulfaguanidine-fed and hypophysectomized rats.[4]

SUMMARY

Various tissues were assayed for their ability to stimulate rat growth, that had been arrested by feeding 1% of sulfaguanidine. In general, tissues related to the digestive tract, the lymphatic system and the female reproductive system, stimulated growth. Liver, blood and a number of other tissues did not stimulate growth. The possibility that thyroid hormones could be the growth-promoting principle was considered.

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[4] See footnote 2.

Beneficial Effects of Pectin in Prevention of Hypercholesterolemia and Increase in Liver Cholesterol in Cholesterol-Fed Rats[1]

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A number of studies indicate that atherosclerosis and coronary artery disease occur more frequently in populations showing a high incidence of hypercholesterolemia than in those having lower serum cholesterol levels. Serum cholesterol levels, as well as the incidence and severity of atherosclerosis, are also lower in populations subsisting on vegetarian diets than in those ingesting diets containing animal protein and fat. Hardinge and Stare ('54) observed that serum cholesterol levels of adults were appreciably lower in a group of pure vegetarians (average 206 mg per 100 ml) than in a comparable group of nonvegetarians (average 291 mg per 100 ml). Similar results were noted by Page et al. ('56). A comparison of the diet of vegetarians with that of nonvegetarians reveals significant differences between the two, not only in respect to type and quantity of lipids and proteins (dietary factors which have been extensively investigated in respect to their effect on serum cholesterol levels), but also of other dietary constituents as well.

Since vegetarian diets are almost invariably of high fiber content (Hardinge et al., '58), interest has been expressed concerning the possible effects of ingested plant fiber material on serum cholesterol levels. Walker and Arvidsson ('54) and Higginson and Pepler ('54) were among the first to call attention to the high fiber content of the Bantu diet as a possible explanation for the low serum cholesterol level observed in the Bantu population. A similar suggestion was also made by Bersohn et al. ('56). The present investigation was undertaken to determine the comparative effects of cellulose and other types of roughage or bulk-forming materials on plasma and liver cholesterol levels in the cholesterol-fed rat.

PROCEDURE

The basal ration consisted of the following: (in per cent) sucrose, 61; casein,[2] 24; cottonseed oil, 10; salt mixture,[3] 5; and the following vitamins per kilogram of diet: (in milligrams) thiamine·HCl, 20; riboflavin, 20; pyridoxine·HCl, 20; Ca pantothenate, 60; nicotinic acid, 100; ascorbic acid, 200; biotin, 4; folic acid, 10; *p*-aminobenzoic acid, 400; inositol, 800; 2-methyl-1,4 naphthoquinone, 5; α-tocopheryl acetate, 100; and vitamin B_{12}, 150 μg; choline chloride, 2 gm; vitamin A, 5000 U.S.P. units; vitamin D_2, 500 U.S.P. units. Cholesterol and the various test supplements were incorporated in the basal ration in the amounts listed in tables 1 and 2, replacing an equal amount of sucrose.

Male rats of the Holtzman strain, 22 to 24 days of age, weighing 45 to 55 gm, were divided into comparable groups of 8 to 12 animals each. The rats were placed in metal cages with raised screen bottoms and were provided the test diets and water ad libitum for 4 to 6 weeks. The animals were fed on alternate days and all food not consumed 48 hours after feeding was discarded. Body weights were recorded weekly. At the end of the feeding period the animals were anesthetized with sodium pentobarbital, and blood was withdrawn from the aorta into a heparanized syringe.

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[1] Supported in part by a research grant (H-4510) from the National Heart Institute, National Institutes of Health, U. S. Public Health Service.

[2] Vitamin-Free Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

[3] Hubbell et al. ('37), salt mixture obtained from General Biochemicals, Inc., Chagrin Falls, Ohio.

TABLE 1

Comparative effects of pectin and other materials on plasma and liver cholesterol and liver total lipid in cholesterol-fed rats[1,2]

Supplements fed with basal ration	Body weight at sacrifice	Plasma cholesterol		Liver weight	Liver total lipid	Liver cholesterol	
		Free	Total			Free	Total
	gm	*mg/100 ml*		*gm*	%	*mg/gm*	*mg/gm*
Experiment 1							
None	221.0	20.6 ± 0.9[3]	108 ± 3	9.92 ± 0.21	4.0 ± 0.08	1.40 ± 0.06	1.68 ± 0.07
1% Cholesterol	223.6	21.3 ± 0.7	126 ± 2	11.59 ± 0.39	9.0 ± 0.03	3.30 ± 0.16	17.2 ± 1.3
1% Cholesterol + following supplements:							
5% Pectin N.F. (citrus)	223.6	17.9 ± 1.3	105 ± 3	11.18 ± 0.53	6.2 ± 0.6	1.98 ± 0.06	5.22 ± 0.57
5% Cellulose	236.4	24.7 ± 1.3	165 ± 6	11.56 ± 0.35	11.0 ± 0.4	3.11 ± 0.14	21.6 ± 1.4
5% Agar	226.0	24.5 ± 2.2	168 ± 8	11.10 ± 0.55	10.5 ± 0.4	4.16 ± 0.24	25.4 ± 2.2
5% Sodium alginate	190.3	23.5 ± 1.1	144 ± 7	9.84 ± 0.35	8.2 ± 0.3	3.35 ± 0.60	15.7 ± 1.0
5% Calcium silicate[4]	211.9	22.0 ± 1.4	140 ± 8	9.76 ± 0.32	7.7 ± 0.6	3.20 ± 0.27	13.6 ± 2.2
Experiment 2							
None	283.5	19.8 ± 0.9	95 ± 4	10.56 ± 0.23	4.5 ± 0.04	1.83 ± 0.03	2.26 ± 0.08
5% Pectin N.F. (citrus)	279.3	18.0 ± 1.9	92 ± 5	10.38 ± 0.27	4.4 ± 0.03	1.79 ± 0.04	2.11 ± 0.05
5% Protopectin	286.7	20.1 ± 1.8	93 ± 4	10.66 ± 0.37	4.7 ± 0.03	1.88 ± 0.05	2.05 ± 0.04
1% Cholesterol	293.8	26.6 ± 2.2	145 ± 8	13.32 ± 0.45	12.6 ± 0.9	4.06 ± 0.25	31.1 ± 3.4
1% Cholesterol + following supplements:							
5% Pectin N.F. (citrus)	292.7	20.0 ± 1.6	104 ± 7	12.45 ± 0.50	7.3 ± 0.4	2.84 ± 0.16	10.8 ± 1.8
5% Protopectin	284.9	26.2 ± 2.3	145 ± 10	13.92 ± 0.41	12.9 ± 0.5	4.60 ± 0.19	37.7 ± 2.6

Experiment 3							
None	207.4	25.1 ± 0.2	94 ± 3	8.36 ± 0.91	4.5 ± 0.2	1.55 ± 0.08	2.03 ± 0.08
1% Cholesterol	197.8	28.0 ± 2.0	131 ± 12	11.29 ± 0.43	9.7 ± 0.3	3.28 ± 0.20	24.7 ± 1.3
1% Cholesterol + following supplements:							
2.5% Pectin N.F. (citrus)	206.9	24.2 ± 1.0	109 ± 3	10.59 ± 0.26	9.4 ± 1.0	2.93 ± 0.18	15.6 ± 2.0
5% Pectin N.F. (citrus)	196.5	23.8 ± 1.2	106 ± 3	10.00 ± 0.45	7.5 ± 0.5	2.64 ± 0.09	11.4 ± 1.3
2.5% Pectin (apple)	196.0	24.3 ± 1.0	113 ± 2	10.20 ± 0.38	8.9 ± 0.3	2.68 ± 0.08	15.9 ± 1.0
5% Pectin (apple)	193.3	24.3 ± 0.6	104 ± 4	9.93 ± 0.42	6.9 ± 0.4	2.33 ± 0.04	8.22 ± 0.79
5% Pectic acid	198.3	24.6 ± 1.0	117 ± 6	10.43 ± 0.32	10.8 ± 0.5	3.34 ± 0.26	24.8 ± 2.2
5% Polygalacturonic acid	221.4	22.1 ± 2.3	114 ± 3	11.72 ± 0.56	10.7 ± 0.6	3.22 ± 0.19	23.3 ± 2.4
5% Galacturonic acid	223.3	21.0 ± 1.7	156 ± 9	11.69 ± 0.34	10.9 ± 1.1	3.41 ± 0.14	24.4 ± 2.1
5% Gastric mucin	219.3	25.2 ± 1.7	129 ± 4	11.75 ± 0.49	10.3 ± 0.3	3.30 ± 0.14	22.6 ± 1.2
10% Gastric mucin	221.0	24.0 ± 1.3	117 ± 4	11.29 ± 0.65	9.8 ± 0.4	3.17 ± 0.13	19.8 ± 1.4
15% Protopectin	197.8	22.5 ± 1.4	119 ± 6	9.75 ± 0.68	12.9 ± 1.0	4.43 ± 0.35	38.7 ± 4.7
1% Sulfasuxidine + 0.05% streptomycin	198.3	27.0 ± 2.0	153 ± 10	9.94 ± 0.40	10.0 ± 0.2	3.47 ± 0.15	24.1 ± 1.7
1% Sulfasuxidine + 0.05% streptomycin + 5% pectin N.F. (citrus)	215.5	21.0 ± 1.3	95 ± 4	11.57 ± 0.53	6.7 ± 0.4	2.61 ± 0.17	10.3 ± 1.1

[1] The test supplements were obtained from the following sources: pectin N.F. (citrus), protopectin, polygalacturonic acid, and galacturonic acid, Sunkist Growers, Ontario, California; apple pectin, Mann Research Laboratories, Inc., New York; pectic acid, Nutritional Biochemicals Corporation, Cleveland; cellulose (Solka Floc BW 200), Brown Company, Boston; Micro-Cel (calcium silicate), Johns-Manville Products, Los Angeles; and agar and sodium alginate, Hathaway Allied Products, Los Angeles. The streptomycin was supplied in the form of streptomycin sulfate.

[2] Eight animals/ group used in experiments 1 and 3; 12 animals/group in experiment 2. Rats were sacrificed after 4 weeks of feeding in experiments 1 and 3 and after 6 weeks in experiment 2.

[3] Including standard error of the mean calculated as follows:

$$\sqrt{\frac{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}{n-1}} \Big/ \sqrt{n}$$

where "x" = individual values and "n" = number of observations.

TABLE 2

Effects of alternate feeding of cholesterol and pectin on plasma and liver cholesterol and liver total lipid in the rat[1]

Diets alternated	Body weight at sacrifice	Plasma cholesterol		Liver weight	Liver total lipid	Liver cholesterol	
		Free	Total			Free	Total
	gm	*mg/100 ml*		*gm*	*%*	*mg/gm*	*mg/gm*
1 Basal ration + 2% cholesterol 2 Basal ration	184.3	11.8 ± 1.1[2]	70 ± 5	10.65 ± 0.66	6.8 ± 0.02	1.96 ± 0.07	13.30 ± 1.51
1 Basal ration + 2% cholesterol 2 Basal ration + 10% pectin N.F. (citrus)	184.1	11.2 ± 1.0	61 ± 3	10.99 ± 0.24	4.6 ± 0.05	1.81 ± 0.06	6.24 ± 0.89

[1] Eight animals used in each of the two series. Rats were sacrificed after 4 weeks of feeding.

[2] Including standard error of the mean; see footnote 3, table 1.

Livers were excised, blotted to remove excess blood, weighed and stored in a freezer until analyzed. Lipid was extracted from the livers by the method of Thompson et al. ('49), and cholesterol was determined on liver and plasma by the method of Nieft and Deuel ('49). Total lipids were determined gravimetrically on an aliquot of the liver extract.

RESULTS

Experiment 1. Comparative effects of cellulose and other roughage or bulk-forming materials on plasma and liver cholesterol in the cholesterol-fed rat. Observations are summarized in table 1. The addition of 1% of cholesterol to the basal purified ration resulted in a highly significant increase in free and total liver cholesterol and a slight increase in total plasma cholesterol over that obtained when the basal ration alone was fed. A marked increase in liver total lipid also occurred following cholesterol feeding. Supplements of cellulose, agar, sodium alginate or calcium silicate,[4] when fed as 5% of the diet in conjunction with a 1% cholesterol supplement, either showed no significant effect or increased plasma and liver cholesterol and liver total lipid over that obtained with the supplement of 1% of cholesterol alone. In contrast with this, 5% of pectin N.F. fed in conjunction with a 1% cholesterol supplement largely counteracted the increment in plasma and liver cholesterol and liver total lipid obtained with the basal ration plus 1% of cholesterol alone.

Experiment 2. Comparative effects of pectin and protopectin on plasma and liver cholesterol when fed with or without cholesterol, in the rat.[5] The animals in experiment 1 were killed after 4 weeks of feeding; those in experiment 2 after 6 weeks. The extra two weeks of feeding resulted in appreciably higher levels of plasma and liver cholesterol and liver total lipid in rats fed the basal ration plus 1% of cholesterol than occurred in the former experiment. In agreement with experiment 1, 5% of pectin N.F. in the diet

[4] Micro-Cel Johns-Manville Products, Los Angeles.

[5] Findings of this experiment were presented in part at the Annual Meeting of the American Institute of Nutrition, Atlantic City, New Jersey, 1959; Federation Proc., *18*: 551, 1959 (abstract).

largely counteracted the increment in plasma and liver cholesterol and liver total lipid induced by cholesterol feeding. Protopectin,[6] however, at a 5% level in the diet showed no activity in this respect. No significant effects on plasma or liver cholesterol or liver total lipid were observed in rats fed the basal ration plus 5% of pectin or 5% of protopectin without the cholesterol supplement (table 1).

Experiment 3. Effects of graded levels of pectin and comparative effects of pectin and pectin derivatives on plasma and liver cholesterol in the rat. Observations indicate that the effects of pectin on plasma and liver cholesterol and liver total lipid in cholesterol-fed rats are dependent on the level fed. Thus, whereas both a 2.5% and 5% pectin supplement were active in reducing plasma and liver cholesterol and liver total lipid in cholesterol-fed rats, the 5% supplement was more active than the 2.5% supplement in this respect.[7] Similar results were obtained with both citrus and apple pectin. In contrast with the results obtained with pectin, supplements of 5% of pectic acid, polygalacturonic acid and galacturonic acid in the diet had no significant activity in counteracting the increase in plasma and liver cholesterol and liver total lipid induced by cholesterol feeding. Similarly, gastric mucin at a 5 or 10% level and protopectin at a 15% level in the diet (containing an amount of pectic material equivalent to 5 to 6% of the diet) was also inactive. Incorporating 1% of sulfasuxidine and 0.05% of streptomycin in the basal ration plus 1% of cholesterol showed no significant effect on the increase in plasma and liver cholesterol and liver total lipid induced by cholesterol feeding. Plasma and liver cholesterol and liver total lipid of rats fed the sulfasuxidine and streptomycin supplements in conjunction with 5% of pectin and 1% of cholesterol were also not significantly different from that of rats fed a similar diet with the sulfa drugs omitted (table 1).

Experiment 4. Effects of alternate feeding of cholesterol and pectin on plasma and liver cholesterol in the rat. Tests were conducted to determine whether the inhibitory effect of pectin on the increase in plasma and liver cholesterol in the cholesterol-fed rat could be demonstrated under conditions in which pectin and cholesterol were fed on alternate days. Male rats were selected at weaning and divided into two comparable groups. One group received the basal ration plus 2% of cholesterol on the first day and the basal ration plus 10% of pectin on the second. This alternate feeding schedule was continued for 28 days. The second group was similarly treated except that in place of the basal ration plus 10% of pectin, these animals were fed the basal ration alone. An 8-hour fast separated each feeding period. Food was placed before the animals each day at 4:00 P.M. At 8:00 A.M. the next day the food was removed until 4:00 P.M. the same day, at which time the alternate diet was fed. Animals were killed after 28 days of feeding, and plasma and liver cholesterol and liver total lipid determined. Observations are summarized in table 2. Liver cholesterol values were significantly lower in rats fed the pectin-containing diet on alternate days than in those fed the basal ration. Liver total lipid was also lower in rats fed the pectin-containing diet. Plasma cholesterol levels in both groups were appreciably lower than values in earlier experiments including those of rats fed the cholesterol-free basal ration. Even under these conditions plasma cholesterol levels were lower, although not significantly so, for rats fed the pectin-containing ration. The inhibitory effect of pectin on the increment in liver cholesterol of cholesterol-fed rats occurred even though pectin and cholesterol were fed on alternate days.

DISCUSSION

Both citrus and apple pectin, fed at a 5% level in the diet, largely counteracted the increase in plasma and liver cholesterol and liver total lipid induced by cholesterol feeding in the rat; other roughage or bulk-forming materials such as cellulose, agar, sodium alginate, protopectin or calcium

[6] The protopectin was prepared by air-drying finely ground citrus albedo which had been dehydrated with isopropyl alcohol.

[7] Subsequent findings indicate that a supplement of citrus pectin N.F. at a 10% level in the diet was even more active in this respect. Even at the 10% level of supplementation, however, liver cholesterol and total liver lipid values were not reduced to those of rats fed the basal cholesterol-free ration.

silicate, when fed at a comparable level, showed no activity in this respect. The effects of pectin were more marked at the 5% than the 2.5% level of feeding. Attempts to identify an active component or fraction were unsuccessful. None of the pectin derivatives such as pectic acid, polygalacturonic acid or galacturonic acid exhibited any activity; nor was protopectin (a material from which pectin is derived commercially) active even when fed at a level of 15% of the diet. In man, Keys et al. ('59) have also reported that pectin is active in reducing hypercholesterolemia.

No data are available as to the mechanism whereby pectin exerts its cholesterol-lowering effect. Lin et al. ('57) have reported that pectin reduced the apparent absorption of cholesterol from the intestinal tract of rats, an observation which could account, at least in part, for the results obtained in the present experiment. Preliminary observations in this laboratory, however, indicate that the cholesterol content of the feces of rats fed a purified diet supplemented with 1% of cholesterol and 5% of pectin N.F. did not differ significantly from that of rats fed a similar diet supplemented with 1% of cholesterol alone. Present observations that pectin inhibited the increase in liver cholesterol following cholesterol feeding, even when fed on alternate days with cholesterol, suggest that more is involved than the simple tying up of cholesterol (possibly by the formation of a nonabsorbable pectin-cholesterol complex). Another possibility is that pectin induces changes in the intestinal flora which result in greater degradation of cholesterol, thereby leaving less of this material available for absorption. The failure of sulfasuxidine and streptomycin to modify the effects of pectin administration, however, raises doubt as to the validity of this hypothesis.

SUMMARY

Increased plasma and liver cholesterol and liver total lipid induced by cholesterol feeding in the male rat was largely counteracted by the concurrent feeding of either apple pectin or citrus pectin N.F. at a 2.5% level and more so at a 5% level in the diet. Other roughage or bulk-forming materials such as cellulose, agar, sodium alginate, protopectin or calcium silicate, or the pectin derivatives, pectic acid, polygalacturonic acid and galacturonic acid, when fed under comparable conditions, showed no such activity. The cholesterol-lowering effect of pectin was not decreased by the concurrent feeding of 1% of sulfasuxidine and 0.05% of streptomycin in the diet, or when the pectin and cholesterol were fed on alternate days.

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Effects of Dietary and Intraperitoneal Excess of L-Lysine and L-Leucine on Rat Pregnancy and Offspring[1]

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The metabolic mechanisms of growth retardation in the growing rat produced by experimental dietary excess of amino acids have been loosely categorized (Harper, '58) as amino acid imbalances, antagonisms and toxicities. Amino acid imbalance has been defined (Harper, '59) as "an adverse effect which can be prevented by supplementing the diet with a relatively small amount of the most limiting amino acid or acids." Adverse effects resulting from antagonism or toxicity are differentiated from imbalance by their failure to respond to supplementation with small amounts of the most limiting amino acid. Amino acid antagonism has been demonstrated (Harper et al., '55) when excess dietary L-leucine was added to the diet of low protein fed weanling rats. The growth retardation observed has been postulated to result from an antimetabolite effect of excess L-leucine inhibiting the utilization of structurally related isoleucine for protein synthesis. Amino acid toxicity was first reported 30 years ago (Lewis, '25). Subsequently growth retardation and other adverse effects have been observed following feeding of excessive amounts of most of the indispensable and many of the dispensable amino acids.

This communication reports the results of experiments devised to explore the effect of amino acid toxicity on rat pregnancy and offspring.

EXPERIMENTAL

CF Wistar strain rats weighing 240 to 260 gm were used. Pregnant animals were kept two in a cage and allowed water ad libitum. The day following the night of mating is considered day 1 of gestation.

The experimental diets were prepared by thoroughly mixing 30 gm of L-leucine or L-lysine with 270 gm of either a low-protein (9% casein) or optimum-protein (27% casein) powdered diet. In each of these mixtures the added amino acid represented 10% of the total diet by weight. Diets with 25% excess amino acid were prepared in a similar fashion. The

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TABLE 1
Effect on rat pregnancy of optimum protein diet (27% casein) supplemented by excess L-lysine and L-leucine[1]

Group	Surviving litters[2]	Maternal weight[3] gain or loss	20-Day fetus weight
		gm	*gm*
L-Lysine, 10% excess[4]	11	+48.4 ± 3.2	4.4 ± 0.2
L-Leucine, 10% excess	12	+41.6 ± 2.4	4.5 ± 0.1
L-Lysine, 25% excess	6	+ 7.0 ± 4.1	3.4 ± 0.2
L-Leucine, 25% excess	8	+11.1 ± 2.9	3.6 ± 0.1
Control	15	+60.2 ± 3.2	4.6 ± 0.1

[1] Supplemented diet fed from 5th to 15th day of gestation.
[2] Number of surviving litters from 15 pregnant rats.
[3] Maternal weight from 5th to 20th day.
[4] Amino acid excess as percentage of total diet.

pregnant animals were allowed to feed from the experimental mixtures from the 5th to 15th day of gestation.

For intraperitoneal administration 1- to 3-ml suspensions of L-leucine or L-lysine in distilled water in varying concentrations from 20 to 30% were injected twice daily. These doses approximate the median lethal dose (Mld/50) for intraperitoneal amino acids in the rat (Gullino et al., '55). Control rats received intraperitoneal aliquots of distilled water. All animals were fed an optimum-protein diet with water ad libitum. As greater increments of the amino acid were introduced, it was necessary to concentrate the suspension, because increasing the dose by increasing the volume injected would have quickly progressed beyond peritoneal tolerance.

RESULTS

In table 1 are summarized the effect on litter survival, maternal weight gain and fetal weight of dietary challenge with a 27% casein base-protein diet containing either 10 or 25% of added L-leucine or L-lysine. An excess of 10% of amino acid produced only a slight decrease in litter survival and expected maternal weight gain. When the amino acid excess was increased to 25%, however, a more pronounced decrease in litter survival and maternal weight gain was observed. The L-lysine group was most severely affected with litter survival reduced to 40% and a maternal weight gain of 7.0 gm as compared with the control gain of 60.2 gm. Average fetal weights in the groups receiving a 10% excess of amino acid were not significantly affected. In the 25% group, however, average fetal weights were decreased by 24%.

The effects of amino acid excess on a 9% casein basal diet are shown in table 2. The decrease in litter survival and maternal weight gain were more marked but follow the same pattern observed in the 27% casein-base diet experiments. It is noteworthy, however, that average fetal weights were remarkably constant with both the 10 and 25% amino acid-excess diets. Moreover, though litter survival and maternal weight gain were severely reduced the average fetal weight was only 5 to 10% less than that of the control.

Since food consumption was not constant but decreased in direct proportion to the increase in amino acid distortion of the diet, an attempt was made to circumvent this variable by administering the amino acid intraperitoneally. Increasing the concentration of either amino acid produced a graded depression of litter survival and maternal weight gain (table 3). However, the average fetal weight of surviving fetuses on the 19th day was apparently unaffected.[2]

No gross fetal malformations were observed in any of the surviving litters in either the dietary or intraperitoneal experiments. Animals were not serially sacrificed to determine whether fetal deaths in the resorbed litters were due to developmental malformations.

[2] Whereas in the oral amino acid excess, food acceptance was impaired within 24 hours, in the intraperitoneal experiments food intake was not grossly disturbed until signs of toxicity were evident.

TABLE 2

Effect on rat pregnancy of low-protein diet (9% casein) supplemented by excess L-lysine and L-leucine[1]

Group	Surviving litters[2]	Maternal weight[3] gain or loss	20-Day fetus weight
		gm	*gm*
L-Lysine, 10% excess[4]	9	+ 1.3 ± 2.6	3.6 ± 0.1
L-Leucine, 10% excess	10	+ 5.6 ± 3.1	3.5 ± 0.1
L-Lysine, 25% excess	3	−26.4 ± 4.1	3.5 ± 0.2
L-Leucine, 25% excess	5	−31.3 ± 2.2	3.5 ± 0.1
Control	12	+40.9 ± 3.4	3.7 ± 0.1

[1] Supplemented diet fed from 5th to 15th day of gestation.
[2] Number of surviving litters from 15 pregnant rats.
[3] Maternal weight from 5th to 20th day.
[4] Amino acid excess as percentage of total diet.

TABLE 3

Effect on rat pregnancy of intraperitoneal administration of excess L-lysine and L-leucine

	L-Lysine					L-Leucine			Control
No. of pregnant rats	20	20	15	15	15	20	20	15	15
Amino acid injected,[1] mg	200	400	400	600	600	200	400	600	—
Amino acid concentration, %	20	20	30	20	30	20	30	20	—
Surviving litters at 19 days	18	16	5	6	0	19	7	7	14
Maternal mortality	0	1	8	5	15	0	11	7	0
Maternal weight[2] gain, gm	+60.4	+58.3	+16.1	+21.4	—	+48.8	+14.6	+12.4	+64.2
19-Day fetus weight, gm	1.3	1.3	1.2	1.3		1.2	1.2	1.3	1.3

[1] Intraperitoneal injections twice daily from 5th to 15th day of gestation.
[2] Maternal weight increment from 5th to 19th day.

DISCUSSION

Supplementing the diet of pregnant rats with toxic amounts of L-leucine or L-lysine results in a depression of litter survival and maternal weight gain. The severity of these effects depend on both the protein content of the basal diet and the amount of specific amino acid excess. Similar to the observations reported in experiments with young growing rats, pregnant rats are most severely affected when a low-protein diet is supplemented with excess amino acid and the degree of maternal toxic effect is directly related to the amount of excess amino acid. Average fetal weights of surviving litters, however, were unaffected by amino acid excess except when the 27% casein-base diet was supplemented with a 25% amino acid excess. Under these conditions diet acceptance was markedly inhibited so that total protein intake was a factor in fetal weight depression. It is noteworthy that the depressed fetal weights observed in this group were comparable to those in the low-protein experiments with either 10 or 25% supplementation. Average fetal weights in pregnant rats fed low-protein diets were unaffected by either amino acid excess or diet acceptance. These data suggest that although maternal weight gain and fetal resorption may be influenced by the effect of amino acid toxicity on maternal metabolism, the fetuses that persevere attain a relatively normal growth pattern consistent with the maternal total protein intake. The intraperitoneal experiments lend added support to this thesis since the average fetal weights of surviving fetuses were likewise unaffected by maternal challenge with Mld/50 amounts of amino acid excess.

SUMMARY

Pregnant rats were supplied with a dietary and intraperitoneal excess of L-lysine and L-leucine. Supplementing a low-protein (9% casein) or optimum-protein (27% casein) diet with 10 or 25% amino acid excess resulted in a decrease in litter survival and expected maternal weight gain. The weights of surviving fetuses, however, were relatively unaffected by the amino acid distortion of the diet. Intraperitoneal administration of suspensions of either amino acid gave similar results.

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Effect of Pyridoxine Deficiency on Fatty Acid Composition of Carcass and Brain Lipids in the Rat[1]

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Previous investigations (Sherman, '50; Witten and Holman, '52) regarding the fatty acid composition of the carcass lipids extracted from pyridoxine-deficient rats have been carried out with animals which had a double deficiency of linoleic acid and pyridoxine or which had received a limited supply of essential fatty acids from hydrogenated fats. Such investigations showed that in the fats of pyridoxine deficient animals there is a relatively greater percentage of arachidonic acid (Carter and Phizackerly, '51) and a higher degree of unsaturation in general (Quackenbush and Steenbock, '42) than in the fats of the rats supplemented with pyridoxine. Recently Brown ('60) has shown that rats fed corn oil as the source of dietary fat were better able to withstand the effects of pyridoxine deficiency than animals fed hydrogenated shortenings.

Although the "essential" fatty acids have been reported to have a sparing effect on pyridoxine (Sherman, '50; Witten and Holman, '52), little is known regarding the relative carcass fatty acid composition of pyridoxine-deficient and pyridoxine-supplemented animals which have been fed corn oil. The protective effects of corn oil noted by Brown ('60) undoubtedly resulted from the presence of large amounts of linoleic acid in the oil. It might be expected, therefore, that pyridoxine-deficient rats fed corn oil would exhibit no difference in the degree of unsaturation of their carcass fat when compared with supplemented controls. The possibility also exists that even when a pyridoxine-deficient animal has received adequate linoleic acid, it may, according to the postulation of Sherman ('50), spare unsaturated fatty acids and preferentially metabolize the saturated acids. In the present study, therefore, we attempted to determine which of these possibilities, if either, appeared to be correct. Furthermore, since Tower ('56) has suggested that studies of neural lipids in pyridoxine-deficient animals may prove important in the elucidation of the role of pyridoxine in neural lipid metabolism, an attempt was made to determine whether any gross changes in brain fatty acid composition appeared to be associated with pyridoxine deficiency.

EXPERIMENTAL

The basal diet used in all experiments consisted of glucose,[2] 680 gm; vitamin-free casein, 180 gm; salt mix, Wesson ('32), 40 gm; and corn oil, 100 gm per kg. Three grams of a water-soluble vitamin mix were added to each kilogram of feed. This mixture was composed of the following: (in grams) inositol, 45.5; choline chloride, 91; niacin, 4.5; thiamine, 0.2; riboflavin, 0.4; *p*-aminobenzoic acid, 0.136; and biotin, 0.19. Fat-soluble vitamins[3] were administered by dropper once per week.

Twenty female albino rats,[4] weighing 40 to 50 gm, were divided into 4 groups, and two groups were fed ad libitum. One of these groups received the basal diet plus

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

[3] Vitamin A, 160 I.U.; vitamin D, 1.6 μg; and vitamin E, 295 μg per rat per week.

[4] Holtzman Rat Company, Madison, Wisconsin.

1.5 mg of pyridoxine per gm of feed (vitamin B_6- or pyridoxine-supplemented group) and the other received the basal diet without pyridoxine (vitamin B_6- or pyridoxine-deficient group). In the remaining two groups the individual daily feed intake was noted and feeding was controlled so that the average feed consumption by the vitamin B_6-supplemented animals was essentially the same as that of the vitamin B_6-deficient animals. The deficient animals consumed an average of 5.9 ± 0.5 gm of feed per day, and the pyridoxine supplemented animals consumed an average of 6.0 ± 0.6 gm per day. The rats were caged individually and were weighed each week.

After 10 weeks, feed was withdrawn and the rats were sacrificed 12 hours later by anesthetization with chloroform. At the termination of the experiment the rats which had received the vitamin B_6-deficient diet showed symptoms of a severe vitamin B_6 deficiency characterized by loss of weight, acrodynia, capillary fragility, and in the case of several rats, convulsions.

Immediately after death two or three brains from each group were pooled and extracted several times by homogenizing with 2:1 chloroform-methanol mixture. The last extract was warmed on a steam bath to insure breakdown of lipoproteins. The combined extracts were washed to remove nonlipid material according to the method of Folch et al. ('51). The washed chloroform-methanol extracts were concentrated by evaporation under vacuum and poured into 4 times their volume of ice-cold acetone containing 1 ml per 100 ml of a 5% magnesium chloride solution in 94% ethanol. This procedure removes acetone-soluble cholesterol and precipitates the remainder of the lipid. The precipitated lipid was filtered off under N_2, washed several times with acetone-magnesium chloride and dried under vacuum. The cholesterol was obtained from the acetone filtrate as the digitonide, washed, dried and weighed. In order to retrieve any lipid which may have dissolved in the acetone, the solvent washings from the digitonide were collected, evaporated to dryness and the residue was added to the precipitated lipid. Only small amounts of nonsterol lipid appeared to have dissolved in the acetone and the precipitated lipid had a negative reaction to the Liebermann-Burchard test for 3 β-ol sterols. The dried lipid was subjected to methanolysis by refluxing for 24 hours with dry methanol which had been saturated with hydrochloric acid gas and diluted to 6N. As an antioxidant during methanolysis 1 ml of 0.1% hydroquinone solution in dry methanol was added per 5 ml of hydrolyzing solution (Bottcher et al., '59). The methyl esters were removed from the solution by 4 extractions with diethyl ether. The combined ether extracts were washed free of mineral acid, dried, and the solvent was removed under vacuum.

The nonhydroxylated (normal) methyl esters were separated from the hydroxy methyl esters by chromatography according to the method described by Kishimoto and Radin ('59). The columns used had a 1.8-cm I.D., and 15 gm of Florisil[5] was used per 100 to 200 mg of methyl esters. The flow rate was 500 to 600 ml per hour.

After excision of the brains, the intestinal tract was removed from the carcass in order to insure that there would be no contamination of the carcass lipid with dietary fat. The individual carcasses were then transferred to large beakers and digested with 50% hydrochloric acid. The beaker and contents were warmed in a steam cabinet for 24 hours; the fat floated on the surface and was readily removed by extraction with petroleum ether (Skellysolve B) and ether. The combined extracts were dried over anhydrous sodium sulfate and freed from solvent under vacuum. The carcass lipids thus obtained were saponified using 1N alcoholic potassium hydroxide solution. The nonsaponifiable material was removed by extracting the soap solution three times with ether. The soaps were acidified with hydrochloric acid, and the free fatty acids were obtained by extracting the solution 4 times with ether followed by evaporation of the dried solvent under vacuum. The methyl esters were obtained by refluxing the fatty acids for three hours with the methanol-hydrochloric acid solution, followed by ex-

[5] Floridin Company, Tallahassee, Florida.

traction with ether and removal of the dried solvent under vacuum. All procedures were carried out quantitatively.

Gas chromatographic analysis of the methyl esters was carried out using a 10-ft. diethylene glycol succinate (DEGS) column with a ¼-inch O.D. The instrument used was a Model 90C Aerograph with thermal conductivity cells as detectors and a Daystrom-Weston recorder with 1 mv full scale sensitivity. Helium was used as the carrier gas at a flow rate of 30-ml per minute. All analyses were carried out at temperatures between 215 and 225°C. Identification of peaks of the unknowns was made by comparison of retention times with known standards, use of internal standards, and in cases where no standards were available, use was made of the "carbon number" as suggested by Woodford and van Gent ('60). Quantitative determinations were made on the basis of peak area measured with a planimeter.

The α-hydroxy methyl esters were analyzed by gas chromatography using the same instrument, but the column used was a 10-ft. silicone rubber column of ¼ inch O.D. The temperature of all runs was 240°C and the helium flow rate was 45 ml per minute. The major α-hydroxy methyl esters eluted were the C_{22}, C_{23}, and C_{24} esters. Small amounts of the C_{18} and C_{20} esters were also observed. The only unsaturated α-hydroxy acid separated under these conditions was the C_{24} (oxynervonic) acid. The C_{18}, C_{20}, C_{22}, and C_{23} unsaturated α-hydroxy acids had coincident retention times with the saturated fatty acids of the same chain length. The peaks obtained by chromatography of these esters were markedly skewed and therefore difficult to measure. This difficulty was largely overcome, however, when the methyl esters of the α-hydroxy methyl ethers, rather than the esters containing the free hydroxyl group, were chromatographed. The methyl ethers were prepared by the method described by Kishimoto and Radin ('59) and the yields obtained were between 96 to 98%.

RESULTS AND DISCUSSION

Carcass fatty acids. Under conditions of ad libitum feeding, the carcass fat of pyridoxine - deficient animals contained 9.72% of stearic and 25.41% of linoleic acid, whereas fat from the pyridoxine-supplemented rats contained 3.74% of stearic and 30.96% of linoleic (table 1). These differences were significant at the 0.1 and 1.0% levels, respectively. The Wijs iodine number of fat from the vitamin B_6-deficient group was 10 units less than that from the vitamin B_6-supplemented rats, thus confirming that fat from deficient animals was more saturated.

Dermal symptoms of pyridoxine deficiency and significant growth depression appeared in both groups of pyridoxine-deficient rats after diets were supplied for three weeks. The dermal symptoms became severe and convulsions were observed after 10 weeks on the dietary regimen. In agreement with reports by others (Sherman, '50) the body weight gain of the vitamin B_6-deficient animals was significantly less than that of the controls Deficient animals gained only 60.2 ± 16.5 gm during the experimental period, whereas the vitamin B_6-supplemented rats gained 167.7 ± 26 gm, a significant difference at the 0.1% level. As previously reported by Beare et al. ('53), body fat was significantly diminished in the vitamin B_6-deficient animals, which in the present study had 5.5 ± 1.1% of fat in the carcass compared with 14.8 ± 3.0% in the rats supplemented with vitamin B_6 ($P = 0.01$). When these differences in the quantity of carcass fat were taken into account and the amounts of fatty acids were calculated on the basis of milligrams of fatty acid per 100 gm of body weight, it was found that the vitamin B_6-supplemented animals contained more of all the fatty acids including stearic acid (table 1).

When the feed consumption of both the vitamin B_6-deficient and vitamin B_6-supplemented animals was the same, somewhat different results were obtained. In this case only the stearic acid content of the carcass fat of deficient animals was increased significantly (table 1). The decrease in the palmitoleic acid content is of rather doubtful significance ($P = 0.05$). Furthermore, the increase in the stearic acid content to 6.97% in vitamin B_6-deficient rats in contrast with 5.57% in the vitamin B_6-supplemented

TABLE 1

Carcass fatty acid composition of pyridoxine-deficient and pyridoxine-supplemented rats

Fatty acid	Ad libitum feeding					Controlled feeding				
	Vitamin B_6-deficient	Vitamin B_6-supplemented	P[1]	Vitamin B_6-deficient	Vitamin B_6-supplemented	Vitamin B_6-deficient	Vitamin B_6-supplemented	P	Vitamin B_6-deficient	Vitamin B_6-supplemented
	%	%		*mg/100 gm body weight*		%	%		*mg/100 gm body weight*	
Below C_{14}	traces	traces	—	—	traces	traces	—	—	—	—
C_{14} Myristic	1.84 ± 0.45[2]	1.80 ± 0.33	—	101	266	1.60 ± 0.10	1.75 ± 0.17	—	72	91
C_{16} Palmitic	26.61 ± 0.82	26.35 ± 1.73	—	1464	3900	25.92 ± 0.31	26.94 ± 0.98	—	1166	1401
C_{16} Monoenoic	4.23 ± 0.57	4.95 ± 0.81	0.20	233	733	4.53 ± 0.49	5.17 ± 0.25	0.05	204	269
C_{18} Stearic	9.72 ± 1.52	3.74 ± 0.14	0.001	534	554	6.97 ± 0.60	5.57 ± 0.34	0.01	314	290
C_{18} Monoenoic	29.72 ± 1.05	31.11 ± 1.57	0.20	1634	4604	35.25 ± 1.59	36.94 ± 0.92	0.10	1586	1921
C_{18} Dienoic	25.41 ± 0.88	30.96 ± 2.0	0.01	1400	4582	24.84 ± 2.22	23.50 ± 0.98	0.30	1118	1222
C_{20} Tetraenoic	0.5	0.5	—	28	74	0.3	0.3	—	14	16

[1] P value calculated from the "t" test.
[2] Standard deviation of the mean.

rats is appreciably smaller than the increase observed when ad libitum feeding was permitted. This difference, however, is still significant ($P = 0.01$). The iodine number of fat from B_6-deficient animals was only 1.0 unit less than the iodine number of fat from B_6-supplemented rats. Thus, no appreciable difference was observed in the degree of unsaturation of carcass fat between the two groups. Pyridoxine-deficient animals gained 49.2 ± 8.3 gm during the experimental period in contrast with 76.1 ± 3.6 gm gained by the supplemented rats, a significant difference at the 0.1% level. The carcasses of the deficient animals yielded 4.5 ± 0.65 gm of fat, whereas the controls contained $5.2 \pm 1.0\%$, a small and statistically insignificant difference. The latter observation is in agreement with the observation of Williams et al. ('59) who reported that the particular level of dietary fat determines whether a difference in the percentage of body fat appears between deficient rats and their controls. Since the difference in carcass fat is small, the higher stearic and lower palmitoleic acid contents of the fat from vitamin B_6-deficient animals are reflected when they are expressed in terms of milligrams per 100 gm of body weight. Deficient animals contained 314 mg of stearic and 204 mg of palmitoleic, whereas the controls contained 290 mg of stearic and 269 mg of palmitoleic acid per 100 gm of body weight (table 1).

A synergistic relationship between pyridoxine and the essential fatty acids has been shown to exist in the maintenance of dermal tissue integrity and in the promotion of growth. One cannot completely replace the other, however, and each must therefore be regarded as an indispensable factor (Sherman, '50). Previous investigations regarding qualitative and quantitative changes in the carcass fat composition of pyridoxine-deficient animals have been carried out on animals which were also deficient in or had received a limited supply of essential fatty acids (Witten and Holman, '52; Quackenbush and Steenbock, '42; Sherman, '50). The results of the present study indicate that when pyridoxine deficiency alone was observed and when the deficient animals and their supplemented controls received

the same amount of feed, the differences in composition of the carcass fat were not great. However, increased stearic acid and decreased palmitoleic in the fat of deficient animals was noted, indicating that in pyridoxine deficiency some aspects of fat utilization are affected. Information derived from the present investigation cannot explain the exact nature of these effects. It can be said, however, that since pyridoxine is apparently not required for the synthesis of palmitoleic from palmitic acid (Bloomfield and Bloch, '60) nor for the synthesis or oxidation of saturated fatty acids (Bloch, '60), the effect presumably is of a secondary nature. It has been previously suggested by Beaton et al. ('54) that the defect in fat metabolism associated with pyridoxine deficiency is a consequence of the impairment of energy yield. The present results tend to confirm this view. The conversion of a saturated long chain fatty acid such as palmitic acid to its monounsaturated analogue requires molecular oxygen, ATP and coenzyme A (Bloomfield and Bloch, '60). Furthermore, pyridoxine deficiency, because of its effects on amino acid metabolism, has a profound effect upon the metabolic interchanges which occur in the tricarboxylic acid cycle (Tower, '56).

Under conditions of ad libitum feeding, the increase in stearic acid in deficient rats was enhanced and a significantly lower linoleic acid content of the carcass fat was noted. The latter observation is no doubt a result of the higher food intake and hence higher intake of corn oil by the pyridoxine-supplemented animals. However, on neither feeding regime was an increase of degree of unsaturation of carcass fat from deficient animals observed. No attempt was made to examine any tissue for its hexaenoic acid content, although measurements were made for arachidonic acid content and in all cases the amounts were small and contributed little to the total iodine number of the fat. It appears, therefore, that the rat tends to spare unsaturated fatty acid only when it is deficient in both pyridoxine and essential fatty acids.

Brain lipid fatty acids The percentage composition of the normal fatty acids from total brain lipids are reported in table 2. An examination of the results re-

TABLE 2
Nonhydroxylated fatty acid composition of brain lipid from pyridoxine-deficient and pyridoxine-supplemented rats

Fatty acid[1]	Ad libitum feeding		Controlled feeding	
	Vitamin B_6-deficient	Vitamin B_6-supplemented	Vitamin B_6-deficient	Vitamin B_6-supplemented
	%	%	%	%
Below C_{14}	1.0[2]	0.9	0.9	1.1
C_{14} Myristic	0.9	0.8	0.9	1.0
C_{15} Saturated	0.9	0.9	1.7	1.6
C_{16} Palmitic	29.9	28.8	27.4	27.0
C_{16} Monoenoic	1.0	1.0	2.0	1.4
*C_{17} Saturated	0.4	0.4	0.8	0.8
*C_{17} Monoenoic	1.1	1.1	0.9	0.8
C_{18} Stearic	30.6	29.6	31.7	31.8
C_{18} Monoenoic	19.1	21.3	21.6	20.6
C_{18} Dienoic	0.3	0.5	0.4	0.4
C_{19} Saturated	trace	0.3	0.2	0.4
C_{20} Arachidic	1.3	1.0	1.1	1.1
*C_{18} Trienoic + C_{20} monoenoic	2.6	2.6	2.5	2.3
*C_{20} Dienoic	0.3	0.1	0.3	0.2
C_{20} Tetraenoic + C_{22} behenic	2.3	2.8	2.7	2.6
*C_{22} Unsaturated	0.4	0.3	trace	0.6
*C_{22} Unsaturated	0.8	0.9	1.3	1.1
*C_{24} Saturated + C_{22} Unsaturated	3.5	3.1	2.9	2.9
*C_{22}, C_{24} Unsaturated	3.0	3.2	2.1	2.4

[1] An asterisk beside a fatty acid indicates that the identification is tentative.
[2] Mean of two pools of two or three brains each.

TABLE 3

α-Hydroxy fatty acid composition of brain lipid from pyridoxine-deficient and pyridoxine-supplemented rats

α-Hydroxy fatty acid	Ad libitum feeding		Controlled feeding	
	Vitamin B_6-deficient	Vitamin B_6-supplemented	Vitamin B_6-deficient	Vitamin B_6-supplemented
	%[1]	%	%	%
C_{22} Saturated + unsaturated	19.83[2]	22.2	20.5	22.6
C_{23} Saturated + unsaturated	6.99	7.8	5.8	7.0
C_{24} Unsaturated	13.77	15.4	12.2	11.5
C_{24} Saturated	59.08	54.8	61.6	59.1

[1] Percentage of total α-hydroxy fatty acids.
[2] Mean of two pools of two or three brains each.

veals that there appear to be no changes in composition of the normal acids associated with pyridoxine deficiency. All of the methyl esters eluted are not reported in table 1; however, those listed represent the major acids which could be positively or tentatively identified and which were eluted sufficiently well to give defined measurable peaks. Other peaks including those corresponding to C_{25} and C_{26} saturated fatty acids were noted but amounts of these were very small.

The α-hydroxy fatty acid composition of brain lipids is reported in table 3. Again no difference appears to be associated with pyridoxine deficiency in the composition of this fatty acid fraction. In the supplemented rats which were fed ad libitum there appeared to be a slight increase in the C_{24} unsaturated and a corresponding decrease in the C_{24} saturated fatty acids. Although these results cannot be treated statistically, such a change might be expected since these rats had a higher intake of corn oil and were more developed in general. The α-hydroxy fatty acids in brain are mainly associated with the cerebroside fraction and the amounts of fatty acids observed in the present study agree with those reported by Kishimoto and Radin ('59) who determined the amounts of α-hydroxy fatty acids in pure cerebrosides from rat brain.

Horwitt et al. ('59) have reported changes in brain lipid fatty acids which were brought about by means of changes in dietary fat. No reports have appeared in which similar changes were brought about by deficiency of a micronutrient. However, since the fatty acids from total brain lipid only were examined, it cannot be concluded from the present study that such changes do not occur in the pyridoxine-deficient animals. These fatty acids are associated with many individual lipid fractions; hence subtle changes in the fatty acid composition of a particular lipid fraction such as a glycerophosphatide or sphingomyelin may not be noted. Examination of the fatty acids from purified individual lipids should be conducted before it could be concluded that pyridoxine deficiency has no effect on neural lipid metabolism.

SUMMARY

Female rats were fed diets containing 10% of corn oil (with or without added pyridoxine). Signs of severe pyridoxine deficiency were observed after 10 weeks in rats that received the diets deficient in pyridoxine. These animals and the pyridoxine-supplemented controls were then sacrificed and the fatty acid composition of the carcass and brain lipids was determined.

Under ad libitum feeding conditions the percentage of stearic acid was higher and of linoleic acid lower in carcass fats of the deficient animals compared with fats of the supplemented controls. When the feed consumption of both groups was the same, the carcass fat of the deficient animals contained a higher percentage of stearic acid and a lower percentage of palmitoleic acid than the carcass fat of the controls, but the degree of unsaturation of the fat from rats in both groups was the same. These results were interpreted to indicate that there is no sparing of unsaturated fatty acids and little change in fatty acid composition of carcass fat in pyridoxine

deficiency unless the rats are also deficient in essential fatty acids. It is suggested that the effects of pyridoxine deficiency on fat metabolism are of a secondary nature mediated by changes in protein and carbohydrate metabolism.

No differences in normal and α-hydroxy fatty acid composition of brain lipids from pyridoxine-deficient and pyridoxine-supplemented rats were observed. If pyridoxine deficiency causes changes in neural lipid metabolism, they are not sufficiently marked to be observable when fatty acids from total brain lipid are examined.

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