THE JOURNAL OF NUTRITION®

OFFICIAL ORGAN OF THE
AMERICAN INSTITUTE OF NUTRITION

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VOLUME 95

MAY – AUGUST 1968

PUBLISHED MONTHLY BY
THE AMERICAN INSTITUTE OF NUTRITION
BETHESDA, MARYLAND

THE JOURNAL OF NUTRITION ®
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GRACE MACLEOD

Grace MacLeod

— A Biographical Sketch

(August 6, 1878 — November 16, 1962)

Grace MacLeod, well known for her research in the field of energy metabolism, spent 25 years of her professional life (1919–1944) in active service at Teachers College, Columbia University. My acquaintance with her dates back to 1926 when I joined the staff as an assistant in nutrition. At that time Mary Swartz Rose and Grace MacLeod, devoted friends and associates, had established a strong program for the training of students in nutrition from all parts of the world. These two women with quite different background training and personalities complemented each other in many ways and worked together, pooling their experience and abilities in a common effort to provide the best possible training for the students. This harmonious relationship continued throughout the years until the retirement of Mary Swartz Rose in 1940 and her death in 1941. It was my privilege to work closely with this illustrious team in the further development of the nutrition program at Teachers College. I knew Grace MacLeod as a friend, student, associate, co-author, and companion on the trail in a walking club. Surely, one of life's greatest gifts is a friendship such as this.

In 1882, a little Scotch lassie by the name of Grace MacLeod, stepped off a steamer from Scotland with her mother, Jessie MacGregor MacLeod. They had come to the United States to join her father, Joseph MacLeod, who had arrived in advance to establish himself here before sending for his family, as was the custom in those days. Grace was born in Rothesay, Scotland, on the Isle of Bute, on August 6, 1878, and was four years old when she arrived in the United States. Who could foresee that this little girl was destined to become an outstanding professor in a great university. In all the years ahead she never ceased to be proud of her Scottish inheritance.

The family settled in Cambridge, Massachusetts where Grace had the advantage of a splendid early education in the public schools. Her parents were desirous of providing every opportunity for the best possible education for their family which undoubtedly prompted their moving to the United States. Her special interest and aptitude for science was evident when she reached high school. Recognizing this, her high school teacher encouraged her to enter the Massachusetts Institute of Technology and major in chemistry. Competition was keen at the Institute but she managed to earn the Marion Hovey Scholarship which she held during the second, third, and fourth years. Ellen H. Richards was one of her professors and was most interested in her. Grace told the story of how Mrs. Richards helped her to get certain books and materials she needed. When Grace tried to repay her for these, Mrs. Richards simply replied, "You cannot repay people who do kind things for you, but you can pass along the kindness." Grace never forgot this.

She received her bachelor's degree from the Massachusetts Institute of Technology in 1901. Her first teaching position was at the Mt. Hermon School for Boys in Massachusetts. She often referred to her enjoyment in teaching the boys in this school and spoke with pride when she heard of the distinguished later achievements of a number of them. In 1903, she accepted a position in the Technical High School in Springfield, Massachusetts, where she continued to teach for the next seven years.

In 1910, she was appointed an instructor in chemistry and physics at Pratt Institute in Brooklyn, New York, a position which she held for seven years. Her former Pratt students still remember her as an excellent teacher, an absolute perfectionist, demanding the most from each of them

and at the same time having the utmost respect of each member of her class. The atmosphere of her laboratory and classroom has been described as formal but her manner was bright and cheerful. The relationship with her students was impersonal but they considered themselves fortunate to have had her as an instructor.

While teaching at Pratt Institute, she took advantage of the opportunity to take advanced courses in chemistry at Columbia University in New York City. Her first interview was with Dr. Henry C. Sherman, who suggested that she discuss the question of registering for the Nutrition Seminar given at Teachers College, Columbia University, with Dr. Mary Swartz Rose. This course was conducted jointly by Professors Sherman and Rose and at that time much of the literature in chemistry and nutrition was published in German. Grace MacLeod had an excellent background in German and was able as a student to make a real contribution to the course. This was the inspiration for her lifetime interest in nutrition as a science. She received her Master's Degree from Columbia University in 1914, and her Doctorate in 1924.

In 1917, she became the assistant editor of the *Journal of Industrial and Engineering Chemistry*, a position which she referred to with great pride and which she held until 1919 when an instructorship in nutrition was established at Teachers College. Dr. Sherman persuaded her to leave her editorial work with the Journal and accept the position as an instructor in nutrition, to work with Professor Rose. This marked the beginning of a relationship which offered a rich opportunity for the nutrition students who were fortunate to study with them. Following the completion of her Doctor's degree in 1924, she was promoted to assistant professor. She told the story of the thoughtfulness of Dean James Russell in having her promotion to professorship waiting on her desk when she returned from the commencement exercises at which time her doctoral degree had been conferred. As the years advanced she became an associate professor of nutrition and eventually a professor of nutrition, a position which she held until her retirement in 1944. At that time she was given the title of Professor Emeritus of Nutrition. In 1940, at the retirement of Professor Mary Swartz Rose, Dr. MacLeod assumed the chief responsibility for the nutrition program at Teachers College.

From the time of her father's untimely death Grace was the head of the family. This responsibility postponed the earning of her doctorate and accounts for her working for it along with her youngest sister, Florence. Grace was very close to her mother and they lived together until her mother's death in the mid-thirties.

Grace was devoted to and extremely proud of the accomplishments of her sisters — Sarah, who held the position of home economist in a Cleveland bank, Society for Savings, and published a column on budgeting in the *Cleveland Press*, and Florence, who was professor of nutrition and head of the nutrition department at the University of Tennessee. Grace MacLeod was conscientious, thorough, patient and a skillful research worker. She spent long hours in the laboratory, starting early in the morning to assist students with their problems, neither the students or the professor paying attention to the time it was taking. "Clock watchers" were not in vogue in those days. However, stop watches for the timing and testing of the equipment were always at hand. The achievement of her students was uppermost in her mind.

Grace MacLeod's most active research was in the field of energy metabolism. Her dissertation, published in 1924, entitled, "Studies of the Normal Basal Energy Requirements," set the stage for many research studies on energy metabolism to be carried out in the Nutrition Laboratory at Teachers College, Columbia University. From 1922 to 1928, in addition to her responsibilities at Teachers College, she served as cooperating investigator of the Nutrition Laboratory of the Carnegie Institution located in Boston. In 1925, she published a paper with E. E. Crofts and F. G. Benedict on the basal metabolism of oriental women living in the United States. Over the years she sponsored a number of doctor's degree projects on the energy metabolism of children, including basal metabolism studies of children of different ages, the energy expenditure for certain activities

and mechanical efficiency studies. She was responsible for setting up the respiration chamber in the Nutrition Laboratory and other special equipment used for this research. She also published a number of research papers with Mrs. Rose and others, dealing with other areas of research including the supplementary values of foods, calcium utilization, vitamin B, availability of iron, and protein utilization. These papers were published in the *Journal of Biological Chemistry, Journal of Nutrition, Journal of the American Home Economics Association* and the *Journal of the American Dietetic Association*. In addition she wrote semi-popular articles and concise reviews of the current literature for which she had a special talent.

Following the death of Mary Swartz Rose, Dr. MacLeod assumed the responsibility of the fourth revision of "Rose's Foundations of Nutrition" with Clara Mae Taylor as co-author, which was published in 1944. She was also co-author with Taylor of the fifth edition of "Foundations of Nutrition," published in 1956 and of the fifth edition of "Rose's Laboratory Handbook for Dietetics," published in 1949.

During World War II, she was Chairman of the Food and Nutrition Council of Greater New York, which organization she helped to establish in the twenties. She also served as chairman of the Nutrition Committee of the Greater New York Area, serving on the National Nutrition Program of the War Food Administration. She was chairman of the Nutrition Advisory Board, New York Chapter of the American Red Cross; member of the Advisory Committee of the East Harlem Health Center; member of the Executive Committee of the New York City Food and Nutrition Program and co-chairman of the Planning Committee; member of the Nutrition Advisory Committee for the Henry Street Visiting Nurse Service; and member of the Advisory Board of the Interstate Dairy Council of Philadelphia.

Dr. MacLeod was active in many professional organizations. She joined The American Chemical Society in 1916. She was a charter member of the American Institute of Nutrition and an associate editor of the *Journal of Nutrition* for three years from 1936–1939. She continued to serve on the editorial board of the *Journal of Nutrition* from 1940–1945. She was a member of the Society of Biological Chemists, the Society for Experimental Biology and Medicine, the American Association for the Advancement of Science, the American Home Economics Association, the American Dietetic Association and served as a member of the editorial board of the *Journal of the American Dietetic Association* from 1940 until she retired. She was also a member of the Columbia University Chapter of Sigma Xi.

Dr. MacLeod was an excellent speaker and was frequently called upon to give a brief review of the current literature for the New York City community organizations. She had a special talent for making crisp, up-to-the minute reports of scientific papers. The community groups counted on her to do this for them. She served on many committees and her advice and counsel were greatly appreciated even though committee meetings were prolonged when she served on the committee because of her high standards of perfection in everything she undertook. On her retirement she gave up all of her associations with community organizations and withdrew from her chairmanships and memberships on advisory committees. The community workers missed her personally and felt the lack of her penetrating judgment and assistance.

The students at Teachers College considered Grace MacLeod a superb teacher, with a warm, friendly, and gracious personality, but with firm convictions and high standards of workmanship. She was meticulous about details and had a frank intolerance for anything classified as second-rate. She was popular with and highly respected by her students. On the top of her desk, she had the following quotation from William James, "No one sees further into a generalization than his own knowledge of details extends."

At the time of her retirement the nutrition students established a scholarship in her honor at Teachers College to show their deep appreciation of her as a teacher. Since then the Grace MacLeod Scholarship Fund has grown throughout the years and

has provided financial assistance to a number of students working for advanced degrees.

After Dr. MacLeod's retirement in 1944, she served as a consultant for a number of years on a United States Department of Agriculture cooperative project on the energy expenditure of children, conducted under the direction of Doctors Taylor and Pye in the Nutrition Laboratory at Teachers College.

Although Grace MacLeod lived in an apartment not far from the University during her years there and always walked back and forth, she actually reveled in the great out-of-doors. As a young woman she spent summer vacations hiking in the Black Forest in Germany and on the Appalachian Trail which she helped measure in Virginia. In 1919, she became a member of the Tramp and Trail Club of New York, a membership which continued 40 years. The long brown trail and woodland way shared with congenial friends was a source of great joy and inspiration to her. Resting on the trail she would often sing "Down in the valley, the valley so low, Hang your head over, hear the wind blow . . . ." She loved the birds and the wild flowers in the spring, and the bold reds and yellows of the autumn leaves. Hiking stimulated her and struggling over rocks and glens in snow, rain, or shine, was a joy and never too great a hurdle.

Many of her summer vacations were spent in her beloved Vermont with her mother and sisters except for a few summers when she traveled in Europe and the British Isles. The highlight of one of these trips was a visit to Dunvegan Castle, the home of the MacLeod clan of the Isle of Skye, in Scotland. While there she had the great privilege of visiting with Dame Flora MacLeod, the chief of the MacLeod clan who welcomed clansmen from all over the world. At a later date when Dame Flora was touring in the United States, Grace MacLeod gave a Scottish tea in her honor at the Columbia University Woman's Faculty Club, inviting many friends, her associates, and advanced students.

Grace MacLeod had a natural curiosity about the origin of things. Her conversation was sparkling, refreshing and full of interrogations. She stressed the importance of being consistent and yet in her personal life she was often quite inconsistent in a delightful way.

She had a genuine appreciation of classical music and thoroughly enjoyed playing the piano but had little time for it until after she retired. At that time she renewed her piano lessons, joining an informal piano class conducted by one of the professors in the Music Department at Teachers College. She practiced faithfully and attended the classes regularly for a number of years. Although she perfected her technique in playing selections from Bach, Chopin, Beethoven and other great composers, she played chiefly for her own pleasure and that of her most intimate friends. She also had a great enthusiasm for grand opera and subscribed for season tickets for the operas at the Metropolitan Opera House for years, sharing her tickets with students and friends when she was unable to attend.

In 1958, she closed her New York apartment, never to return to the great city which had been her home for so many years, and moved to Knoxville, Tennessee, to live with her sister Florence, who was head of the Nutrition Department at the University of Tennessee. She had long anticipated this pleasure. Here she was able to enjoy the great out-of-doors, trips to the moutains, the opportunity to continue her music, and to catch up on her reading which had always meant so much to her. She took great pleasure in her new associations and in seeing past professional friends who found their way to the University to give lectures, attend conferences, or just to make a visit. During her last 2 years, following a fall, she was confined to a wheel chair for periods of time but never lost her cheerful and courageous outlook.

In the summer of 1962 she journeyed with her sisters to New England, making what proved to be her last trip to her favorite countrysides. She passed away on November 16, 1962, after a very short illness at the age of eighty-four and was buried in the Cambridge Cemetery, Cambridge, Massachusetts, in the family plot.

It is the custom of the American Institute of Nutrition to honor the passing of one of their Charter Members by presenting a Resolution at the Annual Spring Meeting. Such a statement was presented by this author and it was entered in the Proceedings of the Annual Meeting in April, 1963.

Her students have held outstanding positions of leadership in universities, colleges, Government services, community agencies, and hospitals throughout the country and around the world. They will remember her dynamic qualities, her kindly interest in their welfare, and will think of her with deep affection and gratitude. She rejoiced in the achievements of her colleagues, students, and friends. "Let me pin a medal on you" was a frequent expression of congratulations and pleasure on hearing the good news of a task well done.

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Severe Zinc Deficiency in Male and Female Rats [1]

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ABSTRACT A severe zinc deficiency was induced in both male and female weanling rats by the use of a purified diet containing EDTA-treated soybean protein and elimination of zinc contamination from the environment. Signs of deficiency included extreme retardation of growth, immature hair or alopecia, and dermal lesions. These symptoms were completely reversed by supplementation with zinc. The concentrations of both ash and zinc were reduced in the femurs of zinc-deficient rats. Histological lesions were observed in certain tissues. There were no significant differences in the activities of the liver enzymes, pyridoxal phosphokinase, glutamic dehydrogenase, or lactic dehydrogenase in zinc-deficient animals as compared with controls.

During the course of studies of the effects of nutritional deficiencies on reproduction (1, 2), it was necessary to demonstrate that a specific and extreme zinc deficiency could be induced in the rat. Although severe states of zinc deficiency have been reported in swine (3), chicks (4), and turkey poults (5), only relatively mild zinc deficiencies have been noted in rats (6–12). Zinc deficiency severe enough to cause extremely marked growth retardation has not previously been reported for this species.

Earlier investigation in this laboratory demonstrated that when young female rats were fed a soybean diet with low zinc content, they showed signs of zinc deficiency and were unable to reproduce (13). Procedures were then developed to lower further the zinc content of the diet, and, especially, to reduce the sources of environmental contamination so that a severe zinc deficiency state could be induced in this species. The present paper reports the effects of this regimen in both male and female rats.

Zinc is known to be an essental component of a number of metalloenzymes, including lactic dehydrogenase and glutamic dehydrogenase (14). Zinc is also a preferred cofactor for pyridoxal phosphokinase (15), essential for the phosphorylation of pyridoxal. Since these enzymes are important in both energy and protein metabolism, their activities were measured in liver of zinc-deficient and control rats. The ash and zinc content of bone were also determined.

METHODS

Animals and diets. Weanling rats, both males and females, of the Sprague-Dawley strain (50 ± 5 g body weight), were maintained with a ration with the following percentage composition: isolated soybean protein,[2] 30.0; sucrose, 57.3; corn oil, 8.0; salts,[3] 4.0; and DL-methionine, 0.7. The zinc content of the soybean protein was reduced by treatment with the tetrasodium salt of ethylenediaminetetraacetic acid (Na_4EDTA), by a modification of the method described by Davis and co-workers (16).[4] Crystalline vitamins were given separately.[5] The basal zinc-free ration contained zero ppm of zinc as determined

Received for publication November 13, 1967.

[1] Supported in part by grant no. GB-2316 from the National Science Foundation and by Public Health Service Research Grant no. HD-01743 from the National Institute of Child Health and Human Development.

[2] ADM C-1 Assay Protein, Archer-Daniels-Midland Company, Cincinnati.

[3] Composition of basal salt mix: (in grams) $CaCO_3$, 600; $Ca(H_2PO_4)_2 \cdot H_2O$, 220; K_2HPO_4, 650; NaCl, 336; $MgSO_4 \cdot 7H_2O$, 250; $FeSO_4 \cdot 7H_2O$, 50; $MnSO_4 \cdot H_2O$, 4.6; KI, 1.6; $CuSO_4 \cdot 5H_2O$, 0.6.

[4] The soybean protein was suspended in approximately 8 volumes of tap water and adjusted to pH 4.3. Na_4EDTA was added to the level of 5 g/kg of protein and the mixture was stirred for 30 minutes without heating. The slurry was allowed to settle and the supernatant was removed by decantation. The protein was treated twice with Na_4EDTA and washed 5 times with deionized water. After the final wash, excess water was removed by filtration on a Büchner funnel. The protein was dried at room temperature under circulating air currents.

[5] A mixture of crystalline vitamins in glucose, to which were added vitamin A palmitate, vitamin D_3 and α-tocopheryl acetate, was given 3 times each week in small glass dishes in amounts to provide the following intake in micrograms, per day: Ca pantothenate, 500; *p*-aminobenzoic acid and riboflavin, each 100; thiamine·HCl, pyridoxine, and nicotinic acid, each 300; menadione, 250; folic acid. 6; biotin, 2.5; vitamin B_{12}, 0.3; and choline chloride, 10 mg; inositol, 5 mg; α-tocopherol and ascorbic acid, each 1 mg; vitamin A, 150, and vitamin D, 15, IU each.

by x-ray fluorescence spectrometry, with an error in the method of ± 2 ppm.[6] Zinc-supplemented animals received the same basal diet except that zinc carbonate was added to the salt mix providing a total content in the diet of 1, 40, 60 or 100 ppm of zinc. One group of females and one group of males received the stock ration.[7]

In one experiment, a control group of weanling male rats was pair-fed with rats given the zinc-deficient diet. That is, they were given the control ration (containing 100 ppm of zinc) in quantities limited to those eaten the previous day by the deficient animals.

The effect of repletion with zinc was studied in severely deficient animals, both males and females. After receiving the diet for 4 or 7 weeks, deficient animals were given either a daily oral supplement of zinc sulfate solution equivalent to 1 mg of zinc per day or a single oral supplement of zinc sulfate solution equivalent to 1 mg of zinc followed by transfer to the control diet (100 ppm of zinc).

Rats were housed individually in stainless steel cages. Deionized water was freely available from Pyrex bottles with vinyl plastic stoppers and stainless steel mouthpieces. Food was provided in aluminum and stainless steel feed cups. Extreme care was taken to eliminate sources of zinc contamination in the environment as well as in the diet. For example, stainless steel, plastic, or glass equipment was used throughout, even for the storage of dietary ingredients; chemicals used for the salt mix were reagent grade and were selected for low heavy metal content; all equipment used was rinsed in deionized water; vinyl plastic stoppers were used on the water bottles instead of rubber stoppers (which have a high zinc content).

The animals were weighed 3 times weekly and were carefully examined for signs of deficiency. After 4 or 6 weeks, rats were killed for tissue analyses. Pyridoxal phosphokinase, lactic dehydrogenase, and glutamic dehydrogenase activities were assayed in the liver. Zinc and ash concentration were determined in the femur.

Analytical procedures. The rats were decapitated. The left hind leg and the liver were removed rapidly, frozen on dry ice, and stored at − 15° until they were analyzed. The leg was skinned, but surrounding muscle tissue was left intact during storage. At the time of analysis, the leg was thawed and the femur was separated from attached muscle and periosteum. The femur was dissolved in 1 ml concentrated nitric acid in a preweighed crucible, dried under an air current in a Pyrex glass drying chamber (17), and ashed in a muffle furnace at 450° for 24 hours. The residue was dissolved in 1 ml concentrated nitric acid, dried, and reashed. The final white ash was resolved in 2 N hydrochloric acid resulting in a clear bone ash extract which was analyzed for zinc content.

Liver samples were homogenized in a Potter-Elvehjem type of mechanical grinder in 10 volumes of appropriate fluid for 1 minute at 0°. Homogenates were prepared in 0.25 M sucrose solution for the pyridoxal phosphokinase assays, in 0.14 M KCl for lactic dehydrogenase assays, and in deionized water for glutamic dehydrogenase assays. All homogenates were centrifuged at 30,000 × *g* for 30 minutes at 0°. Enzyme activities were measured in the supernatant fraction. Because of the stability of the crude pyridoxal phosphokinase preparation (18), these tissue fractions were prepared in advance and stored at − 15° until assayed; tissue fractions for determination of dehydrogenase activities were prepared immediately before assaying.

Zinc content of tissues was determined by atomic absorption spectrophotometry using a Perkin-Elmer Model 303 spectrophotometer. Pyridoxal phosphokinase activity was determined by the McCormick et al. (15) modification of the Wada (19) method. Lactic dehydrogenase and glutamic dehydrogenase activities were assayed with the use of a Gilford recording spectrophotometer. Model 2000, by measuring the change in absorbance at 340 mμ resulting from the oxidation of reduced nicotinamide adenine dinucleotide at 25°. Lactic acid dehydrogenase activity was assayed in a Tris · HCl buffer, pH 7.4, with

[6] Subsequent analysis of the basal zinc-free diet by atomic absorption spectrophotometry has shown that this diet contained 0.20 ± 0.01 ppm of zinc.

[7] Commercial rat chow (Wayne Lab-Blox, Ft. Wayne, Indiana) ad libitum, powdered whole milk ad libitum, and crystalline vitamins as above.

sodium pyruvate as the substrate (20). Glutamic dehydrogenase was assayed in a Tris · HCl buffer, pH 7.4, containing adenosine diphosphate with ammonium sulfate and α-ketoglutarate as substrates (21). Protein was determined by the Lowry (22) method.

RESULTS

The body weights of both male and female rats receiving various levels of zinc are shown in figure 1. Extreme growth retardation was observed in rats fed the zinc-deficient diet containing 0 ± 2 ppm of zinc (no Zn diet). With 1 ppm of zinc added to the diet (low Zn diet) the growth of deficient rats was improved slightly but was still much lower than that of the controls. Rats fed the control diet (+ Zn diet) containing 40 ppm of zinc had growth rates slightly lower than those of stock-fed rats. However, control diets containing either 60 or 100 ppm of zinc resulted in growth that was not significantly different from that of stock-fed rats.

In addition to growth retardation, other signs of deficiency were noted. Deficient animals first displayed immature hair coats, fissures at the corners of the mouth, and scaly feet. As the deficiency progressed, these symptoms became more severe, and alopecia and dermal lesions appeared. In less severely affected rats the early symptoms were not observed, but hair loss was the first sign of deficiency (fig. 2A). The most severely affected animals did not develop alopecia but maintained the soft, immature coat of the weanling. At the height of the deficiency state, the rats were extremely emaciated, they had dermal lesions and edema around the eyes, the mouth, and on the feet (fig. 3), and an abnormal "kangaroo-like" posture (fig. 2B). Such animals died soon thereafter unless zinc supplementation was given. By the end of the fourth week, 9% of the deficient male rats were dead. Pair-fed controls showed none of these signs of zinc deficiency.

Study of daily vaginal smears showed that zinc-deficient females had abnormal

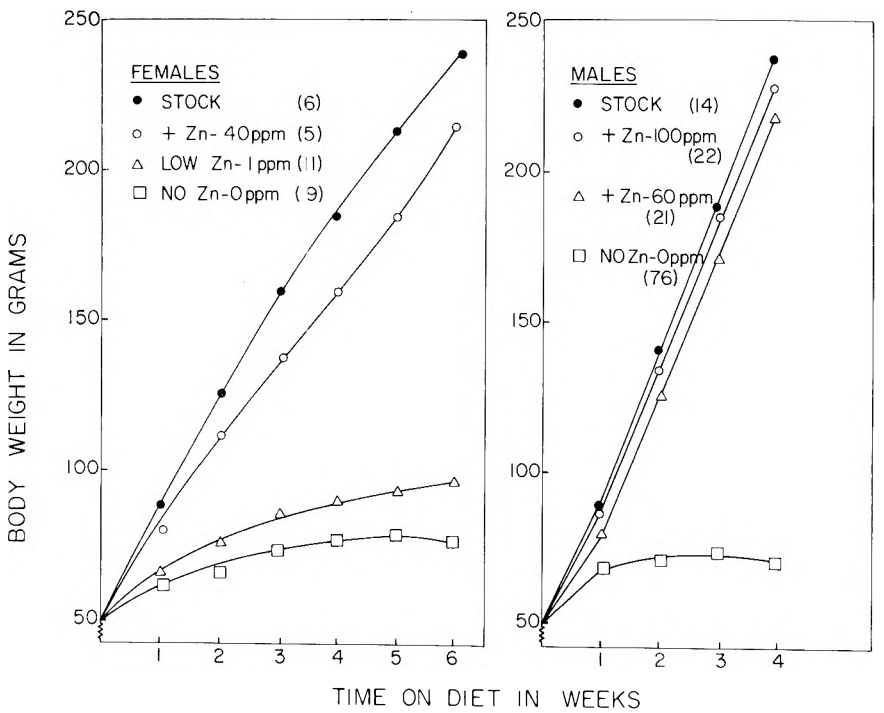

Fig. 1 Growth of male and female rats receiving various levels of dietary zinc. Numbers in parentheses represent number of animals in each group.

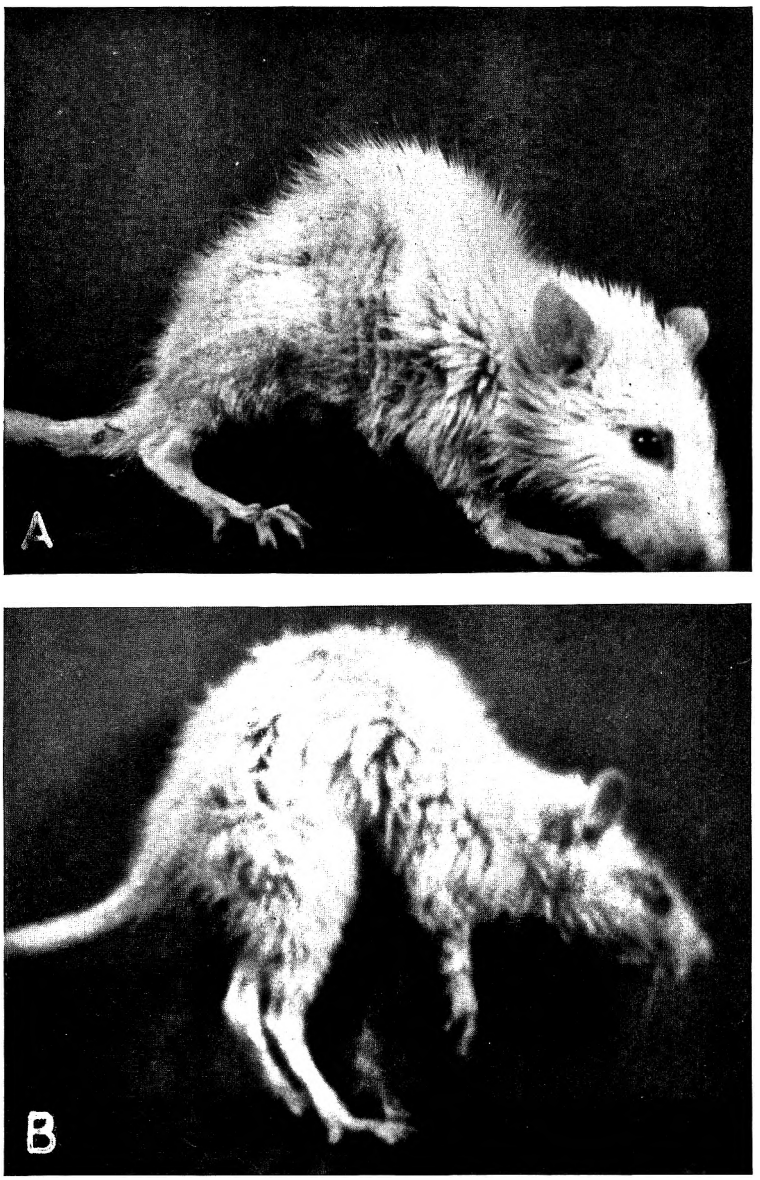

Fig. 2 Examples of female rats showing signs of zinc deficiency after they were fed the experimental diet for 6 weeks. (A) Received diet containing 1 ppm of zinc; note alopecia. (B) Received diet containing 0 ppm of zinc; note extreme emaciation and abnormal posture.

estrous cycles; they were, in fact, in continual anestrus. Histological examination of the esophageal epithelium showed marked hyperplasia and hyperkeratinization in all zinc-deficient groups. Examination of testes showed testicular atrophy and spermatagonic arrest.[8] Male rats receiving 60 ppm of dietary zinc had growth rates comparable to stock-fed rats (fig. 1); nevertheless, after 12 weeks of the diet they showed esophageal and testicular changes similar to those observed in zinc-deficient males. Such alterations were not

[8] We are grateful to Dr. Israel Diamond for the histological studies. Histological findings will be published in detail separately.

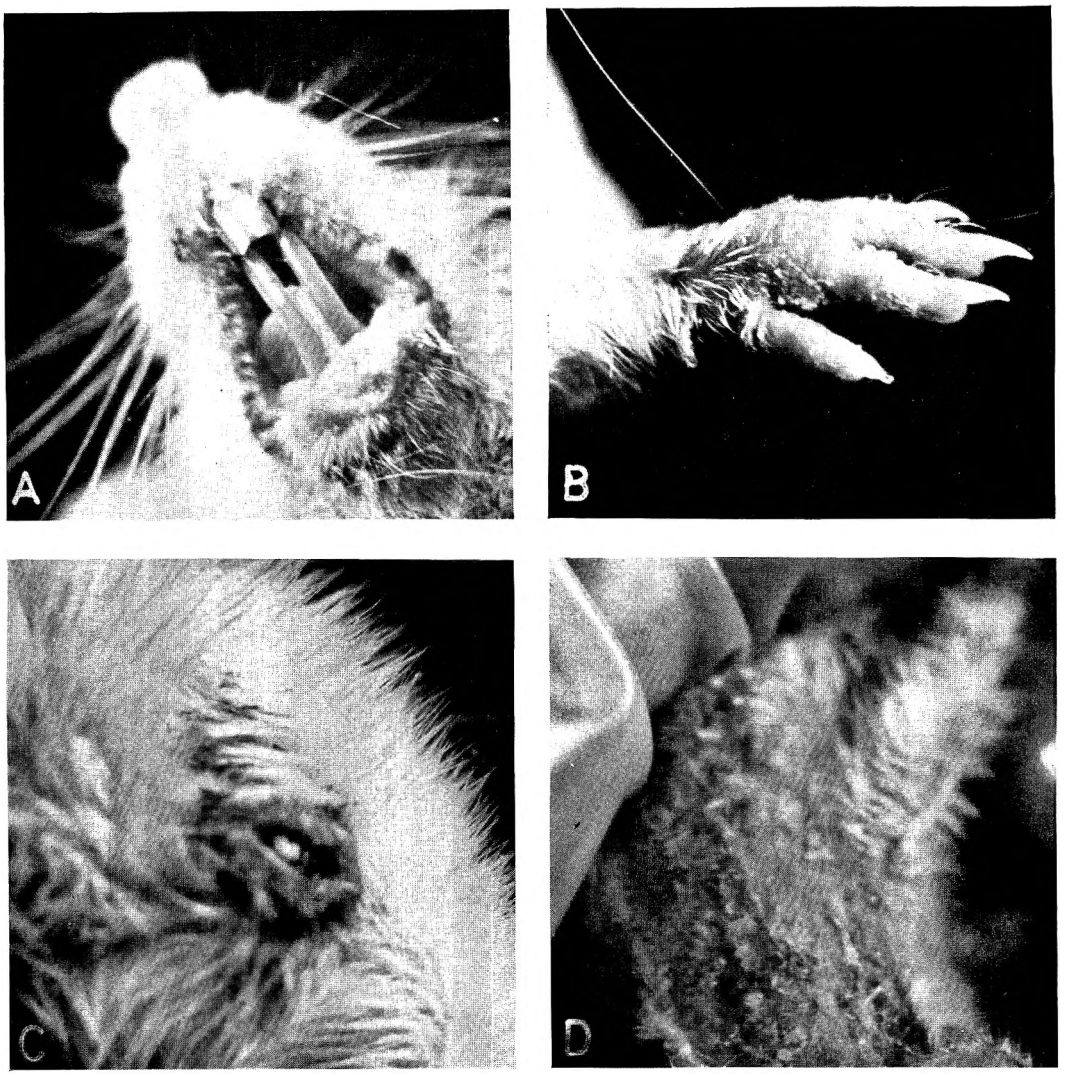

Fig. 3 Signs of severe zinc deficiency in the rat. (A) Oral lesions; (B) dermal lesions and edema of the foot; (C) ocular lesions; and (D) dermal lesions on abdomen.

seen in control rats receiving 100 ppm of dietary zinc nor in stock-fed rats.

The effect of repletion with zinc on the growth rate of zinc-deficient rats is shown in figure 4. Deficient females given the zinc-deficient diet with oral supplements of zinc sulfate (equivalent to 1 mg of zinc/day) showed immediate weight gains. Deficient males showed a similar response when they were supplemented with a single therapeutic dose of zinc sulfate (equivalent to 1 mg of zinc) followed by transfer to the control diet (containing 100 ppm of zinc). Within 2 to 3 days dermal lesions began to improve and by 6 to 8 weeks all overt signs of zinc deficiency disappeared (fig. 5). In addition, the females developed normal estrous cycles and they were able to breed and produce normal young.

The results of the bone analysis are summarized in table 1. Femur weights were lower in both deficient groups, reflecting the general growth retardation. There was a significant decrease in the zinc content

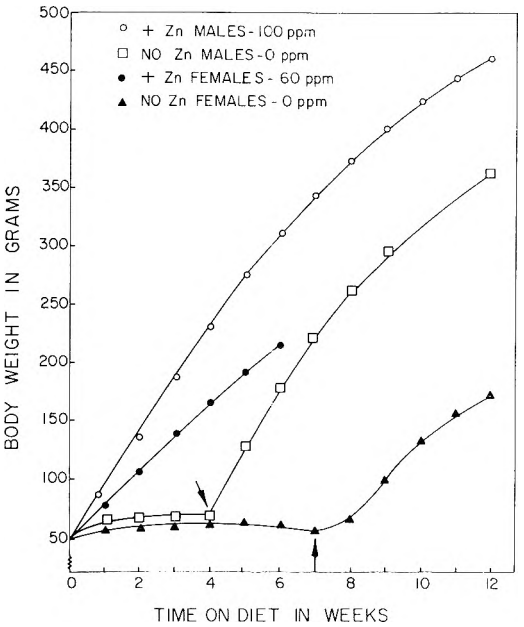

Fig. 4 Effect of zinc repletion on growth of male and female zinc-deficient rats. Curves showing growth of female rats refer to 2 rats: one control and one deficient Arrow shows time at which oral supplementation with zinc sulfate (equivalent to 1 mg of zinc/day) began. Curves showing growth of male rats represent means of 51 zinc-supplemented controls, 29 zinc-deficient rats, and 8 repleted zinc-deficient rats. The arrow shows time at which an oral dose of zinc was given, immediately followed by transfer to the zinc-supplemented control diet.

of the bone ash in both deficient groups; the rats receiving the zinc-supplemented control diet (40 ppm of zinc) had 424 μg of zinc per g ash, whereas the deficient groups had only 102 and 108 μg of zinc per g ash. The percentage of bone ash was also much lower in the deficient groups as compared with controls. There were no significant differences between the two deficient groups (receiving either zero ppm or 1 ppm dietary zinc) in either bone ash or zinc content.

Although deficient rats had lower liver weights than the rats fed the control diet (containing 40 ppm of zinc), there were no significant differences in liver pyridoxal phosphokinase activity on the basis of either liver weight or supernatant protein (table 2).

The results of the liver dehydrogenase assays are summarized in table 3. There was no significant difference in the activity of glutamic dehydrogenase. Lactic acid dehydrogenase activity was significantly lower in the livers of deficient animals than in those of rats fed the control diet (containing 100 ppm of zinc) ad libitum; however, this decrease was also observed in the livers of the pair-fed controls.

DISCUSSION

These studies show that a severe zinc deficiency was induced in rats by the use of a diet containing isolated soybean protein (treated with a chelating agent) when sources of zinc contamination were carefully eliminated from the environment. The specificity of the deficiency was established by demonstrating that the signs and symptoms could be reversed by repletion with zinc either by oral administration of zinc sulfate solution or by the addition of zinc to the diet. Recent findings of very rapid decreases in plasma zinc levels after the institution of zinc-deficient conditions may account for the rapidity of the effect on growth reported here.[9]

Although a diet with 1 ppm of added zinc improved the growth rate in weanling rats, it was not adequate to prevent signs of deficiency. However, the significant

[9] Dreosti, I. E., S. Tao and L. S. Hurley 1968 Plasma zinc and leukocyte changes in weaning and pregnant rats during zinc deficiency, Proc. Soc. Exp. Biol. Med., in press.

TABLE 1

Zinc and bone ash in femurs of normal and zinc-deficient female rats

Group	Dietary Zn	No. rats	Wet wt femur	Bone ash	Zinc	
	ppm		*mg*	*%*	*μg/g ash*	*μg/femur*
+Zn	40 [1]	5	712 ± 31	40.8 ± 0.5	424 ± 10	124 ± 8
Low Zn	1	9	399 ± 33 [2]	32.2 ± 0.8 [2]	102 ± 5 [2]	14 ± 2 [2]
No Zn	0	6	324 ± 25 [2]	30.0 ± 0.5 [2]	108 ± 7 [2]	10 ± 1 [2]

[1] Mean ± SE.
[2] $P < 0.001$ as compared with +Zn controls.

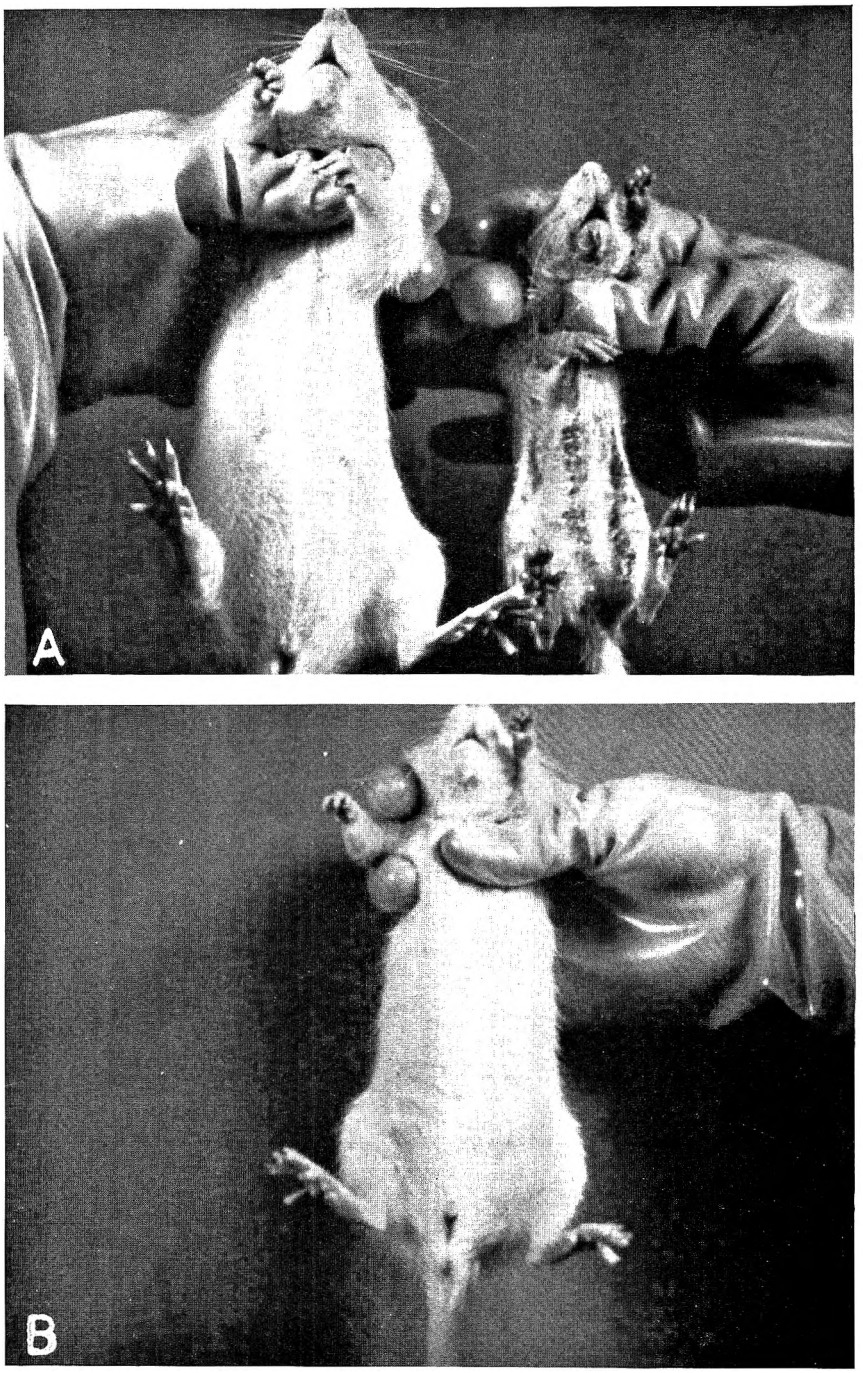

Fig. 5 Effects of zinc depletion and repletion in female rats. (A) Zinc-deficient rat (right) and zinc-supplemented control (left). Note smaller body size, poor hair coat, and dermal lesions in the deficient rat. (B) Zinc-deficient rat (shown in *A*) after 8 weeks of repletion with oral supplements of zinc sulfate solution. (Growth chart of repleted female rat is shown in figure 5.)

TABLE 2

Pyridoxal phosphokinase activity in livers of normal and zinc-deficient female rats

Group	Dietary Zn	No. rats	Wet wt liver	Pyridoxal phosphokinase activity [1]	
					Specific activity [2]
	ppm		*g*	*units/g tissue*	*units*
+Zn	40	5	9.9 ± 0.9 [3]	482 ± 39	3.9 ± 0.3
Low Zn	1	9	4.1 ± 0.4 [4]	349 ± 24	2.7 ± 0.3
No Zn	0	6	2.9 ± 0.3 [4]	394 ± 45	3.3 ± 0.4

[1] Expressed as mμmoles pyridoxal phosphate formed/hour.
[2] Expressed as enzyme units per mg supernatant protein.
[3] Mean ± SE.
[4] $P < 0.001$ as compared with +Zn controls.

growth response to this low level of dietary zinc emphasizes the importance of careful control of the amount of zinc ingested, whether it is from incidental contamination or from the diet itself.

Females fed diets containing 40 ppm of zinc appeared normal. However, males fed diets containing 60 ppm of zinc showed long-term testicular changes, which suggested that the animals were marginally deficient even though their growth was not impaired. It thus appears that the testis is more sensitive to inadequate zinc intake than is growth.

Forbes and Yohe (23) have reported that the zinc requirement for rats is 18 ppm when the diet contains isolated soybean protein. The present work, however, suggests that the requirement for normal growth and development is considerably greater, especially if sources of ingested zinc other than the diet are eliminated. It therefore appears that rat diets containing isolated soybean protein should contain at least 100 ppm of zinc if extraneous sources of the element are minimal. Since rats have been raised for several generations without ill effects on diets with zinc levels as high as 2500 ppm (24), the use of dietary levels of 100 ppm is well within the safety margin.

The severity and consistency of the zinc deficiency in the work reported here can be attributed to 3 factors: 1) use of isolated soybean protein, which appears to increase the requirement for dietary zinc (23, 25); 2) treatment of the protein to reduce the zinc content; and 3) stringent care to eliminate sources of zinc contamination in the environment as well as in the diet. In studies of less severely deficient animals, similar control of environmental sources of zinc might also be helpful if it is important to know the exact quantities of zinc ingested.

The observation of histological changes in the esophagus and testis of zinc-deficient rats confirms the reports of similar lesions described by Follis et al. (26) and Millar et al. (27).

There has been conflicting evidence regarding the effect of zinc deficiency on amount of bone ash in poultry (28–31). In mice, Day and Skidmore (32) reported that there was no decline in bone ash. However, Day and McCollum (8) and Forbes (33) reported that in rats bone ash was lowered. The work described in the present paper confirms the observations by Day and McCollum and Forbes that both the percentage of bone ash and the zinc content of the ash are reduced in zinc-deficient rats. The greater differences in the zinc content reported in the present work, as compared with the earlier reports, further substantiate the conclusion that a more severe zinc deficiency has been produced.

The discovery that zinc is a component of several metalloenzymes has led to the speculation that impairment of enzyme activity may be responsible for the abnormalities seen in zinc-deficient animals. Alterations in enzyme activity have been reported in a number of microorganisms grown in zinc-deficient media (34, 35). There has been little evidence, however, that comparable alterations occur in higher animals (36).

Evidence presented in this paper does not support the hypothesis that reduced activity in the enzymes measured is a cause of the profound physiological and morphological changes associated with zinc defi-

TABLE 3

Glutamic and lactic dehydrogenase activity in livers of normal, zinc-deficient, and pair-fed male rats

Group	Dietary Zn	Glutamic dehydrogenase				Lactic dehydrogenase			
		No. rats	Wet wt liver	Enzyme activity [1]		No. rats	Wet wt liver	Enzyme activity [1]	
					Specific activity [2]				Specific activity [2]
	ppm		*g*	*units/g tissue*	*units*		*g*	*units/g tissue*	*units*
+Zn (ad lib.)	100	10	10.7 ± 0.5 [3]	3.8 ± 0.8	0.027 ± 0.005	14	10.7 ± 0.4	530 ± 43	3.78 ± 0.34
+Zn (pair-fed)	100					4	5.2 ± 0.3 [4]	173 ± 13 [4]	1.28 ± 0.08 [4]
No Zn	0	18	2.7 ± 0.1 [4]	5.1 ± 0.3	0.037 ± 0.003	22	2.7 ± 0.1 [4,5]	200 ± 19 [4]	1.35 ± 0.12 [4]

[1] Expressed as μmoles DPNH converted/minute.
[2] Expressed as enzyme units/mg supernatant protein.
[3] Mean ± SE.
[4] $P < 0.001$ as compared with ad libitum controls.
[5] $P < 0.001$ as compared with pair-fed controls.

ciency. There were no significant differences in the activities of pyridoxal phosphokinase or glutamic dehydrogenase in the livers of deficient animals. Although there was a reduction in the activity of liver lactic dehydrogenase in zinc-deficient rats, a similar decrease was also observed in pair-fed controls. Freedland (21) has also shown a reduction in liver lactic dehydrogenase activity from starvation. Thus, the alteration of liver lactic dehydrogenase activity could have resulted from reduced food intake rather than from zinc deficiency per se.

In studying the effects of zinc deficiency on growth and development, attention must be given to the effect of secondary inanition. Paired-feeding or paired weight-gain experiments are of value in distinguishing between primary effects of zinc deficiency and secondary effects of reduced food intake. However, if alterations in enzyme activities are responsible for the signs of deficiency, such changes in enzyme activity should precede the appearance of the morphological lesions. By the use of a diet extremely low in zinc, metabolic alterations might be detected before gross physical changes are apparent.

ACKNOWLEDGMENTS

We are grateful to the Agricultural Extension Service Laboratory for the use of the x-ray fluorescence spectrometer and the atomic absorption spectrophotometer and to James Quick for his assistance. We are also grateful to Merck Sharp and Dohme, Inc., Rahway, New Jersey, to Hoffmann-LaRoche, Inc., Nutley, New Jersey and to the Dow Chemical Company, San Francisco, for supplies of ascorbic acid α-tocopheryl acetate, DL- methionine, and vitamins A, B_{12}, and D.

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Effect of Calcium and Vitamin D_3 upon the Fecal Excretion of Some Metals in the Mature Male Rat Fed a High Fat, Cholesterol Diet [1]

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ABSTRACT The effect of elevated dietary calcium upon the excretion of 10 metals was studied in 400-day-old male Holtzman albino rats. Increasing dietary calcium from 0.08% to 1.2% increased excretion of lead, nickel, copper, cadium, chromium, iron, manganese, zinc, cobalt and magnesium when the rats were fed a corn-soya diet containing 18% added fat as USP cocoa butter and 2% added cholesterol. Inclusion of 25 units of vitamin D_3 /g of feed partially or completely inhibited the ability of elevated dietary calcium to cause increased excretion of these metals. Although supplementation of a diet with some essential metals may possibly be required, elevated levels of dietary calcium appear to entail the dual effects of lowered serum lipids, without deposition in tissue, and of increased fecal excretion of some potentially deleterious metals.

As part of a continuing study on the hypolipemic action of elevated levels of dietary calcium in man (1) and animals (2–4), it seemed important to study the effect of the elevated dietary calcium upon the absorption and excretion of various trace metals. A study of the possible antagonistic effects in microelement absorption assumes increasing importance in view of the reports by Schroeder et al. (5–12), Tipton et al. (13) and D'Alonzo and Pell (14), among others. D'Alonzo et al. (15) gave a speculative review of the potential role of trace metals in disease, in which the reciprocal relationships of various metals were noted. Various authors have reported on competitive absorption between calcium and other microelements (16–19). Since various trace minerals play a significant role as components of coenzymes, enzyme activators, and transport agents, it is important to determine whether the addition of calcium to a high fat, high cholesterol diet might require increased supplementation of the diet with certain essential microelements. It is also necessary to determine the degree to which elevated dietary calcium might assist in the elimination of potentially toxic microelements and thus exert a second effect in addition to its hypolipemic action.

EXPERIMENTAL

The basic experimental design has been reported previously. Four hundred-day-old Holtzman strain albino rats, mean weight 515 g, were divided into groups of 8 rats and housed two in a cage. All rats were fed a corn soya ration (2) containing 18% fat added as USP cocoa butter and 2% added cholesterol. To the ration of 4 groups, calcium carbonate was added, replacing an equal amount of washed sand, at a level of 0.2% and 1.2% calcium, respectively. Two groups were fed calcium at a level of 0.08%, this level being endogenous to the basal diet. Dietary phosphorus was maintained at 0.35%. Twenty-five units of stabilized vitamin D_3 per g of feed was mixed into the ration of half of the groups. The diets were calculated to contain 15.6% protein, 20.3% fat, and 1.8% fiber.

Feces were collected daily for 3 days by cage during the second and third weeks of

Received for publication November 17, 1967.

[1] This work was supported in part by a grant from the National Institutes of Health, H-5905, a contract in aid from the New Jersey State Department of Health, a grant from the Morris County Heart Association, and a grant from the Sandoz Corporation to one of the investigators (H. Y.).

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the experiments (the collection for a given cage pooled for a 3-day period) and stored frozen until the assay was carried out. The collection was accomplished by permitting the feces to fall through the cage floor onto a paper-covered wire screen from which it was collected. After 21 days the rats were weighed, anesthetized with sodium pentobarbital and exsanguinated by heart puncture. Food was removed 18 hours before death, with fasting time comparable in all groups.

Methods for the analysis of serum, tissues, and fecal total lipids have been reported previously (2, 3). Feces were assayed for trace metals by ashing the entire pooled 3-day fecal collection for a cage according to the method of Tipton et al. (20), dissolving the ash in 25 ml of 3% glass-redistilled nitric acid in glass-redistilled water and determining the metals on an atomic absorption spectrometer.

RESULTS AND DISCUSSION

The mean feed consumption was estimated to be 20.9 g per day (SD ± 0.89) from feed weighback, feed wastage measurements and fecal sand determinations.

In table 1 are shown the results of assays for serum, fecal and liver lipids. As in previous experiments (2–4), elevated dietary calcium significantly lowered all serum lipids without depositing these lipids in the liver. The presence or absence of vitamin D_3 did not influence the hypolipemic action of elevated dietary calcium.

Results of the assays for various elements are shown in table 2. In the absence of vitamin D, increasing dietary calcium to 1.2% of diet was associated with a fivefold increase in the excretion of lead ($P < 0.01$), a doubling in the excretion of nickel ($P < 0.01$), a sixfold increase in cadmium and copper excretion ($P < 0.01$), an apparent fourfold increase in chromium excretion, a fourfold increase in iron excretion ($P < 0.01$), a threefold increase in manganese excretion ($P < 0.01$), and a doubling of excretion of zinc ($P < 0.05$), cobalt and magnesium ($P < 0.01$). No essential differences were noted in fecal metal excretion from those observed when 0.08% calcium was fed and when dietary calcium was raised to 0.2% of diet.

The addition of vitamin D_3 to the diet at the 0.08% calcium level did not influence the excretion of lead, cadmium chromium, iron, zinc, cobalt or magnesium.

TABLE 1

Effects of dietary calcium on body weight,[1] serum, fecal and liver lipids after 3 weeks

Calcium level in diet, %	0.08	1.2	0.08	1.2
Vitamin D_3 level in diet, IU/g	—	—	25	25
Mean wt, g				
Initial	502 ± 8 [2]	521 ± 11	507 ± 10	521 ± 14
Final	527 ± 10	528 ± 10	527 ± 11	542 ± 14
Serum, mg/100 ml				
Total lipids	600 ± 89	300 ± 40** [3]	513 ± 74	325 ± 15*
Phospholipids	172 ± 4	119 ± 3**	178 ± 7	117 ± 7*
Total cholesterol	168 ± 7	93 ± 2**	159 ± 6	100 ± 3**
Triglycerides	126 ± 16	66 ± 3*	99 ± 7	67 ± 3*
Calcium, mEq/liter	5.75 ± 0.04	5.60 ± 0.07	5.90 ± 0.16	5.58 ± 0.16
Liver				
Mean liver wt, g	17.4 ± 0.77	15.9 ± 1.38	15.1	14.8
Total lipids, g/liver	1.83 ± 0.07	1.56 ± 0.16	1.96 ± 0.17	1.82 ± 0.10
Total cholesterol, mg/liver	511 ± 36	540 ± 47	509 ± 68	449 ± 45
Triglycerides, mg/liver	395 ± 105	275 ± 63	541 ± 54	491 ± 30
Feces: dry feces, g/rat/day	3.83	6.56	3.68	5.97
Total lipids, mg/rat/day	962 ± 68	1986 ± 78**	749 ± 51	1969 ± 308**

[1] Eight rats/group.
[2] Mean ± SD.
[3] Significance computed by analysis of variance technique: * significance at $P < 0.05$; ** significance at $P < 0.01$.

TABLE 2

Effect of calcium and vitamin D_3 upon fecal excretion of trace metals

Ca level, % in diet	Feed [1]	0.08	1.2	0.08	1.2
Vitamin D_3, IU/g feed		—	—	25	25
		µg/rat/day	*µg/rat/day*	*µg/rat/day*	*µg/rat/day*
Cobalt	5.9	3.5 ± 0.01 [2]	9.8 ± 0.7**	3.1 ± 0.2	8.0 ± 3.6
Cadmium	5.3	1.3 ± 0.2	3.9 ± 0.4**	1.8 ± 0.2	4.8 ± 0.2**
Chromium	40.0	4.7 ± 1.0	19.3 ± 5.3	19.4 ± 6.3	16.5 ± 3.2
Copper	147.1	25.8 ± 6.1	152.7 ± 6.8**	7.6 ± 1.0	15.2 ± 1.4**
Iron	1500.0	797 ± 248	3193 ± 142**	151 ± 38	246 ± 13
Manganese	882.4	594 ± 122	1705 ± 31**	77 ± 18	127 ± 7
Magnesium	20,000	9277 ± 254	19170 ± 854**	13228 ± 405	22872 ± 406*
Nickel	40.0	34.9 ± 0.3	61.1 ± 4.5**	9.1 ± 0.7	32.9 ± 3.3**
Lead	8.3	1.3 ± 0.2	10.7 ± 0.7**	3.5 ± 0.5	2.3 ± 1.0
Zinc	882.4	75.0 ± 25.3	202.7 ± 8.5*	7.6 ± 1.4	11.5 ± 0.4
		mg/rat/day	*mg/rat/day*	*mg/rat/day*	*mg/rat/day*
Calcium		33.3 ± 4.0	296.0 ± 25.0**	32.3 ± 6.5	241.0 ± 13.5**

[1] Calculated on the basis of 20 g feed exclusive of drinking water.
[2] Mean ± SD of mean.
[3] Significance computed by analysis of variance technique: ** significance at $P < 0.01$; * significance at $P < 0.05$.

Addition of vitamin D_3 to the 1.2% calcium diet reduced the excretion of nickel and iron ($P < 0.01$). The added vitamin D_3 did not affect the increased excretion of magnesium, nickel, or cadmium associated with elevated dietary calcium. The addition of vitamin D_3 to the diet completely nullified the increased excretion of lead, manganese, zinc, and cobalt, in the presence of elevated dietary calcium.

The use of elevated dietary cacium as a hypolipemic agent may require the supplementation of the diet with certain essential elements such as manganese, magnesium, zinc, iron, cobalt, and copper. The quantitative effect of elevated dietary calcium on the requirements for these metals under these dietary conditions is still to be determined.

The enhanced excretion of lead, cadmium, and chromium, associated with elevated dietary calcium, could have a beneficial effect on growth and survival. Schroeder has shown that elevated levels of cadmium are associated with hypertension in both rats (6) and humans (5). The toxic effect of lead is well-known. Schroeder et al. (11) have shown that elevated levels of cadmium and lead exhibit toxicity in mice in terms of survival. Although supplementation of a diet with some essential trace metals may possibly be required, elevated levels of dietary calcium appear to provide the dual effects of lowered serum lipids, without deposition in liver, and of increased fecal excretion of some potentially deleterious trace metals.

ACKNOWLEDGMENT

The authors thank Mrs. M. Hogan, Division of Occupational Health, New Jersey State Department of Health, for her assistance in the assay of the trace elements.

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Influence of Limited Dietary Calcium upon Zinc Absorption, Placental Transfer and Utilization by Swine [1,2]

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ABSTRACT Effect of limited dietary calcium upon zinc absorption and subsequent utilization was studied in 24 gravid gilts and third trimester fetuses. Sows given low calcium excreted less zinc and a lower percentage of endogenous zinc than control gilts. Rate of oral and intravenous ^{65}Zn fecal loss was slower and the quantity was less for sows fed limited calcium diets. The highest tissue zinc concentration was in maternal liver, and somewhat more zinc was in tissues of sows given low calcium. Fetal tissue zinc levels, except for liver, were less than those for dams, and averaged higher for fetuses from sows fed normal calcium rations. Absorbed ^{65}Zn appeared to be more accessible for fetal transfer, and utilization was greater when calcium was limited. However, neither rate of transfer nor movement to and from total organs was significantly affected by dietary calcium intake. Maternal tissue ^{65}Zn decreased, whereas that in fetal livers increased progressively 30 times during 7 days, and ultimately contained nearly 50% of the total fetal ^{65}Zn. Calculated balance tissue data showed that 90% absorbed ^{65}Zn was retained by sows receiving normal calcium rations, and $6.1 \pm 1.8\%$ of this was transferred to the total products of conception and subsequently deposited in the placenta (46.7%), fluids (0.4%) and the developing fetus (52.9%) after 168 hours.

Zinc has been recognized as essential to normal animal growth and development since 1934 (1, 2). However, the significance of its economic importance in swine nutrition first became evident in 1955, when Tucker and Salmon (3) showed zinc deficiency to be the basis of porcine parakeratosis. The discovery and isolation of zinc in many highly purified enzymes has offered an explanation of the mode of action (4), and has showed the diversity of its function in biological systems (5). The wide variety of dietary variables affecting zinc absorption and utilization (6–13) has clearly demonstrated the metabolism of zinc to be closely interrelated with many dietary elements, especially calcium (7–10). Most of these reports, however, have been concerned with zinc antagonism by added dietary calcium (9, 10), and little information is available on zinc behavior when available body and dietary calcium were limited. Likewise, few studies have been involved with zinc retention and subsequent membrane transfer in the epitheliochorial placenta of swine.

Although zinc has been reported to freely cross the membranes of the various placental types (13–19), including that of the sow (20), the apparent physiological relationships between zinc and calcium and their functional and structural contribution to fetal development would indicate a need for more information on the factors affecting placental permeability and fetal zinc utilization. This report is concerned with effects of limited calcium intake upon zinc absorption, and subsequent utilization by gravid gilts and their third trimester fetuses.

MATERIALS AND METHODS

The animals used in this study consisted of 24 gravid and 6 open purebred Poland China and Duroc gilts, maintained with a conventional 17% protein, corn-soybean meal gestation ration (A) (table 1) containing 0.7% calcium, 0.5% phosphorus and 68 ± 7 ppm zinc. After 70 days one-half of the sows were selected at random, removed to separate lots and changed to a

Received for publication November 17, 1967.

[1] Published with the approval of the Director, Louisiana Agricultural Experiment Station, Baton Rouge, Louisiana.

[2] Supported in part by the National Science Foundation (contract GB-6602).

[3] Dr. H. Itoh, International Mineral and Chemical Corporation, Skokie, Illinois, Postdoctorate Research Fellow 1964–65, on leave from Faculty of Agriculture, Tohoku University, Sendai, Japan.

TABLE 1

Gestation rations for gravid gilts (17% protein)

	Control, ration A (0.7 Ca — 0.5 P)	Low calcium, ration B (0.3 Ca — 0.3 P)
	kg	*kg*
Ground yellow corn	765	778.5
Soybean oil meal	200	200.0
Oyster shell flour	10	6.5
Dicalcium phosphate	10	—
Iodized salt	5	5.0
Premix [1]	10	10.0
Total	1000	1000.0

[1] Premix supplied 1.14 million internation units (IU) of vitamin A palmitate, 0.4 million IU vitamin D, 1 g riboflavin, 2 g pantothenic acid, 4.5 g niacin, 10 mg vitamin B_{12}, and 65 g ZnO/1000 kg.

low calcium ration (B) (0.3% Ca and 0.3% P), identical to ration A but without the dicalcium phosphate. Oyster shell flour was reduced to 6.5% to maintain the same Ca:P ratios. During the last 15 days of gestation each animal was placed in a metabolism unit equipped for quantitative separate collection of urine and feces, and given a single oral or intravenous tracer dose (21) of ^{65}Zn citrate. Following 12- to 168-hour blood-balance periods, sows were killed, bled out and maternal-fetal tissues and organs sampled immediately for zinc and ^{65}Zn analyses. Fetuses were weighed individually, and placental membranes and fluids were separated, weighed and sampled. About one-half of the fetuses were alloted at random for whole-body analyses, and others were used for tissue-organ radioactive zinc distribution study. Analytical measurements for zinc and radioactive zinc were made by conventional procedures (19), except that total zinc was determined both chemically (22) and by atomic absorption spectroscopy (23). For comparative purposes all data were standardized for dose, litter size and body weight, and statistically evaluated for differences due to treatment (24).

RESULTS AND DISCUSSION

For the comparative transfer of elements from one metabolic pool to another it was necessary to consider behavior in terms of the total quantity of mineral actually available for transfer, unbiased by differences due to gastrointestinal absorption. In placental transfer studies, therefore excretion values were essential for interpretation of radioisotope behavior patterns for that percentage absorbed and retained in the maternal body.

Zinc excretion and retention. Calculated excretion data from the 7-day balance studies showed that both total zinc and radioactive zinc ingested or injected leave the body primarily via the feces. This applied whether sows were receiving the normal (A) or low calcium (B) rations. Sows consuming 68 ± 7 ppm/zinc/day excreted 86 ± 9%, and differences due to dietary calcium level were nonsignificant. Urinary zinc was not affected by diet and accounted for only 4 to 7 mg/day, which suggests kidney efficiency in sows fed the higher dietary zinc to be less than that of either sheep (18) or cattle (19).

The cumulative percentage of excretions of the orally and intravenously administered tracer dose of ^{65}Zn to sows is shown graphically in figure 1, as a function of time for the two dietary calcium levels. The steep slope observed following oral dose administration denotes fecal excretion of exogenous or unabsorbed ^{65}Zn, and showed peak excretion to occur 48 to 72 hours post-ingestion. Slight elevation in the plateau area of the curves, representing chiefly endogenous ^{65}Zn excretion, included primarily that excreted via pancreas (25). Following intravenous dose administration, fecal ^{65}Zn which was endogenous in origin showed no clear-cut plateau, but rate and quantity were somewhat greater for those sows receiving the higher calcium diets. Neither excretion rate nor quantity of total zinc, however, was significantly affected by treatment. Calculated endogenous losses (20) during the 7-day balance periods were somewhat less for those sows fed the lower

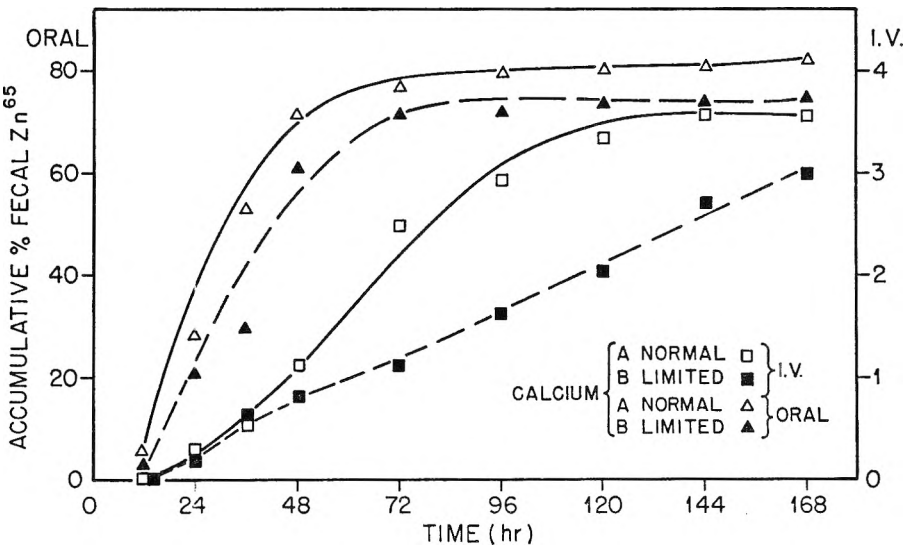

Fig. 1 Accumulative percentage fecal excretion of oral and intravenously administered ^{65}Zn by third trimester sows maintained with normal (*A*) and limited calcium (*B*) diets.

calcium diets, and less total zinc (14 vs. 21%) was retained by sows receiving the higher calcium ration. These data are in agreement with reports by Heth and Hoekstra (26) and Forbes (7) for rats, and that for swine by Michigan workers (27). However, Berry and associates (28), feeding higher increments of dietary calcium to barrows, observed no increase in ^{65}Zn retention, and Spencer's group (29) reported no definite correlations between calcium balance and the intestinal absorption of orally administered ^{65}Zn in man. Conflict in results may reflect growth differences and the lower endogenous calcium losses in young animals, but further emphasizes the current advantage of maintaining experimental animals on the low side of minimum dietary calcium requirements. The present study was unique in that calcium was not added, but reduced, maintaining identical calcium-to-phosphorus ratios under stress of the demands of pregnancy, thereby partially limiting the masking effects of body calcium stores and exogenous calcium upon the blood-balance, and maternal-fetal tissue zinc-^{65}Zn values.

Total blood and plasma zinc. The effects of time and dietary calcium levels upon circulating blood ^{65}Zn, following oral and intravenous administration, are shown graphically in figure 2 for the gravid sows. Pregnancy was without significant effect on ^{65}Zn movement, and values for open gilts were not reported separately. Total circulating ^{65}Zn was calculated from the percentage dose per milliliter values, using a total blood volume of 5 8% of the body weight (30). Red cells and plasma were estimated from the hematocrits. ^{65}Zn entering the blood from the gastrointestinal tract of sows dosed orally reached peak values of less than 3.0% after approximately 72 hours, and rapidly receded to less than 1.0% of the administered dose 120 to 144 hours later, when nearly one-half of that ^{65}Zn remaining was in the erythrocytes. The lower blood ^{65}Zn values, after 144 hours, in those animals dosed orally suggests that ingested zinc is metabolized differently from that injected. Following intravenous dosing, total ^{65}Zn blood levels decreased rapidly from 90% at 5 minutes to 8% after one hour. Equilibrium between erythrocytes and plasma appeared to have been reached about 2 hours following intravenous dosing after which ^{65}Zn gradually increased in the red cells, and plasma levels decreased more rapidly. Differences in ^{65}Zn blood levels due to treatment were not significant, but values decreased more slowly in those sows fed the higher calcium ration (27). Total plasma zinc was higher in the dam (1.4 ± 0.1 mg/

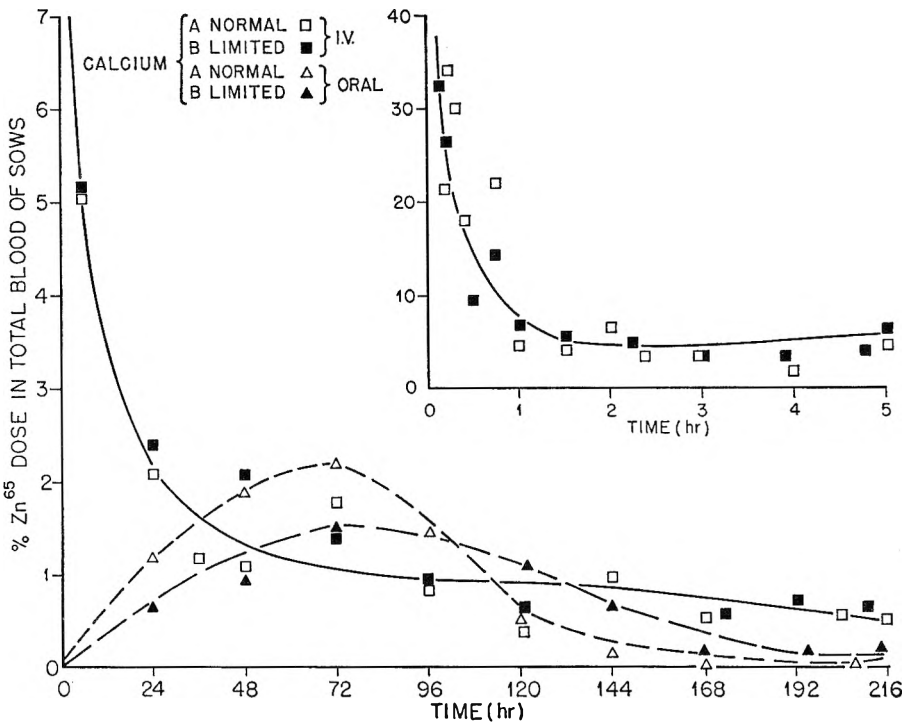

Fig. 2 Disappearance rates of orally and intravenously administered ^{65}Zn from total blood of third trimester sows fed normal (*A*) and limited (*B*) calcium diets.

liter) than the fetus and level was not significantly affected by treatment.

Effects of limited dietary calcium upon maternal and fetal tissue zinc and ^{65}Zn. Average zinc and ^{65}Zn concentrations in selected tissues of the 24 sows and the 224 fetuses are shown in table 2. Of the soft tissue, liver, pancreas, spleen, muscle, kidney and heart contained the most zinc, and in that order. There was a tendency for soft tissue zinc values to be somewhat higher for those sows receiving the limited calcium rations, but differences were not significant. Although liver contained the highest concentration value for these tissues, levels were lower than those for bones of sows on either dietary treatment. Fetal tissue zinc values, except for liver, were generally lower than those for the dam, and concentration in bone was higher in fetuses from sows fed the higher calcium rations.

It was of interest that concentration ratios of calcium to zinc (mg/g) in five selected fetal soft tissues from dams fed normal (A) calcium rations averaged 5.1 ± 0.5, and were consistently higher than those of fetuses from sows fed the limited (B) calcium diets, suggesting a slower zinc turnover rate (26). However, these ratios for fetal femur, ribs, mandible and sternum from sows fed the limited calcium ration averaged 1210 ± 108, and were significantly higher ($P < 0.01$) than those for bones from the normal (A) group. This suggests a greater utilization of zinc by third trimester fetuses from limited calcium sows, and indicate a higher turnover rate for zinc in their soft tissues. These fetal ratios for soft tissues were twice the maternal values, but for most bones, ratios approached one-half the ratio for the dam. Maternal calcium-to-zinc ratios were not significantly affected by dietary calcium levels. Placental calcium averaged 10 times that of zinc, and the total fetus from sows on both treatments contained nearly 400 times more calcium than zinc. Maternal-fetal plasma and whole blood zinc ratios indicate no concentration gradient

TABLE 2

Maternal dietary calcium effects upon zinc [1] and ^{65}Zn concentration [2] in maternal and fetal swine tissues

Tissue	Normal calcium, ration A				Limited calcium, ration B			
	Maternal		Fetal		Maternal		Fetal	
	Zinc	^{65}Zn	Zinc	^{65}Zn	Zinc	^{65}Zn	Zinc	^{65}Zn
Plasma	1.6	1.4	0.9	0.4	1.2	1.9	0.8	0.5
Whole blood	3.2	2.6	1.6	0.6	3.0	3.5	2	0.8
Muscle	26	4.7	10	3.1	24	4.5	9	3.0
Liver	49	62.3	65	71.1	55	58.5	68	71.5
Kidney	19	24.2	12	3.3	18	24.5	12	4.1
Spleen	23	19.2	16	5.4	28	19.4	22	7.3
Heart	16	20.2	15	8.1	18	17.3	17	5.3
Pancreas	35	16.5	26	6.1	34	28.2	27	7.5
Femur shaft	110	4.7	89	9.4	118	4.5	74	8.8
Femur ends	68	8.8	55	5.5	72	8.4	33	6.3
Rib shaft	69	9.0	94	13.0	63	10.3	75	13.2
Rib ends	73	26.0	111	15.0	75	35.0	93	19.0
Mandible	94	7.8	27	5.4	93	12.7	41	7.1
Sternum	74	12.0	20	4.0	53	13.4	29	5.4

[1] Total zinc calculated as mg/kg fresh weight in third trimester sows.
[2] ^{65}Zn calculated as % dose absorbed $^{65}Zn \times 10^{-4}$ after 168 hours, corrected to 150-kg dam.

for fetal zinc transfer, and suggest slightly more favorable relationships for those sows on the limited calcium rations.

Radioactive zinc concentration in soft tissues essentially paralleled that for total zinc and, except for blood and muscle, was usually greater than that for bone. Fetal soft tissue ^{65}Zn levels after 7 days were lower than those for the dam, and values were consistently higher for those fetuses from the limited calcium sows. Fetal bone ^{65}Zn levels, however, approached those of the dam after 168 hours, and values for rib and femur shafts exceeded those of the dam. Concentration in placenta and fetus and in nearly all tissues from the limited calcium group were slightly higher in ^{65}Zn. In third trimester fetuses, therefore, absorbed zinc appears to be more accessible for fetal transfer and utilization when calcium was limited. Further study is needed to learn whether this was related to interference by the available tissue calcium (31), the competitive process for zinc and calcium associated with faster bone growth and calcification (32), or to differences in avidity of the partially depleted tissues of fetuses from sows maintained with limited calcium rations (26, 31).

The magnitude of these differences with treatment, however, was emphasized by the calculated zinc and labeled zinc in total maternal and fetal organs from these gravid third trimester sows. Values in table 3 show liver to be the primary storage organ for both zinc and ^{65}Zn, and suggest the placenta as an active metabolic pool for this element. Liver, heart and fetuses from sows fed limited calcium contained more total zinc ($P < 0.05$), and fetal liver, heart and spleen zinc levels were higher. Fetal livers contained nearly 10% of the total body zinc and 50% of the labeled zinc after 168 hours.

Rate of ^{65}Zn transfer to products of conception. Effects of maternal dietary calcium intake upon the retained dose of ^{65}Zn in placenta, placental fluids and fetuses of third trimester sows, killed at intervals of time to 168 hours, are shown in table 4. At 12 hours the placenta contained 10 and 40 times as much ^{65}Zn as all fetuses from the normal and limited calcium sows. After 48 hours this ratio had decreased to four, with little treatment effect to 168 hours. Labeled zinc in total fetuses, however, increased progressively with time and after 7 days contained 52.9% and 52.4% of that ^{65}Zn in the total placenta complex of sows fed the normal and limited calcium rations, respectively. Placental fluids apparently contributed little to the movement of zinc to the fetus, and levels reached a peak at 12 to 48

TABLE 3

Zinc and ^{65}Zn [1] in total maternal and fetal organs of swine fed normal and limited calcium rations [1]

Organ	Normal calcium, ration A				Limited calcium, ration B			
	Maternal		Fetal		Maternal		Fetal	
	Zinc	^{65}Zn	Zinc	^{65}Zn	Zinc	^{65}Zn	Zinc	^{65}Zn
	mg	*%*	*mg*	*%*	*mg*	*%*	*mg*	*%*
Liver	77.0	9.80	2.30	0.180	86.4	10.0	2.60	0.185
Heart	6.5	0.68	0.11	0.003	7.3	0.75	0.12	0.004
Kidney	3.4	0.43	0.06	0.002	3.2	0.44	0.06	0.002
Spleen	5.9	0.41	0.02	0.001	5.9	0.63	0.03	0.001
Placenta	54.2	2.40	—	—	52.8	2.90	—	—
Placental fluids	1.5	0.02	—	—	1.5	0.01	—	—
Fetus (per kg)	—	—	24.0	0.34	—	—	29.0	0.40
Litter of 8	—	—	192.0	2.72	—	—	232.0	3.20

[1] Third trimester gravid sows killed after 168 hours. Data corrected to 150-kg maternal body weight and to percentage retained ^{65}Zn dose in total organs.

TABLE 4

^{65}Zn in total swine products of conception at time intervals after dose administration [1]

Product	Sow ration	Hours after dosing			
		12	48	72	168
Placenta	A	7.1	6.0	2.2	2.5
Placenta	B	5.9	5.0	3.6	2.9
Placental fluids	A	0.03	0.05	0.03	0.02
Placental fluids	B	0.05	0.02	0.02	0.01
Litter (8 fetuses)	A	0.72	1.44	2.49	2.72
Litter (8 fetuses)	B	0.16	1.36	2.64	3.20

[1] Calculated as ^{65}Zn concentration x total weight, with litters standardized to eight 1-kg pigs from sows fed the normal (A) and limited calcium (B) rations.

TABLE 5

Total ^{65}Zn in whole organs of gravid sows and fetuses killed at intervals after intravenous dosing [1]

Organ	Sow ration	Hours after dosing							
		12		48		72		168	
		Dam	Fetus [2]	Dam	Fetus	Dam	Fetus	Dam	Fetus
Liver	A	18.4	0.006	13.7	0.07	10.8	0.10	9.8	0.180
Liver	B	17.5	0.003	13.1	0.05	10.5	0.12	10.0	0.185
Heart	A	0.5	0.001	0.7	0.002	0.8	0.004	0.7	0.003
Heart	B	0.5	0.001	0.6	0.002	0.7	0.003	0.7	0.004
Kidney	A	0.6	0.0005	0.8	0.002	0.5	0.002	0.4	0.002
Kidney	B	1.1	0.0003	0.8	0.002	0.6	0.002	0.4	0.002
Spleen	A	0.9	0.0002	0.5	0.0010	0.4	0.0010	0.4	0.001
Spleen	B	1.5	0.0001	0.8	0.0005	0.6	0.0009	0.6	0.001

[1] Calculated as ^{65}Zn concentration x total weight of organs from sows killed at intervals of time after dosing during late pregnancy, while maintained with normal (A) and limited calcium (B) ration. Data corrected to 150-kg body weight and to percentage ^{65}Zn dose retained.
[2] Calculated as total percentage dose/organ for one fetus.

hours after maternal ^{65}Zn dosing, decreasing at 7 days to values of less than 0.02% of the retained dose.

Effects of limited calcium and time upon ^{65}Zn in total organs of maternal and fetal swine. The tabulated data in table 5 show the percentage dose ^{65}Zn in whole organs of gravid third trimester sows and fetuses killed at intervals of time to 168 hours. Liver ^{65}Zn levels peaked at about

18% by 12 hours, and decreased progressively to 57% of this value after 168 hours in sows fed both normal and limited calcium rations. Values for maternal kidney and spleens were lower, but rates and pattern of disappearance were similar to those for liver. However, ^{65}Zn levels for total heart peaked at 72 hours, and decreased only slightly to 168 hours after dose administration.

Labeled zinc in total fetal livers, on the other hand, increased progressively with time to 7 days. Most rapid ^{65}Zn accumulation in fetal livers from sows fed low calcium occurred between 12 and 48 hours, when levels increased 17 times and then paralleled rate and level for those of fetuses from the control sows. Values for spleen and kidney increased about 5 and 10%, respectively, and those for heart tripled between 12 hours and 7 days. There were no significant effects of maternal treatment on rate or quantity of total ^{65}Zn accumulation in these fetal organs.

Partition of ^{65}Zn by the gravid sow. Data from balance studies with third trimester sows showed that 90.2 ± 5.1 and 92.1 ± 6.3% of that ^{65}Zn entering the blood was retained and deposited by animals fed the normal and limited calcium dietary, respectively. Subsequent tissue-organ analyses indicated that 5.1 ± 1.1 and 6.1 ± 1.8% of this was transferred to the total products of conception (table 3), and deposited in the placenta (46.7), the fluids (0.4) and the developing fetus (52.9) in sows fed normal calcium diets. Those sows receiving limited calcium deposited the 6.1 in the placenta (47.5), placental fluids (0.1) and the fetus (52.4%) after 168 hours, by essentially the same pattern. More of the total zinc in the placental complex was retained in the placenta itself by sows fed the normal calcium diets (22% vs. 19%), and fetuses from the limited fed sows contained about 4% more of this zinc (81 vs. 77). None of these values were significantly different. Although absorption of labeled zinc was higher for swine, these values for the fetus were lower than those observed for the ewe (18) and gravid bovine (19), and further suggest placental type to be a contributing factor in the placental zinc transfer to the fetus.

ACKNOWLEDGMENTS

Grateful appreciation is expressed to Sharon Nichelson, William Whitfield and Carol Bradley for technical assistance, and to Dr. D. M. Thrasher for furnishing the bred gilts.

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Tissue Cation Content of Rats: Effect of supplemental dietary amino acids in pyridoxine and potassium deficiencies [1]

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ABSTRACT A study was made to determine changes in liver, brain, skeletal muscle and blood concentration of K, Na and Mg resulting from dietary deficiencies of pyridoxine and K in growing male rats. The experimental treatments involved supplementation of the deficient diets with deoxypyridoxine (DOP) or pyridoxine and K, either alone or in combination with supplements of casein or the amino acids glycine, lysine or γ-aminobutyric acid. Effects of treatments on survival time, weight gain, food consumption, and liver and brain weights were also determined. No significant alterations in concentrations of brain or liver cations were observed. Muscle K concentration was reduced by supplemental pyridoxine and increased by supplemental K with one exception—the case in which supplemental casein was present simultaneously. Muscle Na concentration was increased by supplemental casein and reduced by supplemental K. Muscle Mg concentration was reduced by supplemental glycine and K. No significant alterations of blood K concentration were observed. Blood Na concentration was increased by DOP and by supplemental K when supplemental lysine was also present. Blood Mg concentration was increased when DOP was present with supplemental K. Supplemental casein and K reduced survival of pyridoxine-deficient animals—the effect being greatest when both were added simultaneously. Supplemental pyridoxine, glycine and K increased gain, whereas DOP and casein reduced gain. Supplemental pyridoxine and K increased food consumption, whereas DOP reduced food consumption. The importance of pyridoxine and cellular cation fluxes to amino acid transport and protein biosynthesis was discussed.

Since the appearance in the literature of the first reports of convulsive seizures in pyridoxine-deficient experimental animals and infants, much research has been conducted on the metabolism of monovalent and divalent cations in pyridoxine and cation-deficient animals. Recent work has re-evaluated older data in light of newer criteria, for example, the reassessment of the pyridoxine requirement of the rat (1).

Much research has dealt with the effect of pyridoxine deficiency alone—or augmented by the pyridoxine antagonists deoxypyridoxine (DOP) or isonicotinic acid hydrazide (INH)—on tissue cation concentrations and fluxes. Work in this laboratory (2) showed decreased potassium (K) retention in pyridoxine-deficient growing rats; in severe deficiencies decreased sodium (Na) retention was also evident. Further (3), in the pyridoxine-deficient animals an influx of K was apparent in liver, kidney, brain and skeletal muscle, but not in cardiac muscle, blood plasma and blood cells; in the liver a concomitant efflux of Na occurred. Hsu et al. (4) found that pyridoxine deficiency in rats produced marked elevation of muscle K and reduction of muscle Na, but no effect on the levels in heart, liver and kidney.

Studies of Na and K fluxes in tissues demonstrated that variations of membrane potential of skeletal muscle following a high rate of Na efflux were not due to changed ionic strength (5) and that the increased acidity of skeletal muscle of K-deficient rats was related to the chronicity of K restriction but unrelated to unequal replacement of muscle K loss by Na gain (6).

The effect of K flux upon the active transport of amino acids across membranes—and thereby on protein and amino acid metabolism has also received much attention. Potassium supplementation of

Received for publication November 11, 1967.
[1] Authorized for publication on February 24, 1967 as paper no. 3234 in the journal series of the Pennsylvania Agricultural Experiment Station.

swine rations whose protein source was corn and soybean meal resulted in reductions of free basic amino acids of blood plasma, skeletal muscle and kidney (7). Also, arginine and lysine, not present in protein-free skeletal muscle extract of normal rats, was present in such extracts from K-deficient animals (8). The theory that cellular K efflux provides the free energy for active transport of amino acids into the cell has been popular; however, it has been shown in the Ehrlich mouse ascites tumor cell that the free energy available from K efflux is less than the free energy required to pump glycine into the cell (9).

γ-Aminobutyric acid (GABA) and its role in nerve cell metabolism have been subjects of numerous investigations. In a recent review paper (10) in was concluded that exogenous GABA is rapidly distributed throughout the animal body with the exception of the brain and that it is rapidly excreted. Dyablova (11) reported that GABA depressed K-induced contractions of the frog skeletal muscle preparation and suggested that the increased K excretion from cerebral cortex observed with increased GABA concentration might also occur in skeletal muscle, thereby affecting its reactivity to K.

The present study was undertaken to observe in a single experiment changes in liver, brain, skeletal muscle and blood concentrations of K, Na and Mg resulting from deficiencies of pyridoxine and K. Because of the interrelation of ion flux and amino acid transport, data concerning tissue content of K, Na and Mg of animals fed diets supplemented with protein or amino acids were sought. The amino acids chosen for investigation were glycine and lysine, which represented the neutral and the basic amino acids, respectively, and GABA, a compound having a unique function in nerve cell metabolism.

EXPERIMENTAL

Ninety-eight male albino rats of the Wistar strain, ranging in weight from 55 to 94 g, were assigned at random to 7 replicates of 14 experimental treatments. The treatments involved the feeding ad libitum for a 28-day period of the basal diet or the basal diet with additives shown in table 1. The basal diet had the following percentage composition: vitamin-test casein,[2] 20; sucrose, 32.5; glucose monohydrate,[3] 29.5; corn oil,[4] 4; mineral mix,[5] 3; vitamin mix,[6] 5; and cellulose, 2. Additions to the basal diet were made at the expense of glucose monohydrate. The animals, housed in individual screen-bottom cages in a room maintained at 26 ± 2°, were weighed at weekly intervals and individual weekly food consumption was determined by weighing back the left-over food. Distilled water was furnished ad libitum.

At the completion of the feeding period on days 28 and 29, the animals surviving the experimental treatments were anesthetized with ether, decapitated, and the whole blood was collected individually. The brain, gastrocnemius muscles and liver were immediately excised, freed of extraneous tissues and blotted on filter paper. The whole blood and excised organs were placed in beakers, dried, ashed, extracted with hot 0.3 N HCl, and the extracts then assayed for K and Na by flame spectrophotometry as previously described (3). The Mg content of aliquots of the tissue extracts was estimated by flame spectrophotometry (Beckman model B spectrophotometer with photo-multiplier unit) at $\lambda = 366$ mμ. Since K also emitted at 366 mμ, the observed transmittance readings were corrected for the concentration of K in the aliquots. The concentrations of K, Na, and Mg were expressed as millimoles per kilogram of fresh tissue for brain, muscle and liver, and as millimoles per liter of whole blood.

The analyses of variance took into account the disproportion in survival and in consequent numbers of observations per treatment (13, p. 382). Homogeneity of variances was tested (13, pp. 285–289). The homogeneous variances were pooled into an estimate common to all and were used for testing the statistical significance of differences between treatment means

[2] Nutritional Biochemicals Corporation, Cleveland.
[3] Cerelose, Corn Products Company, New York.
[4] Oleum percomorphum (Mead-Johnson and Company, Evansville, Indiana) and α-tocopheryl acetate added to the corn oil so that the final diet contained 2,000 IU of vitamin A, 283 IU of vitamin D, and 10 mg of vitamin E/100 g.
[5] The mineral mixture had the following composition: (in grams) $CaHPO_4$, 816.60; NaCl, 292.50; $MgSO_4$, 120.30; $CaCO_3$, 200.08; $FeSO_4 \cdot 7H_2O$, 56.60; KI, 1.66; $MnSO_4 \cdot 2H_2O$, 9.35; $ZnCl_2$, 0.54; $CuSO_4 \cdot 5H_2O$, 1.00; and $CoCl_2 \cdot 6H_2O$, 0.48.
[6] Hartsook et al. (2).

TABLE 1

Composition of experimental diets [1]

Diet no.	Treatment	Additive(s) per gram of diet [2]						
		10 μg DOP [3]	20 mg GABA [4]	20 mg glycine	20 mg lysine	250 mg casein	3.96 mg K	10 μg pyridoxine·HCl
	−DOP							
	−Pyridoxine							
1	−K							
2	+K						×	
	+Pyridoxine							
3	−K							×
4	+K						×	×
	+DOP							
	−Pyridoxine							
5	−K	×						
6	+K	×					×	
7	−K + GABA	×	×					
8	+K + GABA	×	×				×	
9	−K + glycine	×		×				
10	+K + glycine	×		×			×	
11	−K + lysine	×			×			
12	+K + lysine	×			×		×	
13	−K + casein	×				×		
14	+K + casein	×				×	×	

[1] See text.
[2] With the exception of casein, substances were added to the basal diet as premixes in glucose monohydrate.
[3] Deoxypyridoxine.
[4] γ-Aminobutyric acid.

with Tukey's test (13, p. 251). The heterogeneous variances were left as single estimates. In cases involving 2 treatments having heterogeneous variance, the standard error of the difference between means was based on the pertinent individual heterogeneous variances. In cases involving one treatment with heterogeneous variance, the standard error of the difference between means was based on the individual heterogeneous variance as well as the pooled estimate of the homogeneous variance. Throughout this paper the terms "significant" or "significantly" refer to statistical significance.

RESULTS

Table 2 presents the data observed for survival time, weight gains, food consumption and the weights of brains and livers of the experimental animals. Table 3 indicates the results of comparisons of the effects of the various treatments and the significance to be attached to the observed differences between treatment means.

Survival. One or more animals from treatments 5, 6, 8, 10, 11, 12 and 14 did not survive until day 28 or 29. Two factors were significant in reducing survival: supplemental casein and the increment of K represented by raising the dietary K from a deficient level (0.0005%) to an adequate (0.336%) level. The effect of the increase in dietary K in decreasing survival was most striking when accompanied by the feeding of supplemental casein.

Gain of body weight. Three factors (pyridoxine, supplemental glycine, and K) were significant in increasing gain. Two factors (DOP and supplemental casein) were significant in reducing gain. Although the overall effect of added K was to increase gain (i.e., in the absence of pyridoxine and DOP, and in the presence of pyridoxine), a significant reversal of this effect took place when supplemental casein appeared in the diet.

Food consumption. Two factors (pyridoxine and supplemental K) were significant in increasing food consumption. DOP was significant in decreasing food consumption. The normal quantity of dietary K produced significantly increased food consumption in the absence of pyridoxine and DOP, in the presence of pyridoxine, and in the presence of DOP, supplemental lysine and supplemental casein.

Liver weight. Three factors (pyridoxine, supplemental glycine, and K) were significant in decreasing liver weight. The normal amount of dietary K significantly reduced liver weight in the absence of pyridoxine and DOP.

Brain weight. Two factors (DOP and supplemental casein) were significant in increasing brain weight. Three factors (normal pyridoxine, normal K and supplemental glycine) were significant in decreasing brain weight. The effect of K in this manner was significant in the absence of pyridoxine and DOP, in the presence of pyridoxine, in the presence of DOP, and in the presence of supplemental glycine.

Table 4 presents the data observed for the content of potassium, sodium and magnesium in the livers, brains, skeletal muscles and whole blood of the experimental animals. Table 5 shows the result of comparisons of the effects of the various treatments and the significance to be attached to the observed differences between treatment means.

Brain. No significant alterations in the cation concentrations in the brain were observed. Of interest are the trends evidenced by sizeable though nonsignificant shifts in cation concentrations under certain experimental treatments. Pyridoxine and DOP tended to lower the concentrations of the 3 cations. The amino acids and the supplemental casein tended to increase K and to decrease Na and Mg. Noteworthy is the lower K and Na content of brains when, in the presence of supplemental lysine and casein, K-normal rather than K-deficient diets were fed.

Liver. No significant alteration in liver concentration of the cations was observed under the imposed experimental conditions. However, as was the case with brain tissue, trends toward significant alterations are of interest. When a normal level of K was added to a K-deficient diet in the presence of added GABA, glycine and lysine (but not casein), liver K concentrations tended to decrease, whereas the liver Na concentration increased.

TABLE 2

Effects in male rats of potassium, pyridoxine, deoxypyridoxine, and supplementary amino acids or casein upon survival, weight gain, food consumption, and organ weights [1]

Pyridoxine	−	+	−	−	−	−	−
Deoxypyridoxine	−	−	+	+	+	+	+
Other	−	−	−	GABA	Glycine	Lysine	Casein
K-deficient diet no.	1(7) [2]	3(7)	5(6)	7(7)	9(7)	11(6)	13(7)
Survival, days	28.7 ±0.5	28.7 ±0.5	28.1 ±1.5	28.7 ±0.5	28.7 ±0.5	28.6 ±0.5	28.7 ±0.5
Wt gain, g/day	−0.13 ±0.10	0.13 ±0.13	−0.37 ±0.18	−0.28 ±0.19	−0.09 ±0.10	−0.37 ±0.19	−0.41 ±0.12
Food consumption, g/day	4.5 ±0.5	5.6 ±0.9	4.3 ±0.3	5.1 ±1.2	5.4 ±1.3	4.8 ±0.7	4.4 ±0.8
Liver wt, g dry matter/100 g body wt	1.51 ±0.14	1.32 ±0.11	1.52 ±0.18	1.56 ±0.20	1.19 ±0.06	1.51 ±0.23	1.52 ±0.16
Brain wt, g dry matter/100 g body wt	0.52 ±0.07	0.46 ±0.04	0.52 ±0.04	0.53 ±0.07	0.48 ±0.05	0.50 ±0.04	0.55 ±0.04
K-adequate diet no.	2(7)	4(7)	6(5)	8(6)	10(5)	12(5)	14(1)
Survival, days	28.7 ±0.5	28.7 ±0.5	27.6 ±1.8	28.7 ±0.5	28.3 ±1.1	28.0 ±1.5	23.4 ±3.0
Wt gain, g/day	1.28 ±0.38	3.02 ±0.33	−0.23 ±0.27	−0.37 ±0.26	0.18 ±0.38	−0.38 ±0.18	−0.68 ±0.13
Food consumption, g/day	7.1 ±0.9	10.8 ±0.3	5.6 ±0.9	5.5 ±0.5	5.8 ±1.1	5.7 ±0.6	5.4 ±0.6
Liver wt, g dry matter/100 g body wt	1.26 ±0.19	1.15 ±0.21	1.43 ±0.14	1.50 ±0.11	1.14 ±0.17	1.45 ±0.34	1.16
Brain wt, g dry matter/100 g body wt	0.33 ±0.05	0.22 ±0.01	0.46 ±0.01	0.51 ±0.05	0.39 ±0.06	0.52 ±0.08	0.53

[1] Mean ± SD (SD of individuals in the group).

[2] Numbers in parentheses indicate number of observations.

TABLE 3

Comparisons of the effects of dietary pyridoxine, deoxypyridoxine and supplementary amino acids or casein upon survival, weight gain, food consumption and organ weights of male rats

Comparisons	Basis of comparison: Means of treatment numbers	Differences [1]				
		Survival	Gain/day	Food/day	Liver wt	Brain wt
		days	*g*	*g*	*g dry matter/100 g body wt*	
(Normal pyridoxine) — (low pyridoxine) [2]	(3,4) — (1,2)	0	1.00*	2.4**	—0.14*	—0.09**
(DOP) — (no DOP) [3]	(5,6) — (1,2)	—0.8	—0.87**	—0.8*	0.09	0.06**
(GABA) — (no GABA) [4]	(7,8) — (5,6)	0.8	—0.03	0.3	0.06	0.03
(Supplemental glycine) — (no supplemental glycine)	(9,10) — (5,6)	0.6	0.35**	0.6	—0.30**	—0.05*
(Supplemental lysine) — (no supplemental lysine)	(11,12) — (5,6)	0.4	—0.07	0.2	0.01	0.03
(Supplemental casein) — (no supplemental casein)	(13,14) — (5,6)	—1.8**	—0.25**	—0.1	0.01	0.05*
(Normal K) — (low K)	(2,4,6,8,10,12,14) — (1,3,5,7,9,11,13)	—1.0**	0.62**	1.7**	—0.13**	—0.11**
(Normal K) — (low K):						
In absence of pyridoxine and DOP	(2) — (1)	0	1.41**	2.6**	—0.25*	—0.19**
In presence of pyridoxine	(4) — (3)	0	2.89**	5.2**	—0.17	—0.24**
In presence of DOP	(6) — (5)	—0.5	0.13	1.3**	—0.09	—0.06*
In presence of GABA	(8) — (7)	0	—0.09	0.4	—0.06	—0.02
In presence of supplemental glycine	(10) — (9)	—0.4	0.27	0.3	—0.05	—0.09**
In presence of supplemental lysine	(12) — (11)	—0.6	—0.01	1.0*	—0.06	0.02
In presence of supplemental casein	(14) — (13)	—5.3**	—0.27*	0.9*	—0.36	—0.02

[1] Difference between treatment means.
[2] To be read as follows: Response to diets containing a normal amount of pyridoxine minus response to diets containing a low amount of pyridoxine.
[3] Deoxypyridoxine.
[4] γ-Aminobutyric acid.
* Significant at $P = 0.05$.
** Significant at $P = 0.01$.

TABLE 4

Effects of potassium, pyridoxine, deoxypyridoxine, and supplemental amino acids or casein upon tissue potassium, sodium and magnesium in male rats [1]

Pyridoxine	−	+	−	−	−	−	−
Deoxypyridoxine	−	−	+	+	+	+	+
Other	−	−	−	GABA	Glycine	Lysine	Casein
a. Liver							
K-deficient diet no.	1(7) [2]	3(7)	5(6)	7(7)	9(7)	11(6)	13(7)
K, mmole/kg fresh tissue	75.9 ± 9.0	70.2 ± 13.2	76.2 ± 9.9	82.2 ± 6.6	83.6 ± 7.5	82.5 ± 5.8	78.3 ± 7.3
Na, mmole/kg fresh tissue	39.7 ± 5.4	43.1 ± 5.5	41.4 ± 9.5	42.7 ± 11.7	42.9 ± 8.3	38.9 ± 9.7	44.8 ± 7.8
Mg, mmole/kg fresh tissue	12.7 ± 1.3	11.2 ± 1.8	12.4 ± 1.6	12.4 ± 1.7	12.6 ± 1.7	11.9 ± 2.1	13.0 ± 1.5
K-adequate diet no.	2(7)	4(7)	6(5)	8(6)	10(5)	12(5)	14(1)
K, mmole/kg fresh tissue	74.8 ± 7.2	74.5 ± 6.3	78.5 ± 6.7	80.7 ± 7.5	77.1 ± 6.1	75.5 ± 12.9	84.8
Na, mmole/kg fresh tissue	40.4 ± 6.8	46.9 ± 9.9	40.7 ± 5.6	44.1 ± 9.5	49.4 ± 5.9	41.7 ± 10.6	50.3
Mg, mmole/kg fresh tissue	11.2 ± 1.6	11.1 ± 0.9	12.2 ± 0.8	12.4 ± 1.8	13.7 ± 2.1	11.5 ± 3.0	14.1
b. Brain							
K-deficient diet no.	1(7)	3(7)	5(6)	7(7)	9(7)	11(6)	13(7)
K, mmole/kg fresh tissue	73.7 ± 4.2	69.5 ± 7.4	73.2 ± 5.6	75.0 ± 8.0	74.6 ± 9.9	76.6 ± 5.8	78.4 ± 5.2
Na, mmole/kg fresh tissue	55.5 ± 9.1	51.9 ± 5.2	53.0 ± 6.8	55.0 ± 5.4	53.0 ± 9.0	55.7 ± 8.8	55.8 ± 6.4
Mg, mmole/kg fresh tissue	12.4 ± 2.0	11.2 ± 0.8	11.7 ± 1.5	11.4 ± 0.8	11.0 ± 1.6	11.6 ± 0.6	11.4 ± 1.6
K-adequate diet no.	2(7)	4(7)	6(5)	8(6)	10(5)	12(5)	14(1)
K, mmole/kg fresh tissue	75.5 ± 9.3	70.3 ± 10.7	74.5 ± 11.8	75.8 ± 11.0	75.0 ± 5.8	71.3 ± 8.5	70.7
Na, mmole/kg fresh tissue	56.2 ± 8.4	52.3 ± 11.3	58.2 ± 4.9	55.6 ± 6.5	55.1 ± 5.8	52.7 ± 4.6	50.7
Mg, mmole/kg fresh tissue	11.4 ± 1.7	11.1 ± 1.7	11.8 ± 1.3	11.7 ± 1.4	10.8 ± 0.3	12.0 ± 1.1	11.1
c. Skeletal muscle							
K-deficient diet no.	1(7)	3(7)	5(6)	7(7)	9(7)	11(6)	13(7)
K, mmole/kg fresh tissue	69.0 ± 10.9	57.3 ± 4.0	72.2 ± 14.2	76.2 ± 10.7	68.4 ± 7.4	75.5 ± 4.1	80.8 ± 5.9
Na, mmole/kg fresh tissue	48.4 ± 9.9	53.9 ± 6.0	47.9 ± 8.4	49.8 ± 6.8	51.8 ± 6.3	46.9 ± 9.4	51.2 ± 5.4
Mg, mmole/kg fresh tissue	18.8 ± 8.1	15.0 ± 1.6	20.9 ± 3.1	20.4 ± 7.7	16.3 ± 1.6	18.2 ± 2.9	17.1 ± 3.3
K-adequate diet no.	2(7)	4(7)	6(5)	8(6)	10(5)	12(5)	14(1)
K, mmole/kg fresh tissue	92.3 ± 7.4	82.2 ± 7.6	94.1 ± 10.1	88.9 ± 9.5	89.8 ± 10.3	93.6 ± 7.2	82.0
Na, mmole/kg fresh tissue	34.5 ± 6.8	34.8 ± 7.5	33.9 ± 2.6	38.0 ± 3.5	34.5 ± 6.0	39.1 ± 4.1	62.8
Mg, mmole/kg fresh tissue	14.4 ± 5.1	12.5 ± 1.6	18.1 ± 9.3	17.1 ± 5.9	13.8 ± 1.6	17.8 ± 5.8	15.3
d. Whole blood							
K-deficient diet no.	1(7)	3(7)	5(6)	7(7)	9(7)	11(6)	13(7)
K, mmole/liter	24.1 ± 10.2	20.0 ± 4.7	28.0 ± 6.3	28.6 ± 6.0	29.9 ± 5.0	29.0 ± 5.2	26.3 ± 8.0
Na, mmole/liter	93.6 ± 10.0	96.5 ± 6.7	96.8 ± 8.8	94.1 ± 8.4	88.9 ± 9.8	90.6 ± 8.0	96.1 ± 10.7
Mg, mmole/liter	11.0 ± 3.7	10.9 ± 4.4	9.4 ± 1.5	10.7 ± 2.3	10.9 ± 2.3	10.8 ± 2.6	10.9 ± 2.4
K-adequate diet no.	2(7)	4(7)	6(5)	8(6)	10(5)	12(5)	14(1)
K, mmole/liter	25.0 ± 8.6	27.7 ± 11.5	25.6 ± 4.3	21.7 ± 7.0	25.9 ± 6.2	22.4 ± 7.7	38.7
Na, mmole/liter	87.4 ± 10.2	93.5 ± 10.2	101.5 ± 16.4	105.2 ± 13.3	95.1 ± 8.5	105.7 ± 11.1	102.4
Mg, mmole/liter	10.0 ± 3.3	11.5 ± 1.7	12.9 ± 3.2	11.4 ± 2.5	11.7 ± 1.8	10.7 ± 3.8	7.7

[1] Mean ± SD (SD of individuals in the group).
[2] Numbers in parentheses indicate number of observations.

TABLE 5

Comparisons of the effects of dietary pyridoxine or deoxypyridoxine, potassium, and supplemental amino acids or protein upon the potassium, sodium and magnesium content of rat tissues

Comparisons	Basis of comparison: Means of treatment numbers	Differences of tissue mineral content [1]											
		Brain			Liver			Muscle			Blood		
		K	Na	Mg	K	Na	Mg	K	Na	Mg	K	Na	Mg
(Normal pyridoxine) — (low pyridoxine) [2]	(3,4) — (1,2)	−4.7	−3.7	−0.8	−3.1	5.0	−0.7	−10.9**	2.8	−2.9	−0.8	4.5	0.7
(DOP) — (no DOP) [3]	(5,6) — (1,2)	−0.8	−0.4	−0.1	1.9	1.1	0.4	1.5	0	3.0	2.3	8.4*	0.5
(GABA) — (no GABA) [4]	(7,8) — (5,6)	1.6	−0.1	−0.2	4.2	2.2	0.1	−0.1	2.9	−0.7	−1.5	0.3	0
(Supplemental glycine) — (no supplemental glycine)	(9,10) — (5,6)	1.0	−1.5	−0.9	3.6	4.5	0.7	−4.9	3.1	−4.4*	1.3	−7.4	0.2
(Supplemental lysine) — (no supplemental lysine)	(11,12) — (5,6)	0.4	−1.1	0	2.0	−0.9	−0.6	1.6	1.9	−1.6	−0.9	−1.4	−0.2
(Supplemental casein) — (no supplemental casein)	(13,14) — (5,6)	3.6	−0.3	−0.4	1.8	4.4	0.9	−1.3	11.1**	−2.7	1.0	−2.0	−0.5
(Normal K) — (low K)	(2,4,6,8,10,12,14) — (1,3,5,7,9,11,13)	−0.8	0.5	−0.1	−1.5	2.0	−0.3	18.4**	−13.6**	−2.6*	−1.3	3.7	−0.5
(Normal K) — (low K):													
In absence of pyridoxine and DOP	(2) — (1)	1.8	0.7	−1.0	−1.1	0.7	−1.5	23.3**	−13.9**	−4.4	0.9	−6.2	−1.0
In presence of pyridoxine	(4) — (3)	0.8	0.4	−0.1	4.3	3.8	−0.1	24.9**	−19.1**	−2.5	7.7	−3.0	0.6
In presence of DOP	(6) — (5)	1.3	5.2	0.1	2.3	−0.7	−0.2	21.9**	−14.0**	−2.8	−2.4	4.7	3.5*
In presence of GABA	(8) — (7)	0.8	0.6	0.3	−1.5	1.4	0	12.7*	−11.8**	−3.3	−6.9	11.1	0.7
In presence of supplemental glycine	(10) — (9)	0.4	2.1	−0.2	−6.5	6.5	1.1	21.4**	−17.3**	−2.5	−4.0	6.2	0.8
In presence of supplemental lysine	(12) — (11)	−5.3	−3.0	0.4	−7.0	2.8	−0.4	18.1**	−7.8	−0.4	−6.6	15.1*	−0.1
In presence of supplemental casein	(14) — (13)	−7.7	−5.1	−0.3	6.5	5.5	1.1	1.2	11.6	−1.8	12.4	6.3	−3.2

[1] Differences between treatment means. Concentrations expressed as mmole/kg of fresh tissue for brain, liver, and muscle; as mmole/liter for whole blood.
[2] To be read as follows: Response to diets containing a normal amount of pyridoxine minus response to diets containing a low amount of pyridoxine.
[3] Deoxypyridoxine.
[4] γ-Aminobutyric acid.
* Significant at $P = 0.05$.
** Significant at $P = 0.01$.

Muscle. Several significant alterations in the muscle concentrations of the cations were observed in many of the experimental treatments. The potassium concentration was reduced by pyridoxine, but it was increased in the presence of the normal quantity of dietary K. The effect of the normal K levels in elevating the muscle K was significant in the presence of all remaining treatments except supplemental casein.

Sodium concentration was increased by supplemental casein and reduced by supplemental K. The effect of the normal K in depressing the muscle Na was significant in the absence of pyridoxine and DOP, and in the presence of pyridoxine, DOP, GABA and supplemental glycine.

Magnesium concentration was decreased by supplemental glycine and K.

Blood. No significant alterations in K concentrations were observed, although whole blood K concentration showed a tendency to decrease when adequate K was added to a K-deficient diet in the presence of GABA, glycine and lysine, but not in the presence of casein.

Na concentration in whole blood was significantly increased with DOP treatment. When adequate K was added to a K-deficient diet in the presence of supplemental lysine a significant increase in Na concentration was observed. Nonsignificant changes were observed when adequate K was added to a K-deficient diet in the presence of GABA and supplemental glycine.

Whole-blood Mg concentration was significantly increased when adequate K was added to a K-deficient diet in the presence of DOP.

DISCUSSION

Effects of inanition upon the responses observed. It is recognized that the feeding of experimental diets deficient in varying degrees in a nutrient (s) required for growth results in differing degrees of inanition. Therefore, the observed food intakes, weight gains and survival times are criteria of the adequacy and nutritional balance of the diets. However, such responses are confounded by the accompanying inanition.

Effects of pyridoxine and its antagonist, DOP. The significant decreases in weight gain and food consumption found in both pyridoxine deficiency and DOP treatment are similar to previous observations (2–4). The increase in liver weight in pyridoxine deficiency agrees with previous findings (14). The significant increase in brain weight in pyridoxine deficiency and in DOP treatment is of interest since, to the writers' knowledge, such an effect has not been previously reported. However, inanition cannot be ruled out as causative of this effect, since the feeding of restricted amounts of a nutritionally complete diet has been shown to increase brain weight in the growing rat (15).

The effect of pyridoxine deficiency upon muscle K and Na appears clear — as the deficiency develops, the K concentration increases and the Na concentration decreases. The disparity is enhanced further by the addition of DOP to the diet. These results are in agreement with the observations of Hsu et al. (4). That DOP significantly increased blood Na extends the importance of the findings of Hsu et al. (4) concerning increased serum Na in pyridoxine deficiency.

Effects of amino acids or protein. Neither GABA nor lysine produced significant changes in any criteria. Supplemental glycine significantly increased body weight gain and decreased liver and brain weights. The reduction of muscle Mg by glycine supplementation is of interest, and to the authors' knowledge has not been previously reported.

The stress of added dietary protein imposed on pyridoxine-deficient animals is striking, with survival and body weight gain being significantly reduced and brain weight and muscle Na being significantly increased. These findings of the effect of protein stress with pyridoxine and K-deficient diets parallel those of Bunce et al. (16) who reported growth restriction and increased mortality when dietary protein was increased in diets whose Mg content was inadequate.

Effect of potassium. The principal effect of K deficiency attained significant levels only in the skeletal muscle — the effects were the expected ones, namely, a decrease in K and an increase in Na. The

significant increase in Mg has not been previously reported to our knowledge. The ions moving into the muscle have an ionic strength approximating the K deficit in the tissue. This, in an indirect way, confirms previous work which showed that changes in the membrane potentials of muscle following a high rate of ion extrusion are not due to changes in ionic strength in the muscle fiber (5).

The effects of a K deficiency in the presence of the various dietary additives are of special interest. In general, K added to a K-deficient diet increases food consumption, decreases liver and brain weight, increases weight gain, increases muscle K, decreases muscle Na and has no significant effect on survival. An exception to these generalizations for the various additives is casein. Although added K in the presence of supplemental casein had no significant effect on liver and brain weight, it increased food intake, significantly decreased gain and survival, and completely reversed the usual effect of increased muscle K and reduced muscle Na — with casein there was no significant change in muscle K, while concurrently, muscle Na was significantly increased.

In future experimental work the involvement in protein synthesis of the factors considered here should be carefully considered. Of significance in this respect are the findings that accumulation of cystathionine in brain of pyridoxine-deficient rats was not uniform throughout the organ (17), that rat urinary excretion of leucine, isoleucine and valine were altered in Mg deficiency (16), and that supplementing swine rations with K reduced the free basic amino acids of blood plasma, skeletal muscle and kidney tissue (7).

ACKNOWLEDGMENT

The authors are indebted to Dr. K. R. Bennett for suggestions regarding statistical analysis of the data.

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Effects of Arginine or Serine on the Requirement for Glycine by the Chick

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ABSTRACT Six experiments were conducted to determine the effect of arginine or serine in the diet on the response of the chick to supplementary glycine. With 24% casein diets, growth was improved by additional glycine when zero or 0.4% L-arginine was added to the diet but not when 0.8 or 1.2% was added. No response to glycine was observed when 35% casein or 26.2% of an amino acid mixture was used, regardless of the arginine level. However, when serine was omitted from the amino acid mixture, glycine was needed for maximal growth. In addition, serine improved growth when glycine was omitted from the amino acid mixture. Under these conditions there was no limitation of glycine-serine interconversion in the chick.

The need for glycine in the diet of the growing chick was shown by Almquist et al. in 1940 (1) and Almquist (2). He reported that chicks fed 30% casein diets grew poorly, were weak and had low muscle creatine. These diets supplemented with 1.5% glycine produced optimal growth, whereas higher levels proved to be toxic. Almquist and Grau (3) found that a glycine-free amino acid mixture as a source of nitrogen in the chick diet could not support optimal growth. Their diets, however, contained a racemic mixture of amino acids and the "optimal growth" attained was only about 4 g daily.

Greene et al. (4) observed improved body weight gains in chicks when 2.0% glycine was added to a diet containing an amino acid mixture as a sole source of nitrogen. The diet, however, contained no serine and the experiment was conducted on 9-day-old chicks for a period of 5 days. Klain et al. (5) reported 0.5% glycine to be essential for supporting optimal growth of 7-day-old chicks when a diet containing 30% of an amino acid mixture without serine was fed for an experimental period of 7 days. Dean and Scott (6) also observed a dietary glycine requirement of 1.6% when an amino acid mixture devoid of serine was fed to 7-day-old chicks for the same experimental period. Waterhouse and Scott (7) reported that the supplementation of casein diets with graded levels of glycine (0–4%) produced increased growth at all levels of added dietary arginine (0–1.6%).

An exception to the idea that the growing chick requires glycine was reported by Suguhara and Ariyoshi (8). They observed no growth response to the inclusion of glycine in an amino acid mixture which contained 1.9% serine when it was fed as the only source of nitrogen to 7-day-old chicks.

In our study of factors influencing the glycine requirement of chicks, one-day-old birds were used to avoid the influence which the control diet fed for 7 days might have on the response to dietary treatments during the experimental period. We confirmed the need for glycine in a casein diet but obtained no response to glycine when arginine was adequately supplied. Since casein contains 2.1% glycine (9), this relationship was studied further with a glycine-free amino acid diet. With this diet no glycine requirement was observed, but when serine was absent, glycine improved body weight gains considerably regardless of the level of arginine.

EXPERIMENTAL

One-day-old Arbor Acre chicks were weighed individually, wing-banded and divided into duplicate groups of comparable weight and weight distribution. They were housed in electrically heated batteries with wire floors and were fed ad libitum. Basal

Received for publication December 14, 1967.

diets A and B contained casein, and diet C contained an amino acid mixture (table 1).[1] All amino acid supplements in the first 3 experiments replaced equal amounts of cornstarch. In the first experiment, 4 levels of L-arginine·HCl (0, 0.4, 0.8 and 1.2%) and 3 levels of glycine (0, 2 and 4%) were tested with diet A. In the second experiment, 3 levels of L-arginine · HCl (0.4, 0.8 and 1.2%) and 4 levels of glycine (0, 0.4, 0.8 and 1.2%) were used with diet A. The same levels of L-arginine·HCl and glycine used in experiment 2 were tested in experiment 3 but the level of casein was raised from 24 to 35% (diet B).

In experiments 4, 5 and 6, diet C was used. Different levels of L-arginine·HCl, L-serine and glycine replaced equal amounts of L-glutamic acid. In experiment 4, two levels of L-arginine·HCl (1.1 and 2.2%) and 2 levels of glycine (0 and 1.2%) were tested. At the end of the experiment three whole chickens from each treatment were killed by neck dislocation and their gastrointestinal tract contents were cleaned. Each chicken was then ground, homogenized and frozen for use in nitrogen, fat and moisture determination. Nitrogen retention was determined with chromic oxide as an indicator. Excreta were collected and frozen daily during day 11 through day 13. The combined excreta were homogenized, acidified and processed according to the method of Hill et al. (10). Chromic oxide was determined by the method of Hill and Anderson (11). In experiments 5 and 6, the effect of the presence of L-serine on the response to added glycine in diets with 1.1 or 2.2% L-arginine · HCl was tested. Also in experiment 6, the whole-body analysis was carried out and the percentage nitrogen retention determined. Body weight and feed efficiency data were analyzed statistically for each experiment according to Snedecor (12).

TABLE 1

Percentage composition of basal diets

	A	B	C
	%	%	%
Casein [1]	24.00	35.00	—
Amino acid mixture	—	—	26.20 [2]
Soybean oil	5.00	5.00	5.00
Glycerol	—	—	5.00
Cellulose [3]	3.00	3.00	3.00
$CaHPO_4 \cdot 2H_2O$	3.50	3.50	1.95
$CaCO_3$	1.00	1.00	2.50
Mineral mixture	1.21 [4]	1.21 [4]	3.94 [5]
Vitamin mixture [6]	1.10	1.10	1.10
DL-Methionine	0.30	0.30	—
Chromic bread [7]	—	—	1.00
Cornstarch	60.89	49.89	50.31

[1] Vitamin-free casein, 85.00% protein (N × 6.25).

[2] Amino acid mixture provided the following: (in percent) L-histidine·HCl, 0.7; L-lysine·HCl, 2.1; L-tyrosine, 1.0; L-tryptophan, 0.4; L-phenylalanine, 1.4; L-cystine, 0.6; L-methionine, 0.8; L-threonine, 1.3; L-leucine, 2.3; L-isoleucine, 1.7; L-valine, 1.8; L-aspartic acid, 2.0; L-alanine, 1.0; L-proline, 1.0; and L-glutamic acid, 8.1.

[3] Solka Floc, Brown Company, Berlin, New Hampshire.

[4] Mineral mixture provided the following per kilogram of diet: NaCl, 5 g; KCl, 4 g; $MgSO_4$, 2.6 g; $MnSO_4 \cdot H_2O$, 200 mg; $CuSO_4 \cdot 5H_2O$, 20 mg; $FeSO_4 \cdot 7H_2O$, 200 mg; ZnO, 50 mg; $Co(Ac)_2 \cdot 4H_2O$, 10 mg; $Na_2MoO_4 \cdot 2H_2O$, 10 mg; KI, 1 mg.

[5] Mineral mixture provided the following per kilogram of diet: K_2HPO_4, 17.4 g; $NaHCO_3$, 10 g; Na_2HPO_4, 7 g; $MgSO_4$, 3.6 g; $FeSO_4 \cdot 7H_2O$, 1 g; $MnSO_4 \cdot H_2O$, 240 mg; ZnO, 70 mg, $CuSO_4 \cdot 5H_2O$, 40 mg; $Co(AC)_2 \cdot 4H_2O$, 10 mg; $Na_2MoO_4 \cdot 2H_2O$, 10 mg; KI, 1 mg; $NaSeO_3 \cdot 5H_2O$, 0.35 mg.

[6] Vitamin mixture provided the following per kilogram of diet: vitamin A palmitate, 10,000 IU; vitamin D_3, 1000 ICU; *d*-α-tocopheryl acetate, 882 IU; vitamin K_1, 2.7 mg; thiamine·HCl, 9 mg; riboflavin, 18 mg; pantothenic acid·Ca, 50 mg; niacin, 135 mg; pyridoxine·HCl, 15 mg; choline Cl (44%), 6.7 g; folacin, 6 mg; biotin, 0.46 mg; vitamin B_{12}, 0.045 mg; BHT, 0.5 g.

[7] Made of 70% whole wheat flour and 30% Cr_2O_3.

RESULTS AND DISCUSSION

In our studies using casein diets, we observed a definite relationship between the response to dietary glycine supplementation and the level of arginine in the diet. When no L-arginine·HCl was present in the diet (table 2) the group receiving 2% added glycine had improved body weight gain, although it was not statistically significant. Four percent glycine was not as effective since it depressed body weight gain slightly from that attained with a level of 2%. When 0.4% L-arginine·HCl was added to the diet, the addition of 2 or 4% glycine resulted in increased body weight gain which was statistically significant at the higher level. There was also improved gain in chicks fed the 0.8% L-arginine·HCl-supplemented diet when 2 or 4% glycine was added, but the improvement was not statistically significant. The addition of 2% glycine to the 1.2% L-argin-

[1] The authors thank the Ajinomoto Company, Inc., Takara-cho, chuo-ku, Tokyo, Japan, for their generous contribution of the amino acids used in this study. We are also grateful to Merck and Company, Inc., Rahway, New Jersey and American Cyanamid Company, Pearl River, New York, for gifts of vitamins.

ine·HCl-supplemented diet, however, did not improve body weight gain, and the depression in growth due to the addition of 4% glycine appeared to be not statistically signficant. These data do not agree with those of Waterhouse and Scott (7) since these workers reported improved growth with glycine supplementation at all levels of dietary arginine. Feed efficiency was significantly improved with each glycine level when no arginine was added. When the diets contained 0.4% L-arginine·HCl, the addition of 4% glycine significantly improved feed efficiency. At higher levels of arginine supplementation no significant improvement in feed efficiency was observed with any additional glycine.

In the second experiment somewhat lower levels of glycine were used. The same general body weight gain and feed efficiency responses were obtained with chicks fed the 24% casein diets supplemented with the same level of L-arginine·HCl as in experiment 1. The addition of 0.8% glycine to the 0.4% L-arginine·HCl-supplemented diet improved body weight gain significantly (table 3), but no further improvement was attained with 1.2% gly-

TABLE 2

Average 3-week body weight gain, feed consumption and feed efficiency of chicks fed a 24% casein diet supplemented with different levels of L-arginine·HCl and glycine (exp. 1)

Supplement		Wt gain [1]	Feed consumed	Feed / gain
Arginine	Glycine			
%	%	g	g	
—	—	94 [a]	208	2.23 [a]
—	2	136 [ab]	240	1.78 [b]
—	4	114 [ab]	210	1.84 [bc]
0.4	—	158 [bc]	306	1.96 [bc]
0.4	2	205 [cd]	338	1.65 [cd]
0.4	4	244 [d]	325	1.34 [e]
0.8	—	210 [d]	332	1.59 [cde]
0.8	2	236 [d]	368	1.58 [cde]
0.8	4	244 [d]	323	1.32 [e]
1.2	—	256 [d]	373	1.48 [de]
1.2	2	252 [d]	342	1.36 [e]
1.2	4	218 [d]	300	1.37 [e]

[1] Average of 14 chicks, 7 in each replicate. Numbers with different superscripts are significantly different ($P < 0.05$).

TABLE 3

Average 3-week body weight gain, feed consumption and feed efficiency of chicks fed 24% (exp. 2) or 35% casein diet (exp. 3) supplemented with different levels of L-arginine·HCl and glycine

Supplement		24% casein			35% casein		
Arginine	Glycine	Wt gain [1]	Feed consumed	Feed / gain	Wt gain [2]	Feed consumed	Feed / gain
%	%	g	g		g	g	
0.4	—	140 [a]	264	1.89 [a]	198 [a]	315	1.60 [a]
0.4	0.4	157 [ab]	291	1.86 [ab]	185 [a]	284	1.54 [a]
0.4	0.8	181 [b]	312	1.72 [abc]	198 [a]	286	1.44 [a]
0.4	1.2	180 [b]	302	1.68 [bcd]	208 [a]	319	1.54 [a]
0.8	—	220 [c]	331	1.50 [dc]	220 [a]	322	1.47 [a]
0.8	0.4	238 [c]	364	1.52 [cde]	220 [a]	292	1.33 [a]
0.8	0.8	244 [c]	357	1.46 [e]	218 [a]	301	1.38 [a]
0.8	1.2	240 [c]	356	1.49 [de]	237 [a]	327	1.38 [a]
1.2	—	240 [c]	367	1.54 [cde]	220 [a]	305	1.40 [a]
1.2	0.4	241 [c]	357	1.48 [e]	224 [a]	316	1.42 [a]
1.2	0.8	252 [c]	342	1.36 [e]	245 [a]	312	1.37 [a]
1.2	1.2	244 [c]	350	1.44 [e]	235 [a]	305	1.30 [a]

[1] Average of 20 chicks, 10 in each replicate. Numbers with different superscripts are significantly different ($P < 0.05$).

[2] Average of 20 chicks, 10 in each replicate; no significant differences among treatments ($P < 0.05$).

TABLE 4

Effect of glycine on average 2-week body weight gain, 7- to 14-day daily gain, feed consumption, feed efficiency, whole-body analysis and percentage nitrogen retention of chicks fed amino acid mixture diets containing 1.9% L-serine and two levels of L-arginine·HCl (exp. 4)

Supplement		Wt gain [1]	7- to 14-day daily gain	Feed consumed	Feed/gain	Whole-body analysis [2]			Nitrogen retained
Arginine	Glycine					N × 6.25	Fat	Moisture	
%	%	g	g	g		%	%	%	%
1.1	—	82 [a 3]	6.76 ± 3.01 [4]	144	1.80 [a]	16.18 [a] ± 0.28	13.52 [a] ± 2.67	68.44 [a] ± 2.42	54.57 [a]
1.1	1.2	84 [a]	6.86 ± 2.63	136	1.62 [a]	15.82 [a] ± 0.79	12.48 [a] ± 1.17	69.49 [ab] ± 0.85	60.16 [a]
2.2	—	152 [b]	12.53 ± 1.84	206	1.34 [b]	17.27 [b] ± 0.53	11.65 [ab] ± 1.32	69.43 [a] ± 0.81	71.05 [b]
2.2	1.2	145 [b]	12.41 ± 2.72	196	1.36 [b]	16.81 [b] ± 0.50	9.80 [b] ± 0.32	71.32 [b] ± 0.44	69.28 [b]

[1] Average of 8 chicks, 4 in each replicate.
[2] Whole-body analysis data is based on three determinations in duplicate for each treatment.
[3] Figures with different superscripts are significantly different ($P < 0.05$).
[4] ± SD.

cine. However, the same level of glycine improved body weight gain only slightly when the diet contained 0.8% L-arginine·HCl and did not improve it when the diet was supplemented with 1.2% L-arginine·HCl. The addition of 1.2% glycine to the diets supplemented with 0.4% L-arginine·HCl improved feed efficiency significantly. When the diet contained 35% of casein (exp. 3) there were no statistically significant differences among body weight gains and feed efficiency of groups of chicks fed the various levels of L-arginine·HCl and glycine. By analysis of variance, however, the combined groups of birds supplemented with 0.8 or 1.2% L-arginine·HCl gained significantly better than those receiving 0.4% L-arginine·HCl. These results agree with those of Wietlake et al. (13) who showed a "large" improvement of body weights when 0.5% arginine was added to a 35% casein diet. O'Dell and Savage (14) have also shown improved weight gain of chicks raised with a 35% casein diet, comparing, however, 3.5% glycine with 2% in the basal diets which contained 0.6% L-arginine·HCl.

The results of experiments 1 and 2 show that there was no response to glycine supplementation when the diets were supplemented with 0.8 or 1.2% L-arginine·HCl. Glycine improved growth, however, when the diets contained zero or 0.4% additional L-arginine·HCl.

Sugahara and Ariyoshi (8) had noted no response to glycine using an amino acid mixture, but the 7- to 14-day average gain obtained with their diets was only 7.0 g daily. We attempted to improve this diet by increasing the level of arginine to 2.2% from the 1.9% used by the authors since the lysine content of the diet was relatively high (2.1%). We also changed the mineral mix to eliminate additional chloride and added enough potassium and sodium to balance the chloride provided by the hydrochloride forms of some of the amino acids (15). In experiment 4, the effect of glycine was studied on growth, feed consumption, nitrogen retention and whole body composition with an amino acid diet containing 1.9% L-serine and 1.1% or 2.2% L-arginine·HCl. The addition of glycine did not affect the 2-week or 7- to 14-day average daily weight gain (table 4). It did, however, slightly improve feed efficiency and nitrogen retention when the diets contained 1.1% L-arginine·HCl. Whenever glycine was added to the diets, the nitrogen and fat content of the carcass decreased slightly and the moisture content increased correspondingly. The addition of 1.2% glycine to the diet with 2.2% L-arginine·HCl increased moisture content of the carcass significantly ($P < 0.05$) (table 4). That dietary glycine, regardless of the L-arginine·HCl content of the diet did not improve growth was in contrast with the studies with diets containing casein.

Since glycine and serine are metabolically interconvertible in animal metabolism, we decided to remove L-serine from the amino acid mixture and test the addition of glycine to the diet when it contained 1.1% or 2.2% L-arginine·HCl (exps. 5 and 6). The addition of 1.2% glycine to the diet devoid of serine improved the 2-week body weight gain, average 7- to 14-day daily gain and feed efficiency regardless of the level of arginine in the diet (tables 5 and 6). Also, the addition of serine to the

TABLE 5

Effect of glycine or L-serine on average 2-week body weight gain, 7- to 14-day daily gain, feed consumption and feed efficiency of chicks fed amino acid mixture diets containing 1.1% L-arginine·HCl (exp. 5)

Supplement		Wt gain [1]	7- to 14-day daily gain	Feed consumed	Feed/gain
L-serine	Glycine				
%	%	*g*	*g*	*g*	
—	—	66	4.88 ± 2.38 [2]	126	1.90
—	1.2	87	6.98 ± 2.91	149	1.71
1.9	—	80	6.34 ± 2.60	145	1.84
1.9	1.2	68	5.61 ± 2.60	124	1.86

[1] Average of 8 chicks, 4 in each replicate.
[2] ± SD.

TABLE 6

Effect of glycine or L-*serine on average 2-week body weight gain, 7- to 14-day daily gain, feed consumption, feed efficiency, whole-body analysis and percentage nitrogen retention of chicks fed amino acid mixture diets containing 2.2%* L-*arginine·HCl (exp. 6)*

Supplement		Wt gain [1]	7- to 14-day daily gain	Feed consumed	Feed/gain	Whole-body analysis [2]			Nitrogen retained
L-Serine	Glycine					N × 6.25	Fat	Moisture	
%	%	g	g	g		%	%	%	%
—	—	116 [a 3]	9.61 ± 1.75 [4]	165	1.42 [a]	16.74 [a] ± 0.31	11.61 [bc] ± 1.94	70.06 [ab] ± 1.64	76.82 [a]
—	1.2	144 [b]	11.86 ± 1.44	188	1.32 [a]	17.48 [a] ± 0.81	9.91 [a] ± 0.66	71.78 [c] ± 0.67	79.92 [b]
1.9	—	145 [b]	12.34 ± 0.67	192	1.33 [a]	17.05 [a] ± 0.23	11.94 [c] ± 0.96	69.77 [a] ± 0.63	80.46 [bc]
1.9	1.2	154 [b]	13.34 ± 2.32	192	1.28 [a]	16.85 [a] ± 0.38	10.24 [ab] ± 0.84	71.27 [bc] ± 0.80	81.88 [c]

[1] Average of 8 chicks, 4 in each replicate.

[2] Whole-body analysis is based on 3 determinations in duplicate for each treatment.

[3] Figures with different superscripts are significantly different ($P < 0.05$).

[4] ± SD.

same diet when glycine was lacking improved body weight gain, 7- to 14-day average daily gain and feed efficiency. In experiment 5, the responses, although large, were not statistically significant ($P > 0.05$). This was due to the large variation of the individual weight gain of chicks within the treatment because of the arginine deficiency. In experiment 6, however, when the analysis of variance was run on the individual weight gain of chicks instead of the average of the replicate groups within each treatment, chicks fed the diet with serine, glycine or a combination of both grew significantly better ($P < 0.05$) than those fed the same diet without serine and glycine.

In experiment 6, the addition of either serine, glycine or a combination of both to the diet improved nitrogen retention significantly ($P < 0.05$). The addition of glycine to the diet decreased fat content and increased moisture content of the carcass significantly ($P < 0.05$) regardless of the level of serine. In experiments 4, 5 and 6 the total gains for 14 days were relatively similar to those for the 7- to 14-day period. The effect of another diet during the first week on the second week growth was not tested, however.

It is evident that either serine or glycine is essential for maximal growth rate. Whenever glycine was found to be essential, serine then must have been absent or unavailable in the diet. Except for Almquist and Grau (3) who included a low level (0.4%) of DL-serine in their diets, all the workers who observed a dietary requirement for glycine using amino acid mixtures (4–6) omitted serine completely from their diets.

The response to added glycine whenever workers used casein diets supplemented with no, or low levels of, L-arginine·HCl may be due to the partial or total unavailability of serine in casein compounded by a higher need for glycine. The unavailability of serine may be due to the fact that serine in casein exists in the phospho form (16). The higher need for glycine whenever arginine is deficient may be explained by the very high correlation between total nitrogen and uric acid nitrogen excreted by the chick (17) and the sharp increase in nitrogen excreted per gram of nitrogen ingested during an essential amino acid deficiency. Snetsinger and Scott (18) also observed a higher need for glycine when the diet contained excesses of lysine, histidine or phenylalanine. Both cases may have produced the same end results since, in our case, protein synthesis was limited by the amino acid deficiency and the other amino acids, unable to be used for protein synthesis, were in excess. The higher need for glycine disappeared when casein diets were properly supplemented with L-arginine·HCl and the amount of glycine in casein was enough to support maximal growth.

The results of experiments 4, 5 and 6 indicate clearly that the requirement for glycine disappears whenever the diet is properly supplemented with serine. They also indicate that there is no metabolic limitation on the serine-glycine interconversion in chicks fed the amino acid mixture diet. The metabolic limitation, however, exists in the synthesis of serine from other sources since it becomes essential for optimal growth whenever glycine is absent in the diet.

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Kidney Function of the Progeny of Rats Fed a Low Protein Diet [1]

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ABSTRACT Maternal protein malnutrition in the rat has previously been shown to result in morphological changes in the kidneys of the young. The present study was an investigation of kidney function in these young. Pregnant Sprague-Dawley rats were assigned to a control or pair-bred group fed a diet containing 24% casein, or to the experimental group fed a diet containing 6% casein. The pair-bred females were used as foster mothers for the experimental young. Water diuresis, osmotic diuresis, and inulin clearance were determined in control and deficient young. The results indicate that dams fed a protein-deficient diet during pregnancy produce offspring that have altered kidney function, with marked reduction in glomerular filtration rate and depressed urine excretion during both water diuresis and osmotic diuresis. It is suggested that these effects may be related to previous findings of fewer and more immature glomeruli but may also be attributable to extra-renal factors. The effects may also be influenced by postnatal diet.

The progeny of rats fed a low protein diet during pregnancy show definite morphological and histochemical differences in the kidney (1) when compared with the young of adequately fed females. In newborn young of protein-restricted dams, the kidneys were disproportionately small (2) and had fewer and less well differentiated glomeruli, fewer collecting tubules, and proximal convoluted tubules with fewer convolutions. Reduced activities of acid and alkaline phosphatases were also noted. No morphological or histochemical differences were found between the kidneys of young of control and of pair-fed females (1).

The effects on the function of the kidneys of the protein-deficient young cannot be determined by morphological and histochemical studies alone. Wachstein and Bradshaw (3) suggest, however, that there is good correlation between morphological development, the amount of enzyme present, and the functional adequacy of the growing kidney. Therefore, it was postulated that kidney function of the progeny of protein-deficient females is affected. It was the purpose of this investigation to test the validity of this postulate.

MATERIALS AND METHODS

Virgin female rats of the Sprague-Dawley strain, weighing 180 to 210 g, were housed in single wire-bottom cages and mated with normal males. Pregnancy was assumed to have begun when vaginal plugs or sperm were observed. The day following the night of mating was considered day zero of pregnancy. Each female was then assigned to one of 3 groups. The control group was fed a diet containing 24% casein [2] as the sole source of protein, 8% corn oil, 62% dextrose,[3] and 6% salt mix.[4] A vitamin supplement[5] was given 3 times weekly. The second, or low protein, group was fed a diet containing 6% casein and an additional 18% dextrose. A third group, also fed the control diet, consisted of animals pair-bred to produce litters on the same days as those in the low protein group. These were used as foster mothers for the young in the low protein group. All diets were fed ad libitum

Received for publication November 27, 1967.

[1] Presented in part at the annual meeting of the Federation of American Societies for Experimental Biology, Atlantic City, New Jersey, 1968.

[2] Nutritional Biochemicals Corporation, Cleveland.

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

[4] Salt content of diet: (g/kg) $CaCO_3$, 18.0; K_2HPO_4, 19.5; $CaHPO_4$, 3.6; NaCl, 10.08; $FeSO_4 \cdot 7H_2O$, 1.5; $MgSO_4 \cdot H_2O$, 0.0075; KI, 0.015; $ZnCO_3$, 0.048; $CuSO_4 \cdot 5H_2O$, 0.018; and $MnSO_4 \cdot H_2O$, 0.138.

[5] Calculated on a per day basis, each pregnant or lactating animal received: (in milligrams) choline, 20.0; inositol, 10.0; ascorbic acid, 2.0; Ca pantothenate, 1.0; (in micrograms) *p*-aminobenzoic acid, 200.0; pyridoxine, 600.0; nicotinic acid, 600.0; riboflavin, 200.0; thiamine, 600.0; menadione, 500.0; biotin, 5.0; folic acid, 12.0; vitamin B_{12}, 0.6; vitamin A palmitate, 300 IU; vitamin D, 30 IU; and *dl*-α-tocopherol, 2.2 IU.

throughout pregnancy beginning on day zero of pregnancy. Each animal was also allowed unrestricted amounts of distilled water.

At birth, all young in excess of eight were discarded. The pups to be discarded were chosen at random. The litters of the females in the experimental group were given to the lactating pair-bred female to suckle since protein-deficient rats are usually unable to nurse their young (4–6). The litters of the females in the control group remained with their own dams.

Postnatally, the young remained with the females to which they had been assigned, except during the period when kidney function tests were performed. Dehydration of the pups would obviously introduce a source of serious error; therefore, only those animals were used whose stomachs appeared, by gross examination, to be full of milk.

During the test period, the animals were kept on clean white toweling in a partitioned box so that urination or regurgitation could be easily detected. To prevent cold exposure, which causes rats to increase their urine output and urine osmolality (7), an ambient temperature of 30–32° was maintained with the use of an overhead lamp.

In all tests, a 10 cm (4-inch) piece of no. 10, polyethylene tubing [6] was attached to a one-milliliter syringe for administration of deionized water or saline solution by stomach tube. Urine was obtained by applying suprapubic pressure and quantitatively collecting the sample with a one-milliliter syringe. In a preliminary study to assure complete bladder emptying, animals were dissected and bladders were inspected after urination was thought to be complete. This inspection indicated that the bladders were completely emptied by this method. Samples were stored at − 16°.

Blood was collected from animals anesthetized with 30 mg/kg of body weight of sodium pentobarbital.[7] Blood was taken by cardiac puncture in a one-milliliter heparinized syringe with a 27-gauge needle. Plasma was separated by centrifugation and immediately stored at − 16°.

Water diuresis was measured in newborn, and 2- and 4-day-old young of both control and protein-deficient females. Osmotic diuresis was measured in 2-, 4-, and 6-day-old young. Inulin clearance tests were performed on animals at 6 days of age. Sexes were not separated. Newborn young were not considered one day old until 24 hours after birth. At each age, 11 to 36 animals representing 6 to 8 different litters were tested.

For measurement of water diuresis, subjects were given 5% of their body weight of deionized water by stomach tube after all residual urine was expressed from the bladder. Occasionally the tube punctured the esophagus. Therefore, blue vegetable food coloring [8] was added to the water so that water which did not reach the stomach would be visible. It also provided for easy detection of regurgitation. If the total water load did not reach the stomach or if it was regurgitated, the animal was discarded. Urine samples were collected and the quantities measured 1, 2, and 3 hours after administration of the water.

For measurement of osmotic diuresis, subjects were given, by stomach tube after all residual urine had been expressed, 5% of their body weight of a 0.5 M sodium chloride solution containing 1% blue food coloring. Urine samples were collected from each animal hourly for 5 hours after the test load was given and frozen at − 16° in glass screw-cap vials. Chloride concentration of each sample was determined at low titration rate by automatic titration.[9]

A method adapted from that used by Falk (8) was used to measure inulin clearance in 6-day old rats. After all residual urine had been expressed, male and female rats were given a subcutaneous injection of a 10% solution of alkali-stable inulin in physiological saline followed 30 minutes later by 5% of body weight of water containing blue coloring. The clearance period began one hour after water administration. Urine and blood samples were collected at this time and at one and two hours thereafter. Since it is impossible to obtain hourly blood samples by cardiac puncture from rats of this age without

[6] Intramedic, Clay Adams Company, New York.
[7] Nembutal, Abbott Laboratories, North Chicago, Illinois.
[8] U. S. Certified Blue Food Coloring, McCormick & Company, Inc., San Francisco.
[9] Buchler-Cotlove Chloridometer, Buchler Instruments, Inc., Fort Lee, New Jersey.

serious hemodynamic consequences or death, only one blood sample could be taken from each animal. Therefore, 4 littermates of approximately the same size were used to obtain one clearance rate. One animal was used for the blood sample taken before the injection of inulin. A second animal was used for the urine sample taken before the injection of inulin and also for the blood and urine samples taken one hour after water administration. The third and fourth animals were used for the blood and urine samples taken 2 and 3 hours after water administration, respectively. Inulin concentration in the urine and blood samples was determined by the method of King and Wooten (9), adapted for use with the smaller samples.

The 3/4 power of body weight, previously used as a basis of comparison of animals of different sizes (10), was used for comparison of results of tests of water diuresis and osmotic diuresis. As noted above, 4 animals were used to obtain each value for inulin clearance. Since there were minor variations in body weight for each group, all data were expressed per 100 g rat before interpolation to obtain the clearance rate according to the method of Falk (8). Final results were also expressed on this basis, permitting ease of comparison with Falk's data. Data were analyzed with the Wilcoxon two-sample test for the unpaired case (11).

RESULTS

The effect of a water load on urine volumes in newborn, 2-, and 4-day-old rats during water diuresis is shown in table 1. When newborn rats in both the control and experimental groups were given 5% of their body weight of water, there was some increase in urine flow. The peak of this slight diuresis appeared at 2 hours following ingestion of the water load in both groups. The mean volume of urine excreted by the control group was significantly greater ($P < 0.005$) than in the experimental group at all hours tested.

At the age of 2 days, both groups of rats exhibited a greater response to water load one hour after ingestion than the response found in the newborn. There was a statistically significant difference ($P < 0.05$) between the quantities of urine excreted in the control and experimental groups during the first hour after intake. The differences exhibited between the 2 groups thereafter were not significant. The greatest diuresis for each group was observed in 4-day-old animals the first hour after the water load was given.

Hourly urine excretions during osmotic diuresis in 2-, 4-, and 6-day-old rats are shown in table 2. With the administration of a 0.5 M sodium chloride solution, 2- and 4-day-old rats of both groups responded by increasing their urine flow.

TABLE 1

Urine flow of newborn, two-, and four-day-old rats during water diuresis

Group	No. of animals	Body wt	Urine excretion		
			One-hour	Two-hour	Three-hour
		g		*ml/kg* $^{3/4}$	
			Newborn rats		
Control	14	7.05 ± 0.20 [1]	2.15 ± 0.26	2.84 ± 0.22	2.79 ± 0.26
Protein-restricted	12	4.4 ± 0.20	1.27 ± 0.23 [2]	1.79 ± 0.22 [2]	1.65 ± 0.24 [2]
			Two-day-old rats		
Control	11	7.5 ± 0.37	3.30 ± 0.37	2.29 ± 0.48	1.03 ± 0.22
Protein-restricted	13	4.7 ± 0.07	2.26 ± 0.45 [3]	1.63 ± 0.30	0.92 ± 0.14
			Four-day-old rats		
Control	14	10.1 ± 0.47	3.74 ± 0.57	2.01 ± 0.38	1.03 ± 0.38
Protein-restricted	11	5.7 ± 0.13	2.53 ± 0.36	1.31 ± 0.40	0.83 ± 0.18

[1] Mean ± one SE.
[2] P = < 0.005.
[3] P = < 0.05.

TABLE 2

Urine flow of two-, four-, and six-day-old rats during osmotic diuresis

Group	No. of animals	Body wt	Urine excretion				
			One-hour	Two-hour	Three-hour	Four-hour	Five-hour
		g	*ml/kg* $^{3/4}$				
					Two-day-old rats		
Control	14	7.8 ± 0.29 [1]	0.47 ± 0.09	1.56 ± 0.18	2.37 ± 0.39	2.55 ± 0.26	2.09 ± 0.34
Protein-restricted	13	5.4 ± 0.31	0.50 ± 0.11	1.27 ± 0.19	1.91 ± 0.28	1.83 ± 0.31 [2]	1.57 ± 0.28
					Four-day-old rats		
Control	14	10.8 ± 0.52	0.45 ± 0.12	1.45 ± 0.30	2.22 ± 0.38	2.29 ± 0.22	1.81 ± 0.31
Protein-restricted	14	5.7 ± 0.29	0.43 ± 0.08	1.17 ± 0.23	1.65 ± 0.31	1.58 ± 0.31 [3]	1.81 ± 0.36
					Six-day-old rats		
Control	12	13.8 ± 0.86	0.44 ± 0.06	1.15 ± 0.26	2.68 ± 0.37	3.53 ± 0.44	3.39 ± 0.30
Protein-restricted	18	8.2 ± 0.57	0.34 ± 0.05	0.79 ± 0.15	1.18 ± 0.24 [4]	1.62 ± 0.25 [5]	1.71 ± 0.22 [6]

[1] Mean ± one SE.
[2] $P = < 0.05$.
[3] $P = < 0.01$.
[4] $P = < 0.005$.
[5] $P = < 0.001$.
[6] $P = < 0.0001$.

In the 6-day-old rats, there were pronounced differences in osmotic diuresis between the 2 groups. At 3, 4, and 5 hours after hypertonic saline was given, the control group had a marked increase in urine flow. Urine volume was significantly greater than that from the experimental group.

Urine output 4 hours after saline load in 6-day-old control rats was approximately 1.5 times that found in 2- and 4-day-old animals. Five hours after the load was given, the urine excretion of 6-day-old control rats was twice that found in these animals at 4 days of age. By contrast, the production of urine by 6-day-old young in the experimental group was not significantly different from that found in this group at 2 and 4 days of age.

There were no significant differences in chloride concentration between the 2 groups at any age or within each group at the various ages.

The mean glomerular filtration rate of the experimental group, established by inulin clearance tests, was 3.84 ± 1.15 ml/hour/100 g. When this inulin clearance was compared with that of the control group, the inulin clearance in control animals was about 4 times that observed in the experimental group, or 16.31 ± 5.67 ml/hour/100 g. This difference was found to be highly significant ($P = < 0.001$).

DISCUSSION

The kidney of normal newborn animals may appear to be immature when renal function tests are evaluated by adult standards. By these standards, the function of the kidneys of the newborn is characterized by low maximal tubular excretory capacity, with a relatively high filtration fraction, decreased urine flow rates, limited capacity to concentrate urine, and delayed excretion of water loads. These parameters have been investigated in neonatal rats (8, 12, 13), guinea pigs (14), and human infants (15–19).

The kidneys of infants or young animals appear to function adequately under the usual conditions encountered in neonatal life (20). However, the kidneys may lack reserve capacity to meet unusual conditions (21).

The results of the present study indicate that dams fed a protein-deficient diet during pregnancy produce offspring that have an altered kidney function. Values obtained for glomerular filtration rate in control young in this study agree with the results of Falk (8). The severely depressed glomerular filtration rates in young of protein-deficient females, indicated by the low inulin clearances, may reduce markedly the reserve capacity of the kidney to respond to unusual conditions. These results correlate well with previous findings of fewer and more immature glomeruli in the newborn (1), a difference which has recently been found to persist in these young until at least the fourteenth postpartum day.[10]

During water diuresis, the response of the control group was similar in normal newborn and young rats to that reported previously by Falk (8). At all ages investigated, the results show that animals whose dams received the low protein diet could not adjust to excessive water intake as readily as the young of those that received the control ration. The difference between the 2 groups was apparently not due to a delayed response to water administration, since the greatest increase in urine output occurred during the same time interval following water loading in both groups at all ages studied. Rather, there was a decrease in maximal urine production by the experimental animals.

Histological studies of kidneys of newborn rats produced by females fed low protein diets during pregnancy have suggested that there are fewer glomeruli. Those which do exist appear to be less well differentiated and thus more immature than those of the controls (1). Because of the morphological differences in the glomeruli and the vastly decreased inulin clearances that were observed in the present study, it is suggested that there is less glomerular filtrate, and thus water, being delivered to the tubules.

Experiments with water diuresis using 3- and 4-day-old rats that had been unilaterally nephrectomized from 2 hours to 2 days before testing, have shown that no significant differences in urine flow were found in this group when they were compared with sham-operated littermate controls. Thus, a reduction of approximately half of the nephrons does not significantly change the amount of urine excreted (8). It was concluded that administration of 5% of the body weight of water did not induce maximal urine flow. By increasing the water load to 15%, increased urine output was obtained. Presumably then, a water load of 5% of the body weight should not have created an excessive burden for the young kidney to handle. However, when animals in the experimental group in the present study received 5% of their body weight of water, their response was less than that of the control group. It is possible that in the present study the individual nephron was modified in such a way that the presence of both kidneys still did not permit normal urine filtration. Thus, structural changes observed in the nephrons of these immature kidneys may reflect a more important determinant than the number of nephrons in reducing functional capacity.

Results of experiments with osmotic diuresis demonstrate that the young of both groups respond to a saline load by increasing urine output but do not increase chloride concentration. Since urine volume increased more rapidly and was more marked in control animals, there was a faster elimination of chloride in controls. The volume of urine excreted by animals in the control group during osmotic diuresis at 6 days was similar to that found by Falk (8) in 3- to 7-day-old rats. However, the maximal rate of urine flow appeared earlier. Falk did not give the age distribution within the group. If the average age were younger than 6 days, this might account for the difference.

Because a decrease in the rate of glomerular filtration was observed in the experimental group, it is probable that this was one of the factors which contributed to the depression of osmotic diuresis noted in this group. Changes in the rate of glomerular filtration would also alter the total amount of chloride delivered to the tubules for excretion.

The role of the morphologically altered tubules in production of the abnormal values for both water and osmotic diuresis responses in deficient animals is not clear.

[10] Unpublished data, F. J. Zeman and L. M. Ausman.

Another factor which must be considered is the effect of the postnatal diet. Postnatally, there is a high mortality rate in these young, even though they are suckled by normal dams after birth. It has been suggested that these young lacked sufficient vigor to obtain adequate nourishment by suckling. Postnatal development appeared to be the result of the sum of the direct effects observed in the newborn plus the inability to suckle adequately postnatally (2).

In this study the effects on kidney function in the animals in the experimental group cannot be said, with certainty, to be the effects of the prenatal diet alone. Kidney function must also be affected by the postnatal diet. However, the relative importance of these 2 factors cannot yet be evaluated.

There is no evidence that the postnatal food intake was affected by the process of transferring young from one dam to another. In our experience, foster mother rats accept another litter with no difficulty if the transfer is made soon after parturition. The occasional dam that rejects a litter does not feed the young. The pups die within about 48 hours (2). Litters in which this occurred were not used in this study.

The possibility cannot be dismissed that the influence on the kidney of the young, of protein deficiency during pregnancy, may be at least partially indirect. Protein may influence other factors which affect kidney function such as the rate of water absorption and adrenal medullary or cortical function (8).

ACKNOWLEDGMENT

The authors thank Dr. George A. Baker, Department of Mathematics, for advice on statistical analysis of the data.

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Effects of Dietary Deficiencies of Lipotropic Factors on Plasma Cholesterol Esterification and Tissue Cholesterol in Rats [1]

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ABSTRACT The effects were studied of dietary deficiencies of pyridoxine, pantothenic acid, inositol and choline on the cholesterol-esterifying activity in plasma and on the distribution of cholesterol in the tissues of rats. The rate of cholesterol esterification in plasma was similarly altered by the dietary deficiencies in both male and female rats. It was increased by diets deficient in pantothenic acid or choline, decreased by a diet deficient in inositol and unaffected by a dietary deficiency of pyridoxine. Accompanying the increased esterification rate in the plasma of male rats were specifically increased concentrations of cholesterol esters in liver, kidneys, heart and skeletal muscle of animals subjected to a dietary deficiency of pantothenic acid and in livers of animals subjected to dietary choline deficiency. This specific increase in the concentrations of cholesterol esters in the tissues was not produced by the other dietary deficiencies and did not occur at all in the female rats. The plasma cholesterol concentrations were normal or slightly decreased and the rates of cholesterol esterification in some of the tissues concerned, as measured in vitro, were not increased. The evidence therefore suggests that the cholesterol esters formed in the plasma probably contributed to the increased concentrations of cholesterol esters in the tissues. A possible lack of cholesterol substrate in the plasma of the female rats may explain the sex difference observed.

Sugano and Portman (1) reported that the plasma cholesterol-esterifying activity is increased in essential fatty acid-deficient rats. We (2) have observed that the choline-deficient rats excrete no cholesterol esters in their bile, while excreting the normal amounts of free cholesterol. These observations suggested that the metabolism of cholesterol esters may be altered by dietary deficiencies of lipotropic substances. We have therefore studied the effects of dietary deficiencies of choline, pyridoxine, pantothenic acid and inositol on the cholesterol-esterifying activity in plasma and on the distribution of cholesterol in the tissues of rats.

Sperry (3) was the first to demonstrate clearly that blood serum contains a cholesterol-esterifying enzyme. It is probably produced in the liver (4), but it is not identical to the enzyme which has been measured in hepatocytic organelles (5, 6). Recently, a similar enzyme has been detected in the soluble cell fraction of livers from fasted rats (7). The plasma enzyme is an acyl-transferase and the β-position of lecithin is the source of the required acyl groups (5, 8). Studies with chicken plasma have indicated the possibility that more than one such transferase may occur (9).

MATERIALS AND METHODS

Weanling male and female rats [3] were fed purified diets which were deficient in pyridoxine, pantothenic acid, inositol or choline. Diets deficient in the first 3 factors were prepared by omitting the vitamin in question from diet 1 (table 1). In addition, 0.5 g of succinylsulfathiazole USP was added to each 100 g of the inositol-deficient diet. The choline-deficient diet was prepared by omitting this nutrient from diet 2. Animals fed diet 1 served as controls for those animals fed the pyridoxine-, pantothenic acid- and inositol-deficient diets. Control animals for the choline-deficient diet were fed a commercial rat ra-

Received for publication October 9, 1967.

[1] Submitted in part for presentation at the meeting of the American Society of Biological Chemists, Inc., Chicago, April, 1967. Supported in part by the Nebraska Heart Association and in part by Public Health Service Research Grant no. AM-05235 MET from the National Institute of Arthritis and Metabolic Diseases.

[2] Lederle Corporation student fellow, summer, 1966.

[3] Obtained from Holtzman Rat Company, Madison, Wisconsin.

TABLE 1

Composition of diets

	Diet 1	Diet 2
	g/100 g diet	
Casein, vitamin-free [1]	18	18
Glucose	50	40
Sucrose	20	17
Salts (10) [1]	4	4
Cottonseed oil [2]	2	2
Cod liver oil	1	4
Hydrogenated vegetable oil [3]	5	15
	mg/100 g diet	
Thiamine·HCl	0.4	0.3
Riboflavin	0.5	0.4
Pyridoxine·HCl	0.5	0.3
Ca pantothenate	2.8	1.0
p-Aminobenzoic acid	20.0	—
Inositol	20.0	—
Choline chloride	200.0	200.0
Menadione	0.9	—
α-Tocopherol	7.5	—
β-Carotene	0.1	—
Nicotinamide	10.0	1.0

[1] Nutritional Biochemicals Corporation, Cleveland.
[2] Wesson Oil, Hunt-Wesson Foods, Fullerton, California.
[3] Crisco, Procter and Gamble, Cincinnati.

tion.[4] All groups of control animals of the same sex exhibited the same rate of growth, organ weights, and tissue cholesterol concentrations.

At the end of the feeding period, the animals were anesthetized with diethyl-ether and their abdominal cavities were opened. Blood (1–2 ml) was withdrawn from the abdominal aorta into a plastic syringe which had been rinsed with heparin solution, and was transferred to a cold, glass centrifuge tube containing 2 drops of the heparin solution (1,000 USP units/ml). The blood was thus stored in ice until all samples were collected.

The heart, liver, kidneys and a portion of thigh muscle were removed from the animal, blotted with filter paper and weighed. Samples (0.5–1.0 g) of these tissues were quickly frozen on dry ice and stored at − 40°. Portions of the liver and the kidneys were homogenized for the in vitro determination of cholesterol esterification rates.

After the blood samples were centrifuged at 2°, the plasma layers were removed and the cholesterol-esterifying activity in each plasma sample was determined (11, 12) by incubating 0.50 ml of plasma for 2.0 hours with 2.0 ml of heated rat plasma substrate containing cholesterol-1α-T.[5] The plasma substrate was prepared from pooled, citrated rat plasma which had been heated at 60° for 30 minutes. After cooling, this plasma was mixed with a small quantity of diatomaceous earth [6] coated with tritiated cholesterol and the mixture was incubated with shaking at 37° for 3 hours, at room temperature in vacuo for 20 hours, and then centrifuged. The supernatant fluid was stored at − 40°.

The reaction was stopped by adding 4 volumes of chloroform-methanol (1:1, v/v). The precipitated proteins were removed by filtration and washed with the mixed solvent and with choloform. Filtrate and washings were combined, distilled to dryness and the lipid residue was dissolved in 2.0 ml chloroform.

Cholesterol esters were isolated from 0.4 ml of the lipid extract by means of thin-layer chromatography using silica gel G [7] with gypsum binder as the support, and the solvent mixture *n*-heptane-diethyl-ether-acetic acid (135:15:1.5, v/v). After the cholesterol ester fraction was located with iodine vapor, this area of the chromatography plate was scraped into a counting vial and 15 ml of scintillation fluid [8] added. Radioactivity was measured in a Nuclear-Chicago liquid scintillation spectrometer and counting rates were corrected for quenching by the channels-ratio method. Sufficient counts were collected so that the counting error was less than 5%. A 2.0-ml sample of each heated plasma-cholesterol-1α-T substrate was carried through the entire procedure, and the blank values of radioactivity in the cholesterol ester fractions thus obtained were substracted from corresponding assay samples.

The samples of tissue were thawed, homogenized in ethanol-acetone (1:1, v/v) and filtered. The tissue residues were washed 4 times with the solvent mixture; the filtrate and washings from each sample of tissue were combined and diluted to 25.0 ml with the mixed solvent. The concentrations of total and esterified chol-

[4] Ralston Purina Company, St. Louis.
[5] Nuclear-Chicago Corporation, Des Plaines, Illinois.
[6] Celite, Johns-Manville Products Corporation, New York.
[7] Warner-Chilcott Laboratories Instruments Division, Richmond, California.
[8] Liquifier in toluene, Pilot Chemicals, Inc., Watertown, Massachusetts.

esterol in these extracts and in the remainders of the plasma samples were determined by the ferric chloride method of Rosenthal and Jud (13). The concentrations of free cholesterol were obtained by difference.

In vitro measurements of the cholesterol esterification rates in liver and kidney tissue from male rats fed the diets deficient in pyridoxine, pantothenic acid and choline were carried out according to the procedure of Deykin and Goodman (14).

RESULTS

The feeding of the deficient diets produced growth failure (table 2) and changes in the relative weights of the livers, kidneys and hearts (table 3). A few of the male rats fed the choline-deficient diet had died with hemorrhagic kidney degeneration before the time of killing; the feeding period was extended in the experiment with female rats in an attempt to produce the same degree of choline deficiency but without success.

The rate of cholesterol esterification in the plasma of both male and female rats was unchanged by feeding a diet deficient in pyridoxine, increased by feeding diets deficient in pantothenic acid or choline and decreased by feeding a diet deficient in inositol (table 4).

Cholesterol concentrations in the liver, kidneys, heart, skeletal muscle, and plasma from the animals fed the four deficient diets are shown in tables 5 and 6. Control animals were initially provided for each group of experimental animals; however, since analyses of all control animals of the same sex gave similar results, such results were combined.

In the male rats fed the pantothenic acid-deficient diet, the concentration of total cholesterol was increased in all the tissues examined, due solely to an increase in the concentration of cholesterol esters; the concentrations of free cholesterol were either unchanged or decreased. Thus, the ratio of the free cholesterol concentration to the concentration of cholesterol esters was always decreased. This same pattern was also observed in the livers of the male rats fed the choline-deficient diet.

In those male rats fed the pyridoxine- and inositol-deficient diets, the concentrations of total cholesterol in the tissues were either unchanged or decreased. Decreases were due either to parallel decreases in the concentrations of both free and esterified cholesterol or to a decrease in the concentration of only the free cholesterol. In the tissues, other than the liver, of the male rats fed the choline-deficient diet, the change in the concentration of free cholesterol was responsible for the change in the concentration of total cholesterol.

In contrast with the changes observed in the male animals, the concentration of cholesterol esters in the tissues of females was, with one exception, never increased without a similar or greater increase in the concentration of free cholesterol. The exception was the increased concentration of cholesterol esters found in heart tissue of female rats fed the inositol-deficient diet. Since, in this instance, the concentration of total cholesterol and the ratio of free cholesterol concentration to the concentration of cholesterol esters was the same as in control animals, it is considered that this increased value obtained for the concentra-

TABLE 2

Growth rates of rats fed deficient diets

Diet	Male		Female	
	Days fed diet	Growth rate	Days fed diet	Growth rate
		g/rat/day		*g/rat/day*
Control		5.4 ± 0.2 (17) [1]		4.2 ± 0.1 (30)
Pyridoxine-deficient	17	1.5 ± 0.1 (13) [a]	15	1.5 ± 0.1 (14) [a]
Pantothenate-deficient	21	1.9 ± 0.1 (15) [a]	26	1.9 ± 0.1 (14) [a]
Inositol-deficient	38	3.8 ± 0.2 (10) [a]	41	2.8 ± 0.1 (15) [a]
Choline-deficient	9	1.4 ± 0.3 (15) [a]	21	2.8 ± 0.1 (19) [a]

[1] Mean ± SE of mean. Numbers in parentheses indicate the number of animals.
[a] Value is significantly different from control value, $P \leq 0.02$, Fisher's t test.

tion of cholesterol esters is probably in error.

The concentration of cholesterol esters in heart tissue was increased in all the female experimental animals but, with the exception noted, this increase was accompanied by a similar or greater increase in the concentration of free cholesterol.

In the female rats, the concentrations of cholesterol esters were decreased in the liver, kidneys and skeletal muscle of those animals fed the pantothenic acid- and inosital-deficient diets and in the liver of those rats fed the pyridoxine-deficient diet. In these instances the concentration of free cholesterol increased, decreased or remained unchanged depending on the tissue and the particular diet fed.

The rates of cholesterol esterification, determined in vitro, were increased in the livers of the animals fed the pyridoxine- and the pantothenic acid-deficient diets and in the kidneys of the animals fed the pyridoxine- and choline-deficient diets (table 7). In the kidneys of the animals fed the pantothenic acid-deficient diet the rate was less, whereas in the livers of animals fed the choline deficient-diet the rate was the same as the rates found in the respective tissues of control animals.

DISCUSSION

Sugano and Portman (1) were the first to demonstrate that the cholesterol-esterifying activity of plasma could be influenced by dietary means. The activity was increased in the plasma of male rats fed an essential fatty acid-deficient diet and appeared to reflect an increased enzyme concentration. Increased concentrations of cholesterol esters were previously (15) observed in livers of male rats fed a fat-free, and thus essential fatty acid-deficient, diet.

In the present study, the increased concentration of the enzyme in the plasma of male rats, produced by a dietary deficiency of pantothenic acid, was found to be associated with increased concentrations of cholesterol esters in the tissues, whereas the concentrations of free cholesterol were unchanged. The same relationship was observed in the livers of male rats subjected to a dietary deficiency of choline. This occurrence of specifically increased concentrations of cholesterol esters in the tissues

TABLE 3

Organ weights of rats fed deficient diets

Diet	Male			Female		
	Liver	Kidney	Heart	Liver	Kidney	Heart
	mg/g body weight					
Control	39 ± 1.3 (13) [1]	8.2 ± 0.1 (13)	3.7 ± 0.1 (11)	43 ± 0.7 (31)	8.8 ± 0.1 (31)	3.5 ± 0.1 (31)
Pyridoxine-deficient	56 ± 0.6 (14) [a]	10.6 ± 0.1 (14) [a]	3.9 ± 0.1 (13)	47 ± 1.0 (15) [a]	9.5 ± 0.1 (15) [a]	3.7 ± 0.1 (15)
Pantothenate-deficient	53 ± 1.8 (15) [a]	10.2 ± 0.3 (15) [a]	4.1 ± 0.1 (15) [a]	38 ± 1.1 (14) [a]	8.4 ± 0.1 (14)	3.5 ± 0.1 (14)
Inositol-deficient	42 ± 1.0 (10)	7.6 ± 0.3 (9)	3.5 ± 0.1 (10)	38 ± 0.7 (15) [a]	7.7 ± 0.1 (15) [a]	3.5 ± 0.1 (15)
Choline-deficient	61 ± 1.0 (14) [a]	17.8 ± 1.2 (14) [a]	5.4 ± 0.4 (14) [a]	62 ± 1.2 (19) [a]	8.7 ± 0.3 (19)	3.8 ± 0.1 (19) [a]

[1] Mean ± SE mean. Numbers in parentheses indicate number of animals.
[a] Value is significantly different from control value, $P \leq 0.02$, Fisher's *t* test.

TABLE 4

Cholesterol esterification rates in plasma from rats fed deficient diets

Diet	Esterification rate (cpm in cholesterol esters /0.50 ml plasma/hr) [1]		
	Male		Female
	Exp. 1	Exp. 2	Exp. 3
Control	5,642 ± 348 (6) [2]	1,094 ± 131 (5)	2,661 ± 169 (10)
Pyridoxine-deficient	5,597 ± 456 (13)	1,231 ± 96 (5)	2,753 ± 164 (11)
Control	5,642 ± 348 (6)	1,094 ± 131 (5)	1,231 ± 99 (8)
Pantothenate-deficient	6,959 ± 324 (12) [a]	1,738 ± 205 (5) [a]	1,856 ± 33 (7) [a]
Control	10,342 ± 284 (11)		1,231 ± 99 (8)
Inositol-deficient	7,575 ± 361 (10) [a]		492 ± 63 (15) [a]
Control	10,342 ± 284 (11)	1,449 ± 54 (10)	9,722 ± 310 (10)
Choline-deficient	11,771 ± 428 (11) [a]	2,445 ± 166 (8) [a]	16,080 ± 572 (18) [a]

[1] Two different heated plasma-cholesterol-1α-T substrates with different concentrations of radioactivity were used in these experiments. Experiment 1 was performed during the summer months, and experiments 2 and 3 were carried out during the fall and winter.
[2] Mean ± SE of mean. Numbers in parentheses indicate number of plasma samples.
[a] Value is significantly different from control value, $P \leq 0.02$, Fisher's *t* test.

TABLE 5

Cholesterol analyses of tissues from male rats

Diet	Cholesterol concentration			Free/ester ratio
	Total	Ester	Free	
	mg/g	*mg/g*	*mg/g*	
		Liver		
Control	5.86 ± 0.32 (11) [1]	3.65 ± 0.30 (10)	2.31 ± 0.19 (10)	0.63 ± 0.07 (10)
Pyridoxine-deficient	3.96 ± 0.01 (9) [a]	2.29 ± 0.13 (9) [a]	1.67 ± 0.13 (9) [a]	0.73 ± 0.07 (9)
Pantothenate-deficient	6.83 ± 0.29 (14) [a]	4.76 ± 0.29 (14) [a]	1.99 ± 0.08 (14)	0.42 ± 0.03 (14) [a]
Inositol-deficient	6.18 ± 0.32 (10)	4.30 ± 0.29 (10)	1.88 ± 0.07 (10)	0.44 ± 0.03 (10) [a]
Choline-deficient	7.38 ± 0.30 (14) [a]	4.97 ± 0.30 (14) [a]	2.39 ± 0.08 (14)	0.48 ± 0.03 (14)
		Kidney		
Control	6.61 ± 0.18 (11)	2.24 ± 0.17 (10)	4.38 ± 0.18 (10)	1.96 ± 0.17 (10)
Pyridoxine-deficient	6.67 ± 0.11 (9)	3.02 ± 0.34 (9)	3.66 ± 0.09 (9) [a]	1.21 ± 0.14 (9) [a]
Pantothenate-deficient	7.69 ± 0.21 (12) [a]	3.43 ± 0.24 (12) [a]	4.26 ± 0.16 (12)	1.24 ± 0.10 (12) [a]
Inositol-deficient	6.24 ± 0.18 (10)	2.22 ± 0.23 (9)	4.06 ± 0.12 (9)	1.83 ± 0.20 (9)
Choline-deficient	6.08 ± 0.15 (14) [a]	2.46 ± 0.13 (14)	3.62 ± 0.08 (14) [a]	1.42 ± 0.09 (14) [a]
		Skeletal muscle		
Control	2.26 ± 0.10 (11)	1.32 ± 0.12 (10)	0.96 ± 0.08 (10)	0.73 ± 0.09 (10)
Pyridoxine-deficient	2.06 ± 0.17 (9)	1.16 ± 0.12 (9)	0.90 ± 0.13 (9)	0.78 ± 0.14 (9)
Pantothenate-deficient	2.56 ± 0.08 (14) [a]	1.71 ± 0.11 (14) [a]	0.85 ± 0.07 (14)	0.50 ± 0.05 (14) [a]
Inositol-deficient	1.69 ± 0.07 (8) [a]	0.95 ± 0.09 (8) [a]	0.74 ± 0.03 (8) [a]	0.78 ± 0.08 (8)
Choline-deficient	2.73 ± 0.11 (13) [a]	1.43 ± 0.11 (14)	1.30 ± 0.05 (13) [a]	0.91 ± 0.08 (13)
		Heart		
Control	3.86 ± 0.23 (10)	2.41 ± 0.26 (9)	1.47 ± 0.06 (9)	0.61 ± 0.07 (9)
Pyridoxine-deficient	3.60 ± 0.10 (9)	2.04 ± 0.11 (9)	1.56 ± 0.10 (9)	0.76 ± 0.06 (9)
Pantothenate-deficient	4.71 ± 0.25 (14) [a]	3.51 ± 0.25 (14) [a]	1.20 ± 0.05 (14) [a]	0.34 ± 0.03 (14) [a]
Inositol-deficient	3.29 ± 0.10 (10) [a]	1.98 ± 0.12 (10)	1.30 ± 0.05 (10)	0.66 ± 0.05 (10)
Choline-deficient	3.99 ± 0.13 (14)	2.25 ± 0.11 (14)	1.73 ± 0.10 (14) [a]	0.77 ± 0.06 (14)
		Plasma		
	mg/100 ml	*mg/100 ml*	*mg/100 ml*	
Control	19.3 ± 0.4 (23)	14.8 ± 0.2 (23)	4.5 ± 0.4 (23)	0.31 ± 0.03 (23)
Pyridoxine-deficient	13.9 ± 0.2 (3) [a]	14.2 ± 0.2 (3)	2.7 ± 0.4 (3) [a]	0.19 ± 0.03 (3) [a]
Pantothenate-deficient	19.5 ± 0.8 (6)	14.9 ± 0.5 (6)	4.6 ± 0.7 (6)	0.31 ± 0.05 (6)
Choline-deficient	19.5 ± 1.0 (10)	13.8 ± 0.4 (10)	5.7 ± 0.7 (10)	0.40 ± 0.04 (10)

[1] Mean ± SE of mean. Numbers in parentheses indicate number of animals contributing a sample of tissue.
[a] Value is significantly different from control value, $P \leq 0.02$, Fisher's t test.

TABLE 6

Cholesterol analyses of tissues from female rats

Diet	Cholesterol concentration			Free/ester ratio
	Total	Ester	Free	
	mg/g	*mg/g*	*mg/g*	
		Liver		
Control	6.01±0.09 (31) [1]	2.46±0.08 (31)	3.55±0.08 (31)	1.49±0.06 (31)
Pyridoxine-deficient	3.95±0.14 (15) [a]	1.83±0.06 (15) [a]	2.12±0.10 (15) [a]	1.17±0.05 (15) [a]
Pantothenate-deficient	4.87±0.09 (14) [a]	2.12±0.08 (14) [a]	2.75±0.14 (14) [a]	1.35±0.12 (14)
Inositol-deficient	5.33±0.18 (15) [a]	2.20±0.07 (15) [a]	3.26±0.15 (15)	1.49±0.07 (15)
Choline-deficient	11.05±0.46 (19) [a]	2.86±0.12 (19) [a]	7.20±0.36 (19) [a]	2.52±0.14 (19) [a]
		Kidney		
Control	7.65±0.12 (31)	2.23±0.06 (31)	5.42±0.09 (31)	2.47±0.07 (31)
Pyridoxine-deficient	7.56±0.11 (15)	2.29±0.06 (15)	5.27±0.09 (15)	2.32±0.06 (15)
Pantothenate-deficient	7.99±0.15 (14)	2.04±0.05 (14) [a]	5.95±0.14 (14) [a]	2.93±0.07 (14) [a]
Inositol-deficient	7.27±0.17 (15)	1.82±0.03 (15) [a]	5.46±0.15 (15)	3.00±0.06 (15) [a]
Choline-deficient	7.06±0.12 (19) [a]	2.36±0.06 (18)	4.73±0.13 (18) [a]	2.03±0.08 (18) [a]
		Skeletal muscle		
Control	2.65±0.08 (31)	1.12±0.03 (31)	1.55±0.07 (31)	1.40±0.06 (31)
Pyridoxine-deficient	2.94±0.10 (9) [a]	1.22±0.04 (9)	1.72±0.09 (9)	1.41±0.08 (9)
Pantothenate-deficient	2.59±0.09 (14)	1.01±0.04 (14) [a]	1.59±0.07 (14)	1.60±0.09 (14)
Inositol-deficient	2.10±0.08 (15) [a]	0.93±0.04 (15) [a]	1.17±0.08 (15) [a]	1.29±0.11 (15)
Choline-deficient	3.29±0.12 (19) [a]	1.37±0.09 (19) [a]	1.92±0.09 (19) [a]	1.48±0.10 (19)
		Heart		
Control	4.05±0.09 (31)	2.31±0.03 (31)	1.74±0.08 (31)	0.75±0.04 (31)
Pyridoxine-deficient	5.06±0.11 (15) [a]	2.68±0.05 (15) [a]	2.39±0.10 (15) [a]	0.89±0.02 (15) [a]
Pantothenate-deficient	4.80±0.12 (14) [a]	2.68±0.05 (14) [a]	2.12±0.09 (14) [a]	0.79±0.03 (14)
Inositol-deficient	4.28±0.08 (15)	2.52±0.05 (15) [a]	1.76±0.05 (15)	0.70±0.06 (15)
Choline-deficient	4.72±0.04 (19) [a]	2.72±0.06 (19) [a]	1.99±0.07 (19) [a]	0.73±0.03 (19)
		Plasma		
	mg/100 ml	*mg/100 ml*	*mg/100 ml*	
Control	34.4±1.2 (10)	24.7±0.7 (10)	9.7±1.3 (10)	0.44±0.05 (9)
Pyridoxine-deficient	27.0 [a 2]	21.8 [a 2]	5.2 [a 2]	0.24 [a 2]
Pantothenate-deficient	30.4±3.4 (2)	22.5±0.9 (3)	8.7±2.7 (2)	0.40±0.11 (2)
Inositol-deficient	27.5±0.5 (3) [a]	24.5±0.5 (3)	3.0±0.0 (3) [a]	0.13±0.00 (3) [a]
Choline-deficient	29.0±1.0 (11) [a]	22.1±0.8 (11) [a]	9.0±1.0 (9)	0.44±0.05 (9)

[1] Mean ± SE of mean. Numbers in parentheses indicate number of animals contributing a sample of tissue.
[2] Pooled sample.
[a] Value is significantly different from control value, $P \leq 0.02$, Fisher's *t* test.

TABLE 7

Cholesterol esterification rates in liver and kidney organelles [1] from male rats

Diet	Esterification rate	
	Liver	Kidney
	cpm/mg N/hr	
Control	15,610±1,830 (4) [2]	3,075±407 (5)
Pyridoxine-deficient	38,130±5,450 (3) [a]	6,664±861 (4) [a]
Control	11,893± 975 (4)	3,479±442 (4)
Pantothenate-deficient	25,172±3,567 (4) [a]	1,316± 87 (5) [a]
Control	40,233±5,897 (4)	2,150±139 (4)
Choline-deficient	29,856±4,844 (3)	4,227±599 (5) [a]

[1] Each reaction vessel contained the sediment from 1.0 ml of homogenate centrifuged at 40,000 × *g* for 90 minutes, 3.0 ml buffer, 2.5 mg ATP, 0.5 mg reduced coenzyme A, 3.0 mg bovine serum albumin and cholesterol-1α-T (10.0 μ Ci/19.5 mμmole) in 0.1 ml acetone. The mixtures were incubated in air at 37° for one hour with shaking. The reaction was stopped, the cholesterol esters were isolated, and their radioactivity content was determined as described.
[2] Mean ± SE of mean. Numbers in parentheses indicate number of animals contributing a tissue sample.
[a] Value is significantly different from control value, $P \leq 0.02$, Fisher's *t* test.

was not observed when the plasma enzyme concentration was unchanged or decreased. It was not observed at all in female rats even though the concentration of the plasma enzyme was similarly altered by the same dietary treatments in both the males and females.

In all the dietary deficiencies studied, the concentrations of plasma cholesterol were the same or lower than those of control animals. The ratios in plasma of the concentration of free cholesterol to the concentration of cholesterol esters were decreased in both male and female rats fed the pyridoxine-deficient diet and in female rats fed the inositol-deficient diet. Plasma from male rats fed the inositol-deficient diet was not available for analysis.

Recent evidence (16, 17) strongly suggests that an important fraction of the cholesterol esters in plasma is formed *in situ* from free cholesterol contributed by the liver. The source of the tissue cholesterol esters is in doubt. Studies in humans (18) indicate that a large portion of the tissue free and esterified cholesterol is derived from the plasma. Nevertheless, these studies do not void the possibility that the cholesterol esters were formed in the tissues from free cholesterol, a mechanism which appears to be operative in pigeon aorta during atherogenesis (19). However, studies of the cholesterol ester fatty acid patterns of blood, skin and aorta from cholesterol-fed rabbits suggest that the sterol esters accumulating in the tissues come from the plasma (20).

In the present study the accumulation of cholesterol esters in the tissues of male rats subjected to dietary deficiencies of pantothenic acid and choline cannot be attributed solely to cholesterol esterification in the tissues. The rate measured in vitro of cholesterol esterification in the kidneys of the former was less, and, in the latter case, the rate in the liver was the same as in the controls (table 7).

Thus the evidence at hand suggests that the plasma is probably the source of the increased concentrations of cholesterol esters observed in the tissues of the male rats during dietary deficiencies of pantothenic acid and choline. The deposited cholesterol esters became available in the plasma due to increased concentrations of the cholesterol-esterifying enzyme, phosphatide:cholesterol fatty acid transferase (8).

The failure of cholesterol esters to be deposited in the tissues of female rats, even though the concentration of the plasma transferase was also increased by dietary deficiencies of pantothenic acid and choline, may be due to lack of available cholesterol substrate in the plasma of the female rats.

A sex difference in the distribution of cholesterol between the serum α- and β-lipoproteins has been observed (21) in humans; in the female the plasma cholesterol is predominantly in the α-lipoprotein. The lipoprotein composition of rat serum has not been completely investigated and no differences due to sex have been reported. However, the lipid compositions of rat serum lipoproteins have been found to be similar to those of human serum lipoproteins of the same density (22) though the ratio of α-lipoprotein to β-lipoprotein is 3:1 in rat serum as compared with a ratio of 2:5 in human serum (23). Treatment of male rats with estrogens decreased the cholesterol:phospholipid ratios in both the low and high density serum lipoprotein fractions (22).

That the β-lipoprotein cholesterol is the substrate of the plasma transferase is suggested by the observation (24) that during the incubation of serum at 37° there is a shift of lipids from the β- to the α-lipoprotein fraction. Inhibitors of cholesterol esterification prevented this shift as did heating the serum to 56°.

If a significant portion of cholesterol esters deposited in tissues originates in the plasma by esterification of free cholesterol, as the above evidence suggests, then the plasma enzyme responsible for the esterification would serve a critical role in the overall process. This concept is of even greater interest since the concentration of the enzyme in plasma can be both increased and decreased by suitable manipulation of the composition of the diet. A highly significant positive correlation has been demonstrated in humans (25) between the concentration of free cholesterol in plasma and the rate of cholesterol esterification.

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Effect of Arginine on Weight Gain of Chicks Consuming Diets First-limiting in Lysine or Tryptophan [1]

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ABSTRACT The effect of excess dietary arginine on the weight gain of chicks fed diets first-limiting in lysine or tryptophan was studied. The same basic diet, with amino acids supplied by soybean protein, zein, methionine and glycine was used in all experiments. The gain of chicks fed the basic diet was limited by the lysine content of the diet, and when the diet was supplemented with lysine, by its tryptophan content. When lysine was first-limiting the addition of 0.8% or 1.3% of L-arginine·HCl reduced weight gains somewhat, but the effect was not consistent for all experiments. When tryptophan was first-limiting a marked reduction in weight gain was observed in all trials when arginine was added. This effect was not found when supplemental tryptophan was included with the arginine.

Sauberlich (1) and Harper et al. (2) found that rats grew more slowly when 4 to 5% of arginine was added to their diet. There is also evidence that lower levels of supplemental arginine may be detrimental under certain circumstances. O'Dell and Savage (3) reported that, depending on the mineral balance, 1 to 2% of arginine added to a diet limiting in lysine resulted in reduced weight gains of chicks and Lewis (4) reported a similar effect from 0.2% of arginine added to a diet limiting in tryptophan.

During investigations of a biological assay for lysine (5) we observed a consistent depression in weight gains when an excessive amount of arginine was added to a diet severely deficient in lysine.

To study this observation further we have supplemented a diet based on a mixture of soybean protein and zein with combinations of lysine, tryptophan and arginine. This diet was previously found (6) to be first-limiting in lysine and second-limiting in tryptophan.

EXPERIMENTAL PROCEDURE

The basal diet was similar to one used previously (6). It contained: (in percent) soybean protein,[2] 9.5; zein,[3] 15; corn oil, 4.0; salts,[4] 8.0; vitamins,[5] 2.0; choline chloride,[6] 0.44; DL-methionine, 0.29; glycine, 0.19; cellulose,[7] 7.0; $NaHCO_3$, 1.0; and glucose monohydrate, 52.58.

Methionine, glycine and other amino acids used as supplements were from commercial sources.[8] Additions to the basal diet were made at the expense of the glucose monohydrate.

Preliminary to all experiments, White Plymouth Rock pullets, obtained from a commercial hatchery, were fed a modification of the basal diet from day-old to 2 weeks of age. This modified diet was the same as the basal diet except that the zein and sodium bicarbonate were replaced by glucose monohydrate.

At 2 weeks of age birds whose weights were within a relatively narrow range were

Received for publication November 16, 1967

[1] Supported in part by a grant from the National Research Council of Canada and by financial support from the Ontario Department of Agriculture and Food.

[2] Soybean protein was C-1 Assay Protein purchased from Skidmore Enterprises, Cincinnati.

[3] Purified zein, purchased from Nutritional Biochemicals Corporation, Cleveland.

[4] Contained in g/kg mixture: $CaCO_3$, 252.2; K_2HPO_4, 272.3; $CaHPO_4 \cdot 2H_2O$, 218.8; $MgSo_4 \cdot 7H_2O$, 85.8; NaCl 140.9; ferric ammonium citrate (16.5–18.5% Fe), 17.3; $MnSO_4 \cdot H_2O$, 5.0; $AlNH_4(SO_4)_2$, 2.2; $ZnCl_2$, 1.2; KI conc (90% KI-10% calcium stearate), 0.75; $CuSO_4 \cdot 5H_2O$, 0.75; $CoCl_2 \cdot 6H_2O$, 0.43; $Na_2MoO_4 \cdot 2H_2O$, 0.33; and glucose monohydrate, 2.0.

[5] Contained in g/kg mixture: vitamin E conc (44,000 IU/kg), 40.8; vitamin A conc (250,000 IU/g), 2.12; vitamin D_3 conc (16,500 ICU/g), 2.40; riboflavin premix (35.2 g/kg), 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), 2.03; *p*-aminobenzoic acid, 1.00; inositol, 55.1; glucose monohydrate, 870.7.

[6] A mixture of equal parts of pure choline chloride and a 25% choline chloride concentrate.

[7] Alphafloc, purchased from Lee Chemicals Ltd., Toronto, Ontario.

[8] Amino acids were purchased from Nutritional Biochemicals Corporation, Cleveland, with the exception of L-lysine·HCl which was donated by Merck and Company, Rathway, New Jersey.

assigned to the experimental pens, 10 birds per pen unless otherwise noted, so that in each experiment the average starting weights of the groups were approximately equal.

The experimental diets were assigned to the pens at random, each diet being fed to replicate pens, and feeding was continued ad libitum for one week.

For each experiment the weight gain data were subjected to an analysis of variance (7) and the statistical significance of the pertinent comparisons planned into the experiments are given below each table.

RESULTS AND DISCUSSION

Results of experiments 1 and 2 are summarized in table 1. In experiment 1, 2 levels of supplementation with L-arginine·HCl (0.8% and 1.3%) were tested, both with and without an added 0.8% of L-lysine·HCl. Experiment 2 was a retest of certain observations made in experiment 1 using the lysine-supplemented diet and included an additional level of L-arginine·HCl at 0.4%. Also in experiment 2, the effect of a combination of arginine and tryptophan supplementation was investigated.

Weight gains increased when the basal diet was supplemented with lysine (exp. 1) and the basal diet plus lysine with tryptophan (exp. 2). This result was expected since Hill et al. (6) showed that a similar diet was first-limiting in lysine and second-limiting in tryptophan. It was clear from the results that 0.4% of L-arginine·HCl was tolerated by the chicks but 0.8% and 1.3% reduced weight gains, both in the presence and absence of supplemental lysine. However, the arginine effect was significantly greater in the presence of supplemental lysine than in its absence. Finally, the addition of arginine did not reduce weght gains when both supplemental lysine and tryptophan were added.

Since in experiments 1 and 2 the arginine was added as L-arginine·HCl the possible influence of the chloride ion on weight gain could not be ignored. Consequently, experiment 3 included a group which received arginine as the free base,

TABLE 1

Effect of excess dietary arginine on weight gains and feed consumption of chicks (exps. 1 and 2)

Diet no.	Supplement to basal diet [1]	Experiment 1 [2]		Experiment 2 [3]	
		Avg gain/ chick, 2–3 wk	Feed consumed/ group	Avg gain/ chick, 2–3 wk	Feed consumed/ group
		g	*g*	*g*	*g*
1	None	35	1065	—	—
2	0.8% of Arg·HCl	27	906	—	—
3	1.3% of Arg·HCl	27	992	—	—
4	0.8% of Lys·HCl	66	1415	55	856
5	0.8% of Lys·HCl + 0.4% of Arg·HCl	—	—	52	812
6	0.8% of Lys·HCl + 0.8% of Arg·HCl	38	940	36	654
7	0.8% of Lys·HCl + 1.3% of Arg·HCl	37	943	35	683
8	0.8% of Lys·HCl + 0.12% of Trp	—	—	78	1123
9	0.8% of Lys·HCl + 0.12% of Trp + 0.4% of Arg·HCl	—	—	70	995
10	0.8% of Lys·HCl + 0.12% of Trp + 0.8% of Arg·HCl	—	—	73	989
11	0.8% of Lys·HCl + 0.12% of Trp + 1.3% of Arg·HCl	—	—	70	1039

[1] All amino acid supplements were of L- configuration.
[2] Triplicate pens, 10 birds/pen.
[3] Triplicate pens, 8 birds/pen.

Statistical comparison of diets

Exp. 1		Exp. 2	
2 + 3 + 6 + 7 vs. 1 + 4 (effect of Arg)	$P < 0.01$	5 vs. 4	NS
		6 vs. 4	$P < 0.01$
4 + 6 + 7 vs. 1 + 2 + 3 (effect of Lys)	$P < 0.01$	7 vs. 4	$P < 0.01$
2 + 3 vs. 1 × 6 + 7 vs. 4 (Arg × Lys)	$P < 0.01$	9 + 10 + 11 vs. 8 (effect of Arg in presence of Trp)	

and a group receiving a supplement of sodium chloride at a level equivalent to that which resulted from the neutralization by the sodium bicarbonate of the hydrochloride contributed by the arginine. Furthermore, in experiment 4, in which a second investigation was made of the effect of tryptophan supplementation, all diets were adjusted to the same sodium chloride content.

The results are summarized in table 2. The depression in weight gains which followed arginine supplementation of the diet with lysine was observed regardless of the form of arginine used, and was apparently not influenced by the sodium chloride or by the addition of extra methionine to the diet (group 7). As in experiment 2, arginine did not reduce weight gains when extra tryptophan was included in the diet.

An unexpected observation was the apparent improvement in growth obtained when the basal diet without added lysine was supplemented with 1.3% of L-argin-

TABLE 2

Effect of excess dietary arginine on weight gains and feed consumption of chicks (exps. 3 and 4)

Diet no.	Supplement to basal diet [1]	Experiment 3 [2]		Experiment 4 [2]	
		Avg gain/ chick, 2–3 wk	Feed consumed/ group	Avg gain/ chick, 2–3 wk	Feed consumed/ group
		g	*g*	*g*	*g*
1	None	16	712	—	—
2	1.3% of Arg·HCl	28	935	—	—
3	0.8% of Lys·HCl	56	1180	52	1110
4	0.8% of Lys·HCl + 1.3% of Arg·HCl	34	900	31	869
5	0.8% of Lys·HCl + 1.1% of Arg [3]	26	817	—	—
6	0.8% of Lys·HCl + 0.31% of NaCl [4]	52	1203	—	—
7	0.8% of Lys·HCl + 1.3% of Arg·HCl + 0.2% of Met	—	—	26	761
8	0.8% of Lys·HCl + 0.12% of Trp	—	—	35	1286
9	0.8% of Lys·HCl + 0.12% of Trp + 1.3% of Arg·HCl	—	—	39	1406

[1] Methionine was of DL- configuration, other amino acids were L-.
[2] Triplicate pens, 10 birds/pen.
[3] L-arginine, free base.
[4] Portion of $NaHCO_3$ replaced by an amount of NaCl which would result from neutralization of the hydrochloride in 1.3% of L-arginine·HCl.

Statistical comparison of diets

Exp. 3		Exp. 4	
2 vs. 1	NS	4 vs. 3	$P < 0.01$
5 vs. 3	$P < 0.01$	9 vs. 8	NS
4 vs. 3	$P < 0.05$		
6 vs. 4	$P < 0.05$		

TABLE 3

Effect of excess dietary arginine on weight gains and feed consumption of chicks (exp. 5)

Diet no.	Supplement to basal diet [1]	Avg gain/ chick,[2] 2–3 wk	Feed consumed/ group
		g	*g*
1	None	30	814
2	1.3% of Arg·HCl	20	690
3	0.8% of Lys·HCl	57	1157
4	0.8% of Lys·HCl + 1.3% of Arg·HCl	16	569

[1] All amino acid supplements were of L- configuration.
[2] Triplicate pens, 10 birds/pen.

Statistical comparison of diets

2 + 4 vs. 1 + 3 (effect of Arg)	$P < 0.01$
3 + 4 vs. 1 + 2 (effect of Lys)	$P < 0.01$
2 vs. 1 × 4 vs. 3 (Arg-Lys interaction)	$P < 0.01$

ine·HCl. This effect, although large, was statistically nonsignificant ($P > 0.05$). However, in view of the contradictory data for experiments 1 and 3 in this respect a fifth experiment was conducted which was essentially a repetition of experiment 1, but with the omission of the 0.8% level of L-arginine·HCl. The results (table 3) appeared to confirm the data from experiment 1.

No explanation can be offered for the increased weight gain which followed arginine addition in experiment 3. A qualitative test for the presence of arginine in water extracts of diets 1 and 2 used in this experiment showed conclusively that the 2 diets had not been inadvertently interchanged.

The effect of excess arginine added to the basal diet plus lysine, which was actually a tryptophan-deficient diet, was consistent. Since previous experience with a similar diet (6) indicated it was limiting in lysine, tryptophan and arginine in that order, it is reasonable to suggest that the phenomenon with the lysine-supplemented diet could have resulted from an imbalance brought about by the addition of an amino acid with second-limiting status. This type of imbalance has been demonstrated with rats by Henderson et al. (8), Winje et al. (9) and Morrison et al. (10), most characteristically with a diet deficient in tryptophan. However, in the present series of experiments, there is little evidence from the growth data that arginine was the second-limiting amino acid in the diets. Nevertheless, it is possible that arginine shared a second-limiting status with one or more other amino acids. If this were so, little or no growth response would be anticipated from arginine supplementation alone. An examination of the amino acid composition of the soybean protein-zein diet, based on literature values for amino acid composition, suggested that glycine shared equal status with arginine as the most likely second-limiting amino acid in the basal diet supplemented with lysine, even though 0.19% of glycine was included in all diets as a supplement. However the composition of the tryptophan-deficient diet used by Lewis (4), who reported a growth depression resulting from arginine supplementation, was not such as to suggest that arginine was second-limiting.

It appears that the possibility of a special tryptophan-arginine interrelationship cannot be ignored.

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^{144}Ce-^{144}Pr as a Particulate Digesta Flow Marker in Ruminants [1]

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ABSTRACT The validity of radioactive cerium as a particulate digesta flow marker was tested in sheep by comparing its rate of fecal excretion with residues derived from different feedstuff particles upon which the radioactive cerium had been absorbed and with water-soluble and water-insoluble markers in 3 experiments. The mean retention time for radioactive cerium was related to that for residues derived from feedstuff particles upon which the radioactive cerium had been adsorbed and unrelated to that for residues derived from other concurrently fed feedstuff particles. The mean retention time for radioactive cerium was not significantly correlated with that for a water-soluble and finely divided water-insoluble marker. The mean retention time for radioactive cerium was not markedly different when adsorbed either onto hay particles or readily fermentable starch in a fourth experiment. These results suggested that radioactive cerium remained in close physical association with indigestible residues during their transit of the ruminants' gastrointestinal tract by continued adsorption onto indigestible particles or by readsorption onto other particles. Thus radioactive cerium or other rare earth elements at radiocolloidal concentrations were considered as valid flow markers of digesta particulate matter.

A number of indigestible markers have been used to measure the flow of particulate matter through the gastrointestinal tract of ruminants. The stained particle technique, as early used by German and Finnish investigators and subsequently made quantitative by Balch (1), has been used most frequently (2). However, this technique is laborious and time-consuming. Further, serious questions might be raised concerning the significance which can be attached to such flow measurements in view of the extractive nature of the staining procedure and the selectivity applied to detecting such stained residues in the feces (3).

A number of experiments have demonstrated that at radiocolloidal concentrations the rare earth ions possess strong adsorptive properties for particulate matter (4). Dysprosium and cerium are tenaciously bound to rumen digesta particulate matter in vitro (5–7). These results suggested the potential of radioactive cerium as an indigestible marker of particulate flow which would have a number of operational and interpretive advantages over existent markers. The validity of radioactive cerium as a particulate flow marker was therefore tested in vivo by comparing its flow through the gastrointestinal tract under selected conditions with that for residues derived from different-size stained particles upon which radioactive cerium had been adsorbed and with that for water-soluble and water-insoluble markers.

EXPERIMENTAL

Four experiments were conducted with yearling, wether sheep previously accustomed to the experimental housing and routine. Each experiment involved a 2-week preliminary period during which the animals received a constant, twice daily intake of the forage, a 6-day collection stall adjustment period and a fecal collection period following the administration of the markers at the time of, or admixed with, a single evening meal. Initial fecal collections were made 12 hours later. Subsequent collections were then made at 3-hour intervals for the next 37 hours and at increasingly extended intervals (6, 8, 12 and 24 hours) for the remainder of the 8- to 10-day collection periods.

Received for publication October 20, 1967.
[1] This investigation was supported in part by a grant from the Rockefeller Foundation.

Fresh feces were weighed and an aliquot (10%) was removed, frozen and subsequently thawed for analysis of polyethylene glycol (8). The remainder of each fecal collection was dried for measurement of radioactive cerium (7), chromic oxide (9), and stained particles (3). Mean retention time for stained particles was calculated as described by Castle (10).

Experiment 1. Radioactive cerium was adsorbed onto alfalfa by adding 80 μCi of radioactive cerium (^{144}Ce-^{144}Pr, carrier free) [2] to 360 g of alfalfa, 1 to 8 mm in length, suspended in 500-ml distilled water in a plastic bag. After mixing by kneading for 10 to 15 minutes, water was drained by puncturing the bag. The hay residues were dried under an infra-red lamp (yield, 287 g) and divided into three approximately equal portions.

A single-dose meal was prepared by mixing one portion of the radioactive cerium-labeled hay residues with 410 g alfalfa hay which had been premixed with 10 g polyethylene glycol (PEG,[3] mean molecular weight of 4000) and 5 g chromic oxide.

Experiment 2. Sheep previously used in experiment 1 were fed chopped alfalfa representing particle sizes ranging from approximately 0.2 to 2.5 cm in length. Stained particles of approximately 2.5-cm length were obtained by sieving an aliquot of the chopped alfalfa and staining (1) with brillant green. Radioactive cerium was then adsorbed onto these green stained particles as described in experiment 1. These particles are subsequently referred to as coarse-size dietary particles.

Particles of approximately 2-mm length were obtained by seiving an aliquot of the chopped alfalfa hay and staining with crystal violet. These particles are subsequently referred to as fine-size dietary particles. An aliquot of the chopped alfalfa was stained with basic fuchsin and is subsequently referred to as composite-size dietary particles.

A single meal was prepared by mixing portions of the green-stained and radioactive cerium-labeled coarse particles, and the blue-and-purple-stained particles with sufficient chopped alfalfa hay to compose the usual amount of the evening meal.

Experiment 3. Alfalfa stems were obtained by hand-stripping the leaves and branches from alfalfa hay. These stems were cut into lengths of approximately 3 cm and stained with basic fuchsin. These particles are subsequently referred to as coarse particles. Radioactive cerium was adsorbed onto one-half of these stained coarse particles. Smaller particles of alfalfa were obtained by sieving ground alfalfa hay, and represented particles of approximately 2-mm length. These particles were stained with crystal violet and are subsequently referred to as fine. Radioactive cerium was adsorbed onto one-half of these stained fine particles.

Four sheep, similar to those in experiments 1 and 2, were used in experiment 3 and fed a ration of ground ryegrass hay (particle size range of approximately 0.3–1.00 cm). In the first phase, 2 sheep received a single evening meal consisting of the fine stained and radioactive cerium-labeled particles together with the stained coarse particles, PEG and chromic oxide. The other 2 sheep received at the same time a single meal consisting of the coarse stained and radioactive cerium-labeled particles together with the stained fine particles, PEG and chromic oxide. The same sheep and treatments were used in phase 2 as in phase 1 with treatment-animal combinations reversed.

Although PEG and chromic oxide were fed in phase 2, they were not determined in the feces produced during this phase.

Experiment 4. Seven sheep, similar to those used in the preceding experiments, were fed the ground ryegrass hay as used in experiment 3. Single dose meals were prepared by mixing sufficient $^{144}CeCl$ in 0.05 N hydrochloric acid with aliquots of the ground ryegrass hay to provide a concentration of approximately 1 μCi/454 g. A constant, twice-daily meal consisted of 700 to 800 g of the ground hay which was replaced by a like amount of the radioactive cerium-labeled hay at the evening feeding of the single-dose meal.

Radioactive cerium (approximately 2 μCi) was adsorbed onto starch by pipetting 0.1 ml of a 0.05 N hydrochloric acid

[2] Oak Ridge National Laboratories, Oak Ridge, Tennessee.
[3] Union Carbide Company, New York, New York.

solution onto approximately one gram of cornstarch contained in a no. 000 gelatin capsule. The capsule was sealed and further encapsulated within a 4-g gelatin capsule. This capsule was administered by balling gun to 3 sheep immediately preceding the evening feeding of the single dose, radioactive cerium-labeled hay to the other 4 sheep.

RESULTS AND DISCUSSION

Accumulative excretion curves for PEG, chromic oxide and radioactive cerium during experiment 1 are plotted in figure 1.

In general, chromic oxide and PEG were excreted at a similar rate until 50–60% of either had been excreted, after which PEG was excreted at a faster rate. Radioactive cerium was excreted initially at a slower rate than either chromic oxide or PEG until approximately 10% had been excreted; at a similar but delayed rate until approximately 50 to 60% excretion; and, thereafter, at a slower rate than PEG and at a similar or slightly faster rate than chromic oxide.

The excretion patterns in figure 1 were reduced to mean retention times for statistical evaluation. The overall mean retention time for PEG (26.5 hours) was significantly less ($P < 0.05$) than that for chromic oxide (30.0 hours) which was significantly less ($P < 0.05$) than that for radioactive cerium (32.7 hours).

Recoveries of radioactive cerium in the feces excreted through 129 hours after dosage were 96.8, 109.7 and 102.5% for sheep A, B, and C, respectively. Others have reported cerium at similarly low concentrations to be essentially indigestible by ruminants (11–13).

Experiment 2 was conducted to provide some estimate of the magnitude of transfer of radioactive cerium from the feed particle upon which it was adsorbed before feeding during its transit of the gastrointestinal tract. The experimental approach was based upon the premise that residues from different-size feed particles traverse the gastrointestinal tract at different rates. Thus, if transfer of radioactive cerium to unrelated feed residues was minor, the fecal excretion pattern for radioactive cerium should be similar to that for fecal residues derived from the size of dietary particle

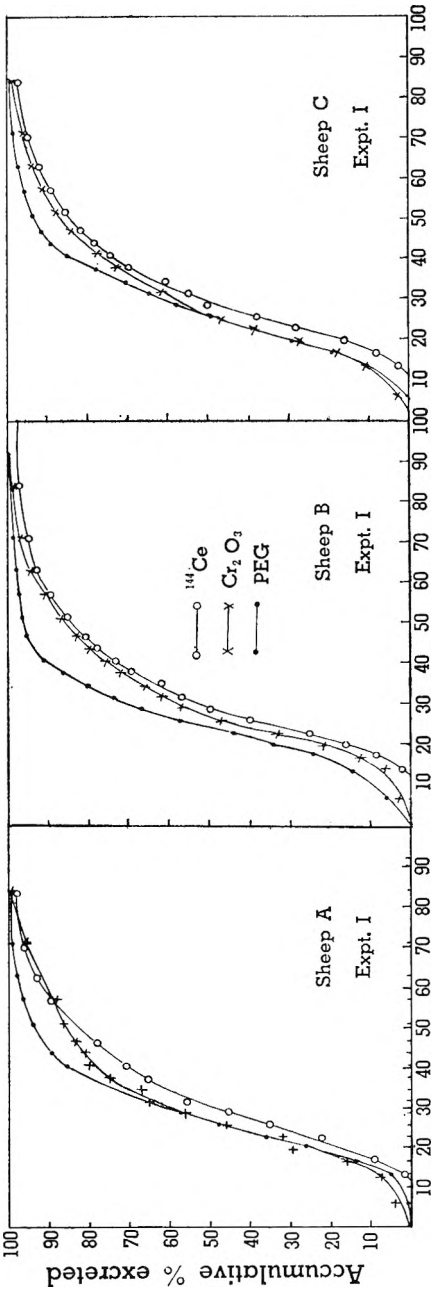

Fig. 1 Accumulative excretion curves for radioactive cerium adsorbed onto hay and for polyethylene glycol (PEG) and chromic oxide. Legend: ○——○ ^{144}Ce; x——x Cr_2O_3; ●——● PEG.

upon which the radionuclide was adsorbed and dissimilar to that for fecal residues derived from dietary particles of other sizes.

In experiment 2 (fig. 2) radioactive cerium tended to be excreted more rapidly than either mesh-size fecal residues derived from the coarse dietary particles upon which the radioactive cerium had been adsorbed until 70 to 80% of the radioactive cerium had been excreted. Thereafter, radioactive cerium was excreted at a slower rate. This change in relative excretion rate after approximately 70 to 80% excretion was in part due to the greater sensitivity for detecting radioactive cerium as compared with stained particles. This explanation is further supported by the lack of such an effect in experiment 3 (fig. 3) where larger-size samples were used for counting stained fecal residues during latter phases of excretion.

The excretion of 0–60 mesh stained fecal residues was consistently more rapid than for 0–40 mesh stained fecal residues derived from the coarse- and fine-stained dietary particles. The more rapid excretion of 0–60 as compared with 0–40 mesh fecal residues must have involved a more rapid excretion by residues smaller than 40 mesh and larger than 60 mesh since the 0–60 mesh residues included those of the 0–40 mesh size. A more rapid excretion of fecal particles smaller than 60 mesh might therefore be inferred. An appreciable, although unmeasured, proportion of the fecal dry matter passed through the 40- and 60-mesh sieves. Therefore, if fecal residues smaller than 60 mesh were excreted at only a slightly faster rate, their exclusion in the stained particle technique and their inclusion in the radioactive cerium technique could account for the differences in excretion pattern observed for radioactive cerium and the stained fecal residues derived from dietary particles upon which the radioactive cerium was adsorbed.

The overall mean retention time for radioactive cerium (table 1) was significantly less ($P < 0.05$) than for residues of either mesh size derived from the composite feed particles and less than that for the 0–40 mesh residues derived from the coarse feed particles upon which the radioactive cerium was adsorbed. The overall mean retention time for radioactive cerium was longer ($P < 0.05$) than that for residues derived from the fine dietary particles. There was no significant difference in the mean retention times of radioactive cerium and the 0–60 mesh residues derived from the coarse dietary particles and the 0–40 mesh residues derived from the fine dietary particles.

These expressions of mean retention time and their statistical significance in general affirm the significance which has been attached to the corresponding differences in excretion pattern (fig. 2) previously discussed. The significant differences in mean retention time between different mesh-size residues derived from the same dietary particles (coarse and fine, table 2) reiterates that the stained particle technique provides only a relative and not an absolute measure of the retention time of all residues derived from a given meal (2). Measures of retention time based upon this technique represent only the retention time for the composite particulate residues collected and, as implied by the results in table 2, may be appreciably different from that for the total of the indigestible residues derived from the feedstuff.

Excretion curves for the various markers used in experiment 3 are reproduced in figure 3. The stained particles excreted during phase 1 appeared unusually erratic compared with those for phase 2 of this experiment and experiment 2. Similarly there was considerable variation in the excretion of PEG in this as compared with experiment 1 (fig. 1). This might be due to the use of stained feedstuff particles of a materially different size and origin (alfalfa) to that of the basal ryegrass hay diet. This will be subsequently discussed in connection with experiment 4. Within the above limitations, the excretion curves in general appear related to each other in a similar fashion as was observed in experiments 1 and 3. Polyethylene glycol was excreted at a more rapid rate than chromic oxide in this, as in experiment 1. Radioactive cerium was consistently excreted more rapidly than were stained residues derived from the coarse (sheep 3 and 4, phase 1; and sheep 1 and 2, phase 2) or fine (sheep 1 and 2, phase 1; and sheep 3 and 4, phase 2) dietary particles upon which the radionuclide had been adsorbed.

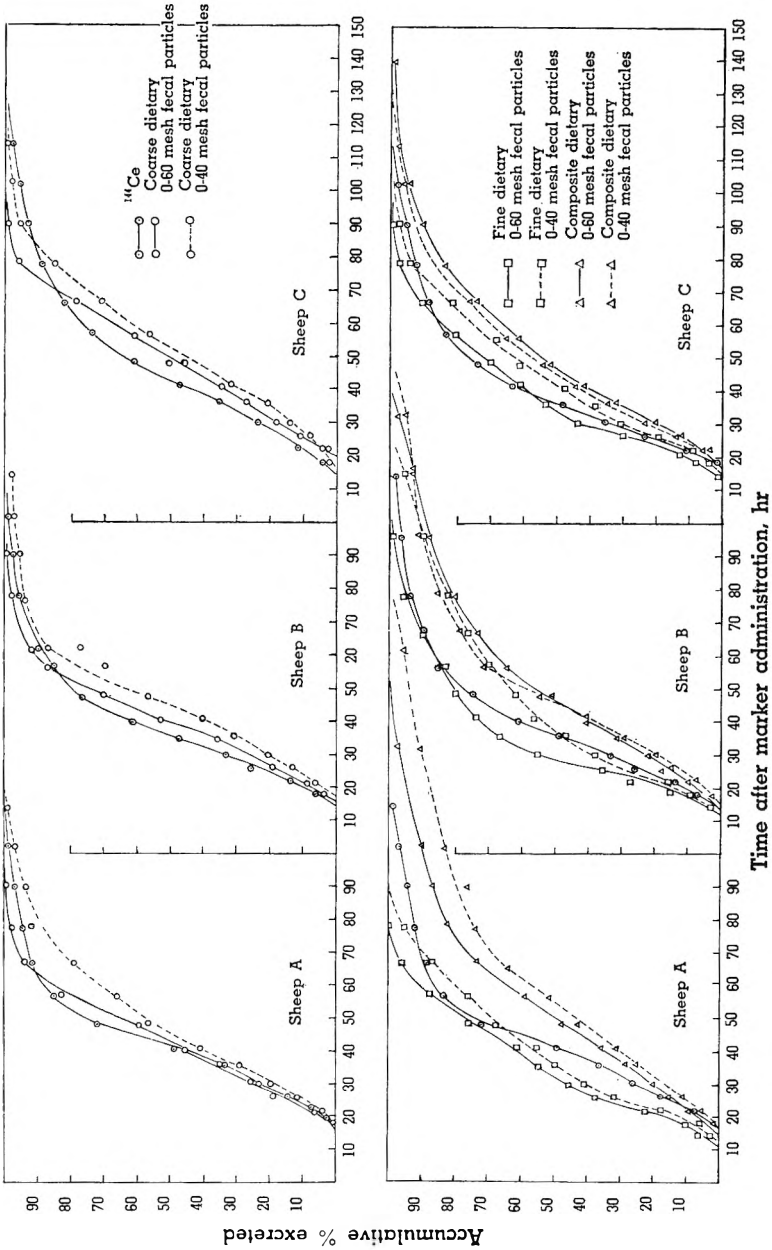

Fig. 2 Accumulative excetion curves for radioactive cerium adsorbed onto coarse, stained dietary particles and for different sizes of fecal particles derived from coarse, fine, and composite-size dietary particles. Legend: ⊙——⊙ ^{144}Ce; ○——○ coarse dietary 0–60 mesh fecal particles; ○- - -○ coarse dietary 0–40 mesh fecal particles; □——□ fine dietary 0–60 mesh fecal particles; □- - -□ fine dietary 0–40 mesh fecal particles; △——△ composite dietary 0–60 mesh fecal particles; △- - -△ composite dietary 0–40 mesh fecal particles.

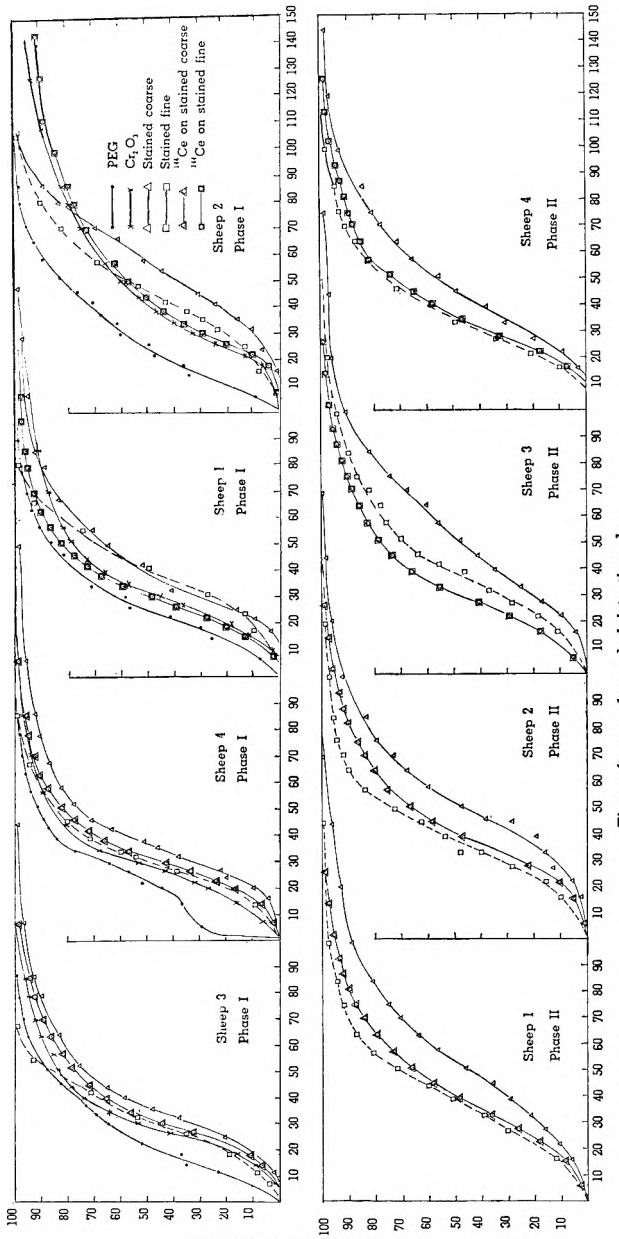

Fig. 3 Accumulative excretion curves for radioactive cerium when adsorbed onto fine or coarse stained feed particles and for fecal residues derived from such and for polyethylene glycol (PEG). Legend: ●——● PEG; x——x Cr_2O_3; △——△ stained coarse; □——□ stained fine; △——△ ^{144}Ce on stained coarse; □——□ ^{144}Ce on stained fine.

TABLE 1

Mean retention time for different-size residues derived from different-size dietary particles and for radioactive cerium adsorbed onto coarse dietary particles (exp. 3)

Dietary particle stained	Fecal marker counted	Fecal particles counted	Mean retention time			Overall mean [1]
			Sheep A	Sheep B	Sheep C	
		mesh	*hr*	*hr*	*hr*	*hr*
Coarse stem, 2.5 cm	green	0–40	50.1	46.1	53 6	49.9 [a]
	green	0–60	42.4	40.1	49 2	43.9 [b]
	^{144}Ce	total	42.0	38.4	43 1	41.1 [b]
Composite, 0.2–2.5 cm	purple	0–40	61.7	50.1	50 7	54.2 [a]
	purple	0–60	54.1	51.2	51 4	52.2 [a]
Fine, 0.2 mm	blue	0–40	40.8	41.9	46 8	43.2 [b]
	blue	0–60	37.0	33.8	39 2	36.7 [c]

[1] Means with dissimilar superscripts significantly different ($P < 0.05$) as indicated by t test using paired data.

TABLE 2

Mean retention times for radioactive cerium when adsorbed onto fine or coarse hay particles, for residues derived from the fine and coarse hay particles, and for polyethylene glycol (PEG) and chromic oxide (exp. 3)

Sheep no.	Associate markers [1]				Unassociated markers			
	^{144}Ce* fine	^{144}Ce* coarse	Fine* ^{144}Ce	Coarse* ^{144}Ce	Fine	Coarse	PEG	Cr_2O_3
	hr	*hr*	*hr*	*hr*	*hr*	*hr*	*hr*	*hr*
Phase 1								
1	29.0	—	38.0	—	—	40.0	24.6	32.6
2	45.7	—	42.8	—	—	53.3	25.8	36.3
3	—	34.6	—	34.6	29.9	—	22.8	28.9
4	—	30.6	—	37.5	30.1	—	20.0	27.3
Phase 2								
1	—	39.6	—	49.8	36.0	—	—	—
2	—	40.0	—	49.4	35.4	—	—	—
3	31.3	—	38.3	—		49.4	—	—
4	34.7	—	33.6	—		44.9	—	—
Mean	35.2	35.9	38.2	42.8	32.8	46.9	23.3	31.3

Marker	Statistical significance [2] of means from paired data								
	Phase 1					Phases 1 and 2 paired			
	PEG	Cr_2O_3	^{144}Ce* fine + ^{144}Ce* coarse	Fine	Coarse	^{144}Ce* fine	^{144}Ce* coarse	Fine* ^{144}Ce	Coarse* ^{144}Ce
Mean [3]	23.3	31.3	34.9	35.2	41.4	35.2	35.9	38.2	42.8

[1] Radioactive cerium adsorbed onto stained hay particles. Mean retention times are for marker indicated by asterisk (*).
[2] Means within each category not underscored by the same line are significantly different ($P < 0.05$) by paired t test.
[3] N = 4 animals in each mean.

Mean retention times for the various markers are summarized in table 3. The overall mean retention time for PEG was significantly less ($P < 0.05$) than that for chromic oxide. The overall mean retention time for chromic oxide was not significantly lower than that for radioactive cerium adsorbed on either fine or coarse dietary particles but was significantly less ($P < 0.05$) than that for stained residues derived from these dietary particles. These results are in general agreement with those of experiment 1 in which the increasing order of mean retention time was PEG, chromic oxide, and radioactive cerium adsorbed onto dietary particles.

TABLE 3

Mean retention time for radioactive cerium when fed adsorbed either onto hay or encapsulated starch (exp. 4)

Hay adsorbent		Starch adsorbent	
Animal no.	Mean retention time	Animal no.	Mean retention time
	hr		*hr*
1	32.5	5	35.9
2	33.7	6	30.4
3	36.7	8	32.5
4	40.4		
Mean	35.8		32.9

The mean retention time for radioactive cerium adsorbed onto coarse dietary particles (35.9 hours) was significantly less ($P < 0.05$) than that for the residues derived from these particles (42.8 hours), whereas there was no significant difference in the mean retention time for radioactive cerium adsorbed onto fine dietary particles (35.2 hours) and the residues derived from these particles (38.2 hours). There was no significant difference in mean retention time for residues derived from the two different feedstuff particles nor for radioactive cerium adsorbed onto the two different feedstuff particles.

The similarity in mean retention time in experiment 3 for the differently stained residues, radioactive cerium, and chromic oxide suggested that the flow of residues derived from the stained and radioactive cerium-labeled alfalfa particles, rather than being determined by their intrinsic properties, was largely determined by the flow of a greater mass of residues derived from the basal ryegrass diet which acted as a matrix within which the stained residues were suspended and transported. It would not be unreasonable to expect that the flow of a relatively limited number of residues within the digesta (such as derived from the stained alfalfa portion of a single meal) would be largely determined by a matrix of more numerous residues derived from a number of meals of unrelated (ryegrass hay) feedstuff particles. This is further supported by the similarity in mean retention times for the stained and radioactive cerium-labeled alfalfa hay particles in this experiment as compared with that for the radioactive cerium-labeled ryegrass hay used in experiment 4 (table 4). The same basal ryegrass hay was used in both experiments.

These differences in experiment 3 in origin and size between stained and radioactive cerium-labeled particles and the basal diet (and the relative numbers of residues derived from each) might also explain the more erratic excretion of stained residues which characterized this as compared with the other 3 experiments.

That feedstuff particle size, within the range used here, can in fact influence the mean retention time of adsorbed radioactive cerium is supported by comparing the results of experiments 1 and 2. These experiments involved the same animals and alfalfa hay, and were similarly conducted except for time and feedstuff particle size. The mean retention time for radioactive cerium adsorbed onto and fed with finely ground alfalfa (exp. 1, 32. 7 hours) was significantly less ($P < 0.01$) than when it was adsorbed onto and fed with more coarsely ground alfalfa (exp. 2, 41.1 hours).

Correlative evidence for a related flow by radioactive cerium and residues derived from radioactive cerium-labeled feedstuff was obtained from the composited results of experiments 1, 2 and 3. The mean retention times for radioactive cerium in these experiments was significantly correlated ($r = 0.60$, $P < 0.05$, $n = 11$) with the retention times for residues derived from the radioactive cerium-labeled feedstuff particles but not ($r = 0.09$) for residues derived from different-size feedstuff particles without adsorbed radiocerium. In contrast, the mean retention time for radioactive cerium in experiments 2 and 3 ($n = 9$) was not significantly correlated with that for chromic oxide ($r = 0.23$) or PEG (-0.50). The correlation between the mean retention times for chromic oxide and PEG ($r = 0.43$) was not signficant.

The collective results of experiments 1, 2, and 3 were interpreted to indicate that, when adsorbed onto feedstuff particles, radioactive cerium traverses the gastrointestinal tract at a rate more rapid than, but related to, that for 0–60 mesh residues derived from such feedstuff particles and at a rate which differs from, and is unrelated to, that for residues derived from

other feedstuff particles. Further, this rate of passage is less than, and unrelated to, that for a water-soluble marker (PEG) and a finely divided, water-insoluble marker (chromic oxide). This suggests that the flow of radioactive cerium through the gastrointestinal tract was the combined result of (a) a continued adsorption by radioactive cerium onto indigestible feedstuff residues during their transit of the gastrointestinal tract, and (b) a readsorption onto indigestible residues derived from the radioactive cerium-labeled feedstuff by any ionic radioactive cerium released through digestive solution of radioactive cerium-labeled feedstuff particles.

The order of correlation between retention times for radioactive cerium and related stained fecal residues ($r = 0.60$) indicates that only 36% of the variation in mean retention time of radioactive cerium could be attributed to variation in mean retention time of feedstuff particles ultimately excreted in the feces as residues larger than 60 mesh. Results of experiment 2 suggest that fecal particles smaller than 60 mesh may represent residues having a shorter mean retention time than for residues ultimately represented by fecal particles larger than 60 mesh. Thus, much of the variation unaccounted for in radioactive cerium retention time might be related to variaton in retention time of residues represented by fecal particles smaller than 60 mesh which were not detected by the stained particle technique. In vitro experiments (7) have shown an increased distribution within and concentration (cpm/g) of radioactive cerium on residues smaller than 60 mesh after a 16-hour incubation of radioactive cerium-labeled particles of a similar size (0.16–0.32 cm) as used in the present in vivo experiments. These considerations further support the above suggested bases for a related flow by marker and residue.

The preceding experiments provide only indirect evidence for the continued association of released radioactive cerium with other residues derived from the adsorbent. The effect of complete fermentation of the adsorbent was studied in experiment 4, in which radioactive cerium was adsorbed onto readily fermentable starch. These results are summarized in figure 4 and table 3.

Accumulative excretion curves for radioactive cerium initially adsorbed onto hay or starch were essentially superimposable with respect to their shape (fig. 4). The overall mean retention time for radioactive cerium adsorbed onto starch was not significantly different from that for radioactive cerium originally adsorbed onto hay (table 3). These results indicate that the digesta particulate matter onto which the radioactive cerium was adsorbed following its fermentation release from starch flowed through the gastrointestinal tract in a quantitatively similar fashion as the radioactive cerium originally adsorbed onto the basal hay particles.

Presumably, much of the radioactive cerium previously adsorbed onto starch was subsequently adsorbed onto mircoorganisms involved in the hydrolysis of the starch due to such organisms' proximity to the release site and their large surface area. In vitro experiments (7) have demonstrated that, with increasing time of incubation, the distribution and concentration of radioactive cerium within the microbial fraction decreases with compensatory increases in larger particulate digesta fractions. This was suggested to be the result of migration of the radioactive cerium-labeled microorganisms to, and close physical association with, the larger digesta particles. A similar process appears to have occurred within the rumen, resulting in a similar rate of excretion by the cerium regardless of its original adsorbent.

Results of experiment 4 were interpreted to indicate that radioactive cerium released by partial or complete digestion of adsorbent flowed at a rate similar to that for radioactive cerium adsorbed onto the array of residues derived from the diet. Thus radioactive cerium adsorbed onto a readily fermentable adsorbent or onto the entire diet would be a valid flow-rate marker for the total residues derived from that diet. Adsorbed onto a relatively indigestible particle of markedly different flow rate from the remaining diet, the radioactive cerium would be a valid flow marker for residues derived from that particle. Radioactive cerium would be a less valid

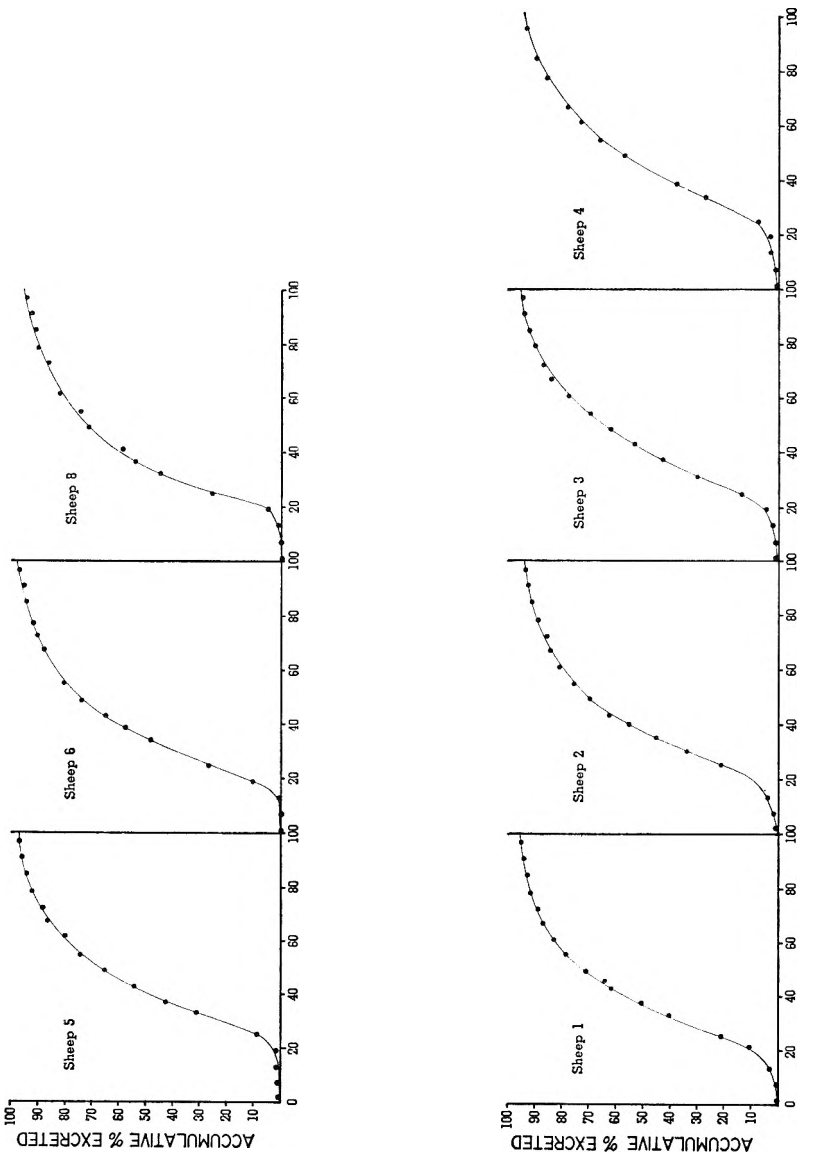

Fig. 4 Accumulative excretion curves for radioactive cerium when adsorbed onto the ration forage (sheep 1, 2, 3, and 4) and when absorbed onto encapsulated starch (sheep 5, 6, and 8).

flow-rate marker for specific particles when such particles were more digestible, yielded residues having flow rates markedly different from matrix residues, and made up smaller proportions of the diet. The latter such flow rates would be intermediate between the flow rate for undigestible residues from a specific particle and residues derived from the total diet (labeled as the result of digestion of the specific particle originally labeled). The greatest potential use for radioactive cerium as a particulate flow marker thus appears to reside in the measurement of flow by the total array of residues derived from the total diet.

Evidence has been presented for the adsorption of radioactive yttrium onto digesta particulate matter in the small intestine of rats (16) and onto rumen contents in vitro and in vivo by dysprosium (5). Radioactive colloidal properties are exhibited by all the rare earths (4). Thus radioisotopes of rare earths other than cerium or dysprosium could be used. Alternatively, stable isotopes could be used for those rare earths which could be analyzed at the low concentrations required for radiocolloidal properties (5). Radiocolloidal properties generally occur in concentrations below 10^{-8} M for most rare earths (4), although there is some evidence for adsorption onto rumen contents by dysprosium at concentrations of 10^{-5} M (5). In contrast, the mass of radioactive cerium used in the present experiments was calculated to be of order of 10^{-10} M in rumen contents.

The rare earths offer a number of advantages as flow rate markers over other techniques. The limitations of the stained particle technique with respect to selectivity of the fecal residues detected has been mentioned. One of the major advantages of a rare earth marker is its ease of application without extracting soluble plant material and thereby possibly influencing rate of fermentation and, as a consequence, rate of passage. For example, the staining technique used in experiment 1 resulted in the removal of approximately 20% of the dry matter. In contrast, the rare earth marker can be adsorbed onto the intact feedstuff particle. Further, depending upon the measurement desired, the rare earth marker may be applied independently of the feedstuff as in experiment 4.

ACKNOWLEDGMENTS

The assistance of Freddie Blackwell with the animals and collections, and Mrs. Dorothy Dunn with certain analytical determinations is gratefully acknowledged.

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Kidney Arginase Activity and Lysine Tolerance in Strains of Chickens Selected for a High or Low Requirement of Arginine [1]

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ABSTRACT Kidney arginase activity was investigated in 2 strains of White Leghorn chickens differing in dietary requirement of arginine. Arginase activity in kidneys of chicks from the high-requirement (HA) strain rose to a level 3 to 4 times that of chicks from the low-requirement (LA) strain following several days of feeding an arginine-deficient diet containing casein. Excretion of activity following a dose of guanido-labeled ^{14}C-arginine increased in excreta in conjunction with the elevated kidney arginase activity of the high-requirement strain. Dietary excesses of lysine, histidine, phenylalanine, ornithine and arginine caused a marked increase in kidney arginase activity of both strains, but the high requirement strain appeared to be particularly sensitive to excess lysine. Growth was depressed in chicks of the HA strain at lower dietary levels of lysine than were growth-depressing for the LA strain. Increasing the lysine content of the diet increased the arginine requirement of the HA strain considerably more than for the LA strain. The data suggest that the 2 strains studied differ in their ability to metabolize excess dietary lysine.

The dietary requirement of arginine for chickens is markedly affected by the type of diet fed and by the genetic background of the chickens. Two strains of White Leghorn chickens selected for a high (HA) or a low (LA) requirement of dietary arginine have been described by Hutt and Nesheim (1) and Nesheim et al. (2). These strains differ markedly in their ability to grow when fed a diet deficient in arginine, and containing casein as a source of protein. Although they differ in response to an arginine deficiency when fed other arginine-deficient diets, the magnitude of the difference is greatest when casein is used.

The reason for the differences in arginine requirement of the 2 strains has not been determined. Chickens are apparently unable to synthesize arginine since they lack the ability to synthesize ornithine (3, 4) and since nearly all the urea cycle enzymes necessary for arginine synthesis are lacking in chick liver (5). Differences in ability to grow on a given dietary level of arginine, therefore, must be due to differences in utilization. Lysine is known to interfere with the chick's utilization of arginine (6). The LA and HA strains may differ in lysine utilization, since consistently higher amounts of this amino acid are present in the amino acid pools in plasma, liver and muscle of the HA than of the LA strain (2).

In chicks the initial step in the degradation of arginine is apparently accomplished to a large extent by the action of the enzyme arginase, which is present in highest concentration in chick kidney (5). O'Dell (7) showed that levels of this enzyme in kidney were negatively correlated with growth of chicks fed an arginine-deficient diet containing casein. Chicks that grew well with this diet tended to have low levels, whereas those that grew poorly had high kidney arginase activity.

The present paper describes studies of the arginase activity in kidneys of chickens of the LA and HA strains, factors that appear to affect this activity, and investigations of the effect of lysine on the arginine requirement of the 2 strains.

EXPERIMENTAL PROCEDURES

Chicks from the S_4 and S_5 generations of the LA and HA strains developed by Hutt and Nesheim (1) were used. The LA

Received for publication August 21, 1967.

[1] This investigation was supported in part by Public Health Service Research Grant no. AM-06850 from the National Institute of Arthritis and Metabolic Diseases.

strain has been shown to have a low requirement for arginine and the HA strain a high requirement. The chicks were housed in battery brooders with raised wire floors. Feed and water were supplied ad libitum except where indicated.

The experimental diets are shown in table 1. The individual diets used will be discussed for each experiment.

Arginase activity in kidneys was measured with assay conditions identical to those reported by Tamir and Ratner (5). Urea was determined colorimetrically by the use of 1-phenyl-1,2-propanedione-2-oxime as reagent, according to the method of Van Slyke and Archibald (6). The sulfuric-phosphoric acid mixture was modified to be 10^{-2} M with respect to ferric ions, as suggested by O'Dell et al. (7). The assay was found to be reproducible and dependent on enzyme concentration.

TABLE 1

Basal diets

	A	B	C
	%	%	%
Isolated soybean protein	—	25.00	—
Casein	25.00	—	—
Amino acid premix	—	—	23.68 [1]
Glucose	59.02	61.15	—
Sucrose	—	—	47.66
Cellulose	3.00	3.00	4.00
Corn oil	4.00	3.00	15.00
Glycine	1.00	0.40	—
Methionine	0.40	0.60	—
Vitamin premix [2]	1.22	1.22	1.20
Mineral mix	6.36 [1]	5.63 [2]	8.46 [1]

[1] Supplied the following amino acids in g/100 g diet: L-arginine·HCl, varied; L-histidine·HCl, 0.62; L-lysine·HCl, 1.40; L-tyrosine, 0.63; L-tryptophan, 0.23; L-phenylalanine, 0.68; L-cystine, 0.35; DL-methionine, 0.55; L-threonine, 0.85; L-leucine, 1.20; L-isoleucine, 0.80; L-valine, 1.04; L-glutamic acid, 12.0; glycine, 1.60; and L-proline, 1.00.

[2] For composition see Nesheim et al. (8).

Kidneys were removed immediately after chicks were killed, and frozen quickly with dry ice, wrapped in paraffin paper and aluminum foil, and stored at − 20° until analysis was performed.

Initial studies of arginase activity in kidneys of chicks of the HA and LA strains showed no appreciable differences between strains. When an arginine-deficient diet was fed for a longer period of time, substantial differences were observed. In this experiment, following one week of feeding a commercial chick starter ration, groups of chicks of each strain were fed basal diet A (table 1), the basal diet supplemented with 1.20% L-arginine, or were continued with the practical diet. After 2 weeks, the chicks were weighed and killed; kidney arginase activity was determined (table 2).

The chicks of the HA and LA strains grew at markedly different rates with the arginine-deficient diet. Kidney arginase levels in the HA strain were about 4 times those of the LA strain when the chicks were fed the basal diet. There was relatively little difference between the 2 strains when they were fed the arginine-supplemented or practical diet. The supplemental arginine increased kidney arginase activity in chicks of the LA strain and appeared to reduce it in chicks of the HA strain, compared with those fed the basal diet.

This experiment suggested that the HA and LA strains differed in their response in kidney arginase activity to the feeding

TABLE 2

Arginase activity of kidneys of chicks from high-requirement (HA) and low-requirement (LA) strains [1]

	Basal diet (Diet A, table 1)	Basal diet + 1.2% L-arginine	Commercial chick starter
HA			
Avg wt, g [2]	87	212	215
Arginase activity [3]	10600 ± 2200 [4]	7000 ± 3000	2700 ± 700
LA			
Avg wt, g [2]	156	212	215
Arginase activity [3]	2600 ± 400	7300 ± 2400	2200 ± 800

[1] All chicks were fed the commercial chick starter for 8 days at which time they were changed to diets indicated for a subsequent 14-day period.

[2] Average weight of chicks on which arginase activity was determined.

[3] Expressed as micromoles of urea formed per gram of fresh tissue per hour at 37° by method described in procedures.

[4] Each value is the average of determinations on 5 chicks with its standard error.

of an arginine-deficient diet. The deficient diet appeared to increase kidney arginase activity of the HA strain to high levels.

An additional experiment was conducted in which the kidney arginase activity was measured at zero, 5, 10 and 15 days after chicks were given the arginine-deficient basal diet (diet A, table 1). The chicks were fed a practical chick starter ration for the first 12 days after hatching and then fed the arginine-deficient diet for an additional 15 days.

Arginase activity was also measured in chicks receiving the arginine-supplemented diets (table 3). Kidney arginase activity was similar in both strains on day zero, but after the arginine-deficient diet was fed for 5, 10 or 15 days, that of chicks of the HA strain had increased markedly, whereas that of the LA strain did not change. The activity in both strains was increased after 5, 10 or 15 days of feeding the diet supplemented with arginine.

The differences in response of kidney arginase activity of the LA and HA strains to an arginine deficiency suggested that chicks of the HA strain used the arginine in the deficient diet less efficiently because of the high kidney arginase activity induced by the diet.

An experiment was conducted to determine whether the increase in kidney arginase activity of the HA strain was also associated with an increase in the catabolism of arginine. Chicks were fed a practical chick starter until they were 10 days of age, and they were changed to the arginine-deficient diet containing casein (diet A, table 1). At this time chicks of each strain were given an oral dose of guanido-^{14}C-labeled L-arginine and the appearance of the label in excreta was measured. Since the ^{14}C in the arginine would be found in the urea carbon, excretion of ^{14}C could be followed as an indication of urea production.

Chicks of each strain, fasted over night, were allowed to consume the arginine-deficient diet for approximately 30 minutes. Those consuming an inadequate quantity of feed at this time were force-fed until they received the same amount as the other chicks from their strain. Approximately 20 minutes after they were fed, the chicks were given an oral dose of the ^{14}C arginine and placed in individual cages; excreta were collected in pans containing 0.1 N HCl. After 6 hours the chicks were killed, excreta were collected, and kidneys removed and frozen for later determination of arginase activity. A blood sample was also taken. The excreta were homogenized, diluted to a given volume (usually 250 ml); one-milliliter aliquots were transferred to a counting vial. Ten milliliters of Bray's solution were added and the radioactivity was measured in a liquid scintillation counter. Blood plasma activity was measured by counting 0.1 ml of plasma after dissolving it in one milliliter of hyamine hydroxide and mixing with 10 ml of Bray's solution.

TABLE 3

Effect of length of time of feeding an arginine-deficient diet on arginase activity of chick kidney [1]

Time fed experimental diet	Basal diet (diet A, table 1)				Basal diet + 1.2% L-arginine			
	HA [2]		LA		HA		LA	
	Body wt	Arginase activity [3]	Body wt	Arginase activity [3]	Body wt	Arginase activity [3]	Body wt	Arginase activity [3]
days	*g*	*moles urea/g fresh tissue/hr*	*g*	*μmoles urea/g fresh tissue/hr*	*g*	*μmoles urea/g fresh tissue/hr*	*g*	*μmoles urea/g fresh tissue/hr*
0	107	3500 ± 700 [4]	101	2500 ± 500	—	—	—	—
5	125	7200 ± 4600	144	2300 ± 1300	150	7700 ± 4000	152	6400 ± 2100
10	139	8200 ± 4400	178	2400 ± 400	215	9500 ± 2300	206	9000 ± 2200
15	199	6300 ± 2700	295	2400 ± 600	285	7500 ± 2100	292	6000 ± 1200

[1] All chicks were fed a commercial chick starter until 12 days of age. At day zero chicks were changed to diets indicated.
[2] HA indicates high-requirement strain; LA, low-requirement strain.
[3] Kidneys from 5 chicks of each strain assayed at each time period and for each diet. Average weights of chicks assayed are shown.
[4] SE.

TABLE 4

Excretion of ^{14}C activity from guanido-labeled arginine by chicks from high-requirement (HA) and low-requirement (LA) strains at various times after feeding an arginine-deficient diet (diet A, table 1)

Days fed diet	Avg wt		Food consumed		Dose in excreta [1]		Dose in plasma		Arginase activity of kidney	
	HA	LA	HA	LA	HA	LA	HA	LA	HA	LA
	g		*g*		*%*		*%/ml*		*μmoles urea/g fresh tissue/hr*	
0	116	116	3.2	3.7	35 ± 14 [2]	42 ± 8	0.54	0.45	3200 ± 1200	3700 ± 1100
5	155	173	3.5	4.6	33 ± 6	22 ± 3	0.38	0.34	6500 ± 3600	3400 ± 1200
14	160	257	3.8	5.5	24 ± 8	12 ± 2	0.35	0.21	11500 ± 4800	2200 ± 500

[1] Each chick was orally given 2.5 μCi ^{14}C activity/100 g body weight of guanido ^{14}C-L-arginine. Five chicks from each strain were studied at zero and 5 days, and 6 chicks at 14 days.
[2] SE.

This experiment was repeated at 5 and 14 days after the chicks were transferred to the arginine-deficient diet. The arginase activity increased in kidneys of chicks from the HA strain at the time they were fed the arginine-deficient diet (table 4). At the beginning of the experiment the percentage of dose of the ^{14}C-labeled arginine recovered in the excreta was similar for both strains, but after 5 and 14 days of feeding the diet, a significantly greater amount was recovered from the HA strain than from the LA strain. The relative breakdown of arginine appeared to follow the difference in kidney arginase activity. Blood levels of ^{14}C activity were somewhat higher in chicks of the HA strain at each period.

One consequence of an arginine deficiency in the HA and LA strains is the marked difference in feed consumption of an arginine-deficient diet containing casein. The HA strain, which grows very poorly under these conditions, also consumes much less feed than the LA strain. To determine whether the apparent difference in arginase activity was due merely to the differences in feed consumption, a controlled feeding experiment was conducted, the design and results of which are shown in table 5.

Chicks of the LA strain were restricted to the amount of feed the chicks of the HA strain consumed ad libitum. Appropriate ad libitum controls were also used. The paired feeding was on a group basis. Kidneys from 5 chicks in each pen were pooled and homogenized for arginase determination.

Two pens of 10 chicks were fed each dietary treatment. The chicks of the HA strain consumed only about 40% as much of the basal diet ad libitum as those of the LA strain. When chicks of the LA strain were restricted to this level of feed intake, the kidney arginase activity was essentially the same as when they were fed ad libitum. The kidney arginase activity in the HA strain, however, showed the marked rise that was characteristically observed in all these experiments when this strain was fed the arginine-deficient casein diet.

Nesheim et al. (2) have shown that the response of the HA and LA strains to an arginine-deficient diet was influenced by

TABLE 5

Effect of paired feeding on arginase activity in kidney of high-requirement (HA) and low-requirement (LA) strains [1]

	Basal diet		Basal diet + 1.2% L-arginine ad libitum
	Ad libitum	Pair-fed	
HA			
Avg wt, g	74	—	173
Feed consumed, g	81	—	192
Arginase activity [2]	12,300 [3]	—	10,000
LA			
Average weight, g	153	87	200
Feed consumed, g	188	85	217
Arginase activity [2]	3,700	4,374	7,200

[1] All chicks were fed to 8 days of age a commercial chick starting diet. The paired-feeding of experimental diets lasted for 12 days. Each treatment was fed to 2 pens of 10 chicks each for each strain. The basal diet was diet A, table 1.
[2] Expressed as micromoles of urea produced per gram fresh kidney per hour. Determination was run on 2 pooled samples of 5 chicks each for each experimental treatment.
[3] Analysis of variance and application of Tukey's HSD (10) showed this value as significantly different from values of chicks of the LA strain pair-fed or fed ad libitum.

type of diet. When an arginine-deficient diet containing casein (such as diet A, table 1) was used, the differentiation of the 2 strains was very great. When a diet containing amino acids as a source of protein was fed, the differentiation of the 2 strains was considerably less. An experiment was conducted, therefore, to determine the arginase activity of kidneys in the HA and LA strains of chickens when they were fed the casein basal diet (A, table 1), a basal diet containing soybean protein (B, table 1) and a basal diet in which the protein was supplied by crystalline amino acids (C, table 1).

High levels of dietary lysine have been reported to increase the arginine requirement of the chick (6). Jones et al. (11) reported that high levels of lysine would markedly increase kidney arginase levels. Boorman and Fisher (12) reported that excesses of several other amino acids besides lysine, particularly phenylalanine and histidine, will increase the arginine requirement. Several amino acids were added to the soy protein basal diet to determine their influence on kidney arginase activity. The design and results of this experiment are shown in table 6.

The chicks from the HA strain had a kidney arginase level approximately 3 times that of chicks of the LA strain when they were fed the casein diet. Supplementing this diet with arginine equalized the kidney arginase levels in both strains, although this involved an increase in activity in chicks from the LA strain, and lowering in the HA strain. Supplementing the basal diet with 3% glutamic acid had no effect on kidney arginase levels.

When the soy protein basal diet was fed, there was no difference in kidney arginase levels in chicks from the 2 strains. Kidney arginase levels, however, of chicks of the HA strain were elevated when graded levels of lysine were added to the diet. Growth rate of these chicks was depressed by 1% added L-lysine·HCl, whereas the same level had no effect on chicks of the LA strain. A level of 1.5% L-lysine·HCl

TABLE 6

Effect of different diets and excesses of single amino acids on kidney arginase activity of chicks from high-requirement (HA) and low-requirement (LA) strains

Diet	HA		LA	
	Avg wt [1]	Kidney arginase	Avg wt [1]	Kidney arginase
	g	*μmoles urea/g fresh tissue/hr*	*g*	*μmoles urea/g fresh tissue/hr*
1) Casein basal diet (diet A, table 1)	68	9500±2500 [2](5) [3]	171	2800±1000(6)
2) +3.0% glutamic acid	98	8900±5300(8)	163	2600± 300(5)
3) +1.2% L-arginine	205	7300±1300(5)	221	7400±1600(5)
4) Soy protein basal diet (B, table 1)	191	3900±1100(7)	189	4000± 600(7)
5) +0.5% L-lysine·HCl	190	6600±2200(7)	199	4400±1400(7)
6) +1.0% L-lysine·HCl	168	8400±1600(8)	191	5000±3100(9)
7) +1.5% L-lysine·HCl	162	5600± 400(8)	168	4900±1500(8)
8) +1.5% L-lysine·HCl +1.5% L-arginine·HCl	189	6900±1200(8)	184	6100±2400(8)
9) +1.5% L-histidine·HCl	174	7000±2200(5)	179	7400±2400(5)
10) +1.5% L-histidine·HCl +1.5% L-arginine·HCl	188	15400±2700(7)	178	11400±2100(7)
11) +3.0% DL-phenylalanine	84	9000±1300(7)	82	7000±1400(5)
12) +3.0% DL-phenylalanine +1.5% L-arginine·HCl	104	12200±1400(5)	94	15800± 200(6)
13) +3.0% glutamic acid	173	4400±1100(5)	178	5100±1500(3)
14) +1.5% L-ornithine	187	8000±1200(5)	168	6400±2500(7)
15) Amino acid basal diet (B, table 1) (0.85% L-arginine)	111	2100±1200(5)	149	1400± 300(5)

[1] Each value is average of a single pen of 10 male chicks/pen except for treatments 4, 5, 6, 7, 8, 9, 10, where 2 pens of 10 chicks were used/treatment.

[2] SE.

[3] Figures in parentheses are number of determinations on individual chicks.

added to the diet had less effect on the kidney arginase activity of chicks of the HA strain. This level of added lysine depressed growth rate in both the HA and LA strains to approximately the same extent. Supplementing the high lysine diet with 1.5% L-arginine·HCl overcame the growth depression from the added lysine. The kidney arginase level in both strains was increased by arginine supplementation.

Supplementing the soy basal diet with histidine and phenylalanine increased kidney arginase activity in both strains. This was increased still further when, in addition to high levels of these amino acids, arginine hydrochloride was added to the basal diet. Glutamic acid had essentially no effect on kidney arginase activity of chicks fed the soy basal diet, whereas L-ornithine increased activity of both strains, particularly the HA strain. Kidney arginase levels in chicks of both strains were low when the amino acid basal diet was fed. Chicks of the HA strain fed the arginine-deficient, amino acid basal diet had a somewhat higher arginase activity in the kidney than those of the LA strain, but this difference was smaller than that observed with the casein basal diet.

The data obtained in the previous experiment showed that growth rate of chicks of the HA strain was depressed with lower levels of lysine (added to diet B, table 1) than that of chicks of the LA strain. Two experiments were conducted to confirm this observation. The design and results of these experiments are shown in table 7. In both experiments, all levels of added lysine were growth-depressing for chicks from the HA strain, whereas only 1.5% added lysine·HCl, in experiment 2, depressed growth to any significant extent in the LA strain.

The kidney arginase levels were increased in both strains by the added dietary lysine.

In a separate study the arginine and lysine content of plasma from chicks fed either the basal diet, or the basal + 1.0% lysine, as in the above experiment, was determined (2). Two pooled samples from 4 chicks of each strain were analyzed from chicks fed each diet. The results are shown in table 8. The chicks of the HA strain had elevated lysine levels in plasma with either diet, but especially so when extra lysine was fed. The arginine levels in plasma were depressed by the added levels of lysine, especially in the HA strain.

The arginine level in the basal diet containing isolated soybean protein was considerably higher than that in diets containing casein, with which differences in the ability of these 2 strains to grow with an

TABLE 7

Effect of excesses of dietary lysine on growth of chicks from the high-requirement (HA) and low-requirement (LA) strains

Diet	HA		LA	
	Avg wt [1] 28 days	Kidney arginase activity [2]	Avg wt [1] 28 days	Kidney arginase activity [2]
	g	*μmoles urea/g fresh tissue/hr*	*g*	*μmoles urea/g fresh tissue/hr*
		Experiment 1 [3]		
Basal (diet A, table 1)	314	—	324	—
+0.75% L-lysine·HCl	267	—	314	—
+1.0% L-lysine·HCl	253	—	303	—
+1.25% L-lysine·HCl	247	—	297	—
		Experiment 2		
Basal (diet A, table 1)	324	3700 ± 1200(9) [4]	331	4200 ± 1400(6)
+1.0% L-lysine·HCl	274	6300 ± 3300(9)	326	5300 ± 1700(9)
+1.5% L-lysine·HCl	236	7050 ± 3100(9)	285	9700 ± 5200(7)

[1] Average of 3 groups of 8 chicks each. In experiment 1 the diets were fed from 1 week of age, in experiment 2 from day-old.

[2] Nine determinations expressed by each mean.

[3] Analysis of variance indicated significant ($P < 0.05$) differences in body weight gains resulting from strain effects, lysine level, and a significant strain × lysine interaction.

[4] Means are given with associated standard error; number of determinations in parentheses.

arginine-deficient diet are best demonstrated. Therefore it seemed important to study the influence of levels of lysine on growth and kidney arginase activity at several levels of arginine. To control the dietary levels of lysine and arginine precisely, diets containing crystalline amino acids were used. The design and results of this experiment are shown in table 9.

The basal diet (C, table 1) was formulated to contain 0.85% arginine. This is the calculated level of arginine in the basal diet containing casein (A, table 1). Two levels of lysine were used, 1.1% and 1.75%. The high level is the same as that calculated to be in the diet with casein as the protein source, whereas the lower level is much closer to the minimal requirement for lysine. Several levels of arginine were included at each level of lysine.

TABLE 8

Plasma lysine and arginine levels in high-requirement (HA) and low-requirement (LA) chicks when fed diets containing excess lysine [1]

	HA		LA	
	Basal	1.0% lysine	Basal	1.0% lysine
	μg/ml	*μg/ml*	*μg/ml*	*μg/ml*
Arginine	51	30	69	47
Lysine	156	316	70	164

[1] Values represent an average of determinations on two pooled samples of 4 chicks each from each strain fed each diet.

Each diet was fed for a 10-day period to 2 pens of 3 male and 3 female chicks per lot, beginning when the chicks were 9 days of age. Before starting the experiment the weights of chicks in all groups were kept to a narrow range by discarding both high and low extremes of weight. Kidney arginase was measured in pooled homogenates from each group of males and females in each pen.

When the lysine content of the diet was 1.1%, 1.0% arginine in the diet appeared to be sufficient for maximal rate of gain for chicks of the HA strain, whereas no response to any level of arginine above 0.85% was observed with chicks of the LA strain. At this level of lysine and the lowest level of arginine, arginase activity was slightly higher in kidneys of chicks of the HA strain than of the LA strain. The arginase activity in kidneys of both strains increased with dietary arginine level.

When the lysine level in the diet was 1.75%, chicks of the HA strain required more than 1.45% arginine in their diet to grow at a normal rate. Chicks of the LA

TABLE 9

Effect of lysine level in the diet on response of chicks to levels of arginine [1]

Level of arginine	HA [2]		LA	
	Wt gain [3]	Kidney-arginase activity [4]	Wt gain [3]	Kidney-arginase activity [4]
%	*g*	*μmoles urea/g fresh tissue/hr*	*g*	*μmoles urea/g fresh tissue/hr*
		1.1% L-lysine		
0.85	53	2000	74	1400
1.00	68	2000	77	1100
1.15	69	1100	78	1300
1.45	70	1700	74	3100
2.05	72	3100	78	4200
		1.75% L-lysine		
0.85	33	3830	62	1700
1.00	47	2900	71	1600
1.15	53	3600	71	2500
1.45	59	3100	73	3700
2.05	66	3500	74	4300

[1] Analysis of variance of these data showed that there were significant ($P < 0.05$) effects from strain, arginine and lysine on weight gains and kidney arginase activity, and significant strain × arginine and strain × lysine interactions for weight gain and arginase activity.
[2] HA indicates high-requirement strain; LA, low-requirement strain.
[3] Represents average 10 day gain of 2 groups of 3 male and 3 female chicks each per treatment.
[4] Represents average of determination on 4 pooled samples of kidneys from 3 chicks each for each value shown.

strain appeared to require no more than 1% arginine in the diet under these conditions. The chicks of the HA strain had a considerably higher level of kidney arginase activity at all levels of arginine when the diet contained 1.75% lysine compared with the activity found in chicks receiving only 1.1% L-lysine in the diet. The kidney arginase activity in the LA strain was only slightly higher at the 1.75% level of lysine. These data show that chicks of the HA strain appear to respond markedly to excesses of dietary lysine both in terms of growth depression and of elevated kidney arginase activity.

DISCUSSION

In many respects, the differences in response of the HA and LA strains of chickens to an arginine deficiency appear to be related to differences in the susceptibility of the strains to excesses of lysine. We showed previously (2) that high levels of lysine are found in body amino acid pools of the HA strain and the data in this paper confirm this and show the marked effect of lysine on the growth of this strain. Jones et al. (11) recently published data showing that excesses of lysine in diets for chicks cause a reduction in growth rate and an increase in kidney arginase level. The effects of the excess lysine can be counteracted by higher levels of dietary arginine, so that, in effect, high levels of lysine increase the arginine requirement. The HA strain of chickens appears to be particularly sensitive to excesses of lysine, and possibly for this reason, has a high requirement of arginine. This greater sensitivity to high levels of dietary lysine may be due to an inability of the HA strain to metabolize lysine as rapidly as chicks of the LA strain.

Since the HA and LA strains were selected on the basis of their ability to grow with a diet containing casein as the source of protein, they were differentiated in the presence of rather high amounts of dietary lysine. The selection may have served primarily to affect enzyme systems responsible for lysine degradation, although other metabolic systems affecting arginine metabolism directly could have been influenced as well.

The response observed with kidney arginase activity in the 2 strains is unusual. In one strain (LA), the kidney arginase activity parallels closely the arginine content of the diet. This confirms the effect of dietary arginine on chick kidney arginase levels reported by O'Dell et al. (7). However, in the HA strain, quite the opposite is true. When the arginine content of the diet is low, the kidney arginase activity increases. Several amino acids appear to be able to induce high levels of kidney arginase activity in chickens. These include lysine, histidine, ornithine and phenylalanine (see table 6). This is in good agreement with respect to lysine, with the data of Jones et al. (11). Since considerable data suggest that the HA strains metabolize lysine rather slowly, the elevated kidney arginase level in response to an arginine deficiency in the HA strain may be caused by the high levels of lysine that accumulate in body pools of the HA strain. The elevated kidney arginase level in the HA strain is accentuated when the casein is fed, unsupplemented with arginine. This diet contains a rather high level of lysine supplied by the casein.

Elevation of kidney arginase activity in the HA strain in response to the arginine deficiency may not be the only reason for the high requirement of arginine by the HA strain. Jones et al. (11) suggest that the effects of excess lysine on arginine metabolism occur within a short time, before kidney arginase activity responds to the excess lysine. Our own data tend to agree with this interpretation since excess lysine appears to depress growth rate of chicks of the LA strain, rather independent of kidney arginase levels (table 7). However, since an increase in kidney arginase activity appeared to be paralleled by an increased catabolism of arginine (table 4), the combination of the excess lysine and increased kidney arginase activity may accentuate still further the arginine deficiency in the HA strain.

Recently, a disorder in children has been described, characterized by high plasma lysine levels (13). This hyperlysinemia is accompanied by numerous developmental disturbances. Bürgi et al. (14) have suggested that a NAD-requiring enzyme that they called "lysine dehydrogenase"

was low in a child with this disorder. We have not been able to demonstrate the presence of this enzyme in chick liver homogenates of either strain, and it has not been described in other species.

The selection program, aimed at differentiating strains of chickens for their dietary requirement of arginine, has had an unexpected result since the selection apparently had considerable effect on lysine metabolism which has indirectly affected arginine metabolism. This is perhaps not so unexpected in light of present knowledge of lysine-arginine relationships.

The HA and LA strains of chickens should be valuable for studies of lysine degradation. Selection programs designed to produce animals with specific nutritional characteristics may be valuable in studying a number of nutritional problems.

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Total Lipid and Cholesterol Levels in Plasma and Liver of Rats Fed Diets Supplemented with Sulfaguanidine or Succinylsulfathiazole

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ABSTRACT The influence of dietary supplements of succinylsulfathiazole and sulfaguanidine on plasma and liver total lipid and cholesterol levels was studied in rats. These 2 sulfonamides were evaluated in diets devoid of cholesterol or supplemented with either cholesterol alone or cholesterol plus cholic acid. The 2 drugs had little influence on liver and plasma lipids and cholesterol in rats fed cholesterol-free diets. In animals fed cholesterol-supplemented diets succinylsulfathiazole had a cholesterol-elevating effect, whereas sulfaguanidine depressed liver cholesterol and lipid levels. When these drugs were tested in diets supplemented with both cholesterol and cholic acid the cholestrol- and lipid-enhancing effects of succinylsulfathiazole were no longer apparent, whereas the depressing action of sulfaguanidine was still evident. The cholesterolemic effect of these 2 sulfonamides could not be related to their goitrogenicity. Possible mechanisms available to explain the results observed were considered and a hypothesis in accord with the reported observations was proposed.

Succinylsulfathiazole is one of several sulfonamide drugs commonly used to reduce the intestinal microflora. This drug has been reported to increase cholesterol levels in plasma (1) and liver (2) of cholesterol-fed rats. This compound has also been found to be hypercholesterolemic in mice (3).

The mechanism by which this drug elevates plasma cholesterol levels is not clear. The conclusion which seems most logical is that succinylsulfathiazole exerts its hypercholesterolemic effect through the suppression of the intestinal flora. Such a conclusion is supported by the observation of Danielsson and Gustafsson (4) that germfree rats have higher concentrations of plasma cholesterol than animals reared under conventional conditions. Further, the fact that the half-life of cholic acid-^{14}C is increased in germfree rats (5) or rats fed a diet supplemented with a mixture of oxytetracycline and phthalylsulfathiazole (6) also appears to support the conclusion that the sulfonamides alter plasma cholesterol levels by influencing the intestinal microbial population.

The studies cited, however, do not rule out the possibility that succinylsulfathiazole may exert its hypercholesterolemic effect through some other mechanism. In fact, studies of the influence of various dietary antibiotics on plasma cholesterol levels suggest that this is the case. Chlortetracycline has been shown to be hypocholesterolemic when administered as a dietary supplement to mice (3). Heat inactivation of this antibiotic almost completely destroys its antibiotic activity but does not alter its blood cholesterol-lowering capacity (7). Orally administered neomycin has been found to decrease blood cholesterol levels in humans (8) and chicks (9) but is ineffective in rats (10) and hypercholesterolemic in the rabbit (11). In the chick, N-methylated neomycin, a neomycin derivative without antibiotic activity, is as effective as neomycin in lowering serum cholesterol (9). These studies might suggest that neomycin and chlortetracycline exert their cholesterol-lowering effects independently of any influence on the intestinal microflora.

The well-known goitrogenicity of sulfonamides (12, 13) presented what appeared to be a reasonable explanation for the observed hypercholesterolemic effects of succinylsulfathiazole. Since it is generally conceded that serum cholesterol levels are inversely related to thyroid function (14),

Received for publication December 18, 1967.

it is reasonable to expect that a drug which depresses thyroid function would elevate blood cholesterol levels. The data to be presented were obtained in an attempt to test this hypothesis. As these data show, the effect of 2 sulfonamides on liver and plasma cholesterol levels could not be related to their goitrogenicity. Also the 2 drugs studied were found to have opposite effects on cholesterol levels.

EXPERIMENTAL

Rats of the Sprague-Dawley strain were used in all studies. Female rats weighing 204 ± 1 g were used in one experiment; in all other experiments males weighing from 72 to 182 g were used. The sex and initial body weight of the rats used for each experiment are indicated in the tables of results. The animals were housed individually in metal cages having raised wire floors and in a temperature-regulated room (21°). The animals were fed a commercial diet [1] until they were used for these experiments. They then received the experimental diets for 3 to 4 weeks. Water was available at all times. Food consumption and body weights were determined weekly.

The diet used had the following composition in g/100 g diet: vitamin-free casein, 18; L-cystine, 0.3; vitamin mixture,[2] 2.2; mineral mixture,[3] 4; nonnutritive fiber, 4; corn oil, 5; and glucose, to 100. Cholesterol, cholic acid, succinylsulfathiazole, sulfaguanidine, and desiccated thyroid were added to the basal diet as indicated in the tables of results. All supplements were added to the basal diet at the expense of glucose.

At the termination of each experiment the rats were anesthetized with ether, and blood was obtained from the abdominal aorta with a heparinized syringe. The animals were exsanguinated, and the liver was removed, weighed and frozen and stored at −20° until analysis was performed. In some experiments the thyroid glands were carefully removed and weighed on a torsion balance. The weight of the thyroids (expressed per 100 g body weight) was taken as an indication of the goitrogenicity of the treatments under study.

Plasma and liver cholesterol and liver total lipids were determined as previously described (17, 18). Plasma total lipid was determined by the method of Huerga et al. (19). The data were analyzed statistically by means of the *t* test, with *P* values of 5% or less being considered as significant.

RESULTS

The experiments shown in table 1 were conducted in an attempt to relate the goitrogenic effect of the 2 sulfonamides tested to plasma and liver cholesterol levels. As the data for these show, such a relationship did not exist. Succinylsulfathiazole was not goitrogenic but produced increased liver and plasma total lipid and cholesterol levels when added to the cholesterol-supplemented diet. However, only the plasma cholesterol difference attained statistical significance. The suggested trends were, nevertheless, in accord with previous studies (1, 2). In the absence of dietary cholesterol, succinylsulfathiazole was without effect on plasma and liver lipids. Furthermore, succinylsulfathiazole did not influence body weight.

Sulfaguanidine, however, was definitely goitrogenic and the level of desiccated thyroid used more than overcame this effect (table 1, exp. 2). Thyroid weights were increased significantly in rats fed sulfaguanidine and depressed below control values in animals fed both sulfaguanidine and desiccated thyroid. Body weight was not influenced by sulfaguanidine but was reduced in animals fed desiccated thyroid. Sulfaguanidine and desiccated thyroid did not influence plasma lipids or cholesterol in this experiment. In rats fed the cholesterol-free diet, sulfaguanidine supplementation significantly reduced liver cholesterol levels and this depression was overcome by adding desiccated thyroid to the sulfaguanidine-supplemented diet. A similar trend was observed for liver total lipid; however, the difference was not statistically significant. In animals fed the cholesterol-containing diet, liver total lipids and cholesterol were markedly reduced in sulfaguanidine-fed rats as compared with control animals, a difference which was

[1] Rockland Mouse/Rat diet (complete), Teklad, Inc., Monmouth, Illinois.
[2] For composition of the vitamin mixture see Leveille (15).
[3] For composition of the mineral mixture see Leveille and O'Hea (16).

TABLE 1

Influence of dietary succinylsulfathiazole, sulfaguanidine and desiccated thyroid on thyroid weight and plasma and liver lipids of rats fed cholesterol-free or cholesterol-supplemented diets [1]

Dietary supplements [2]	Final body wt	Thyroid wt	Liver [3]		Plasma	
			Total lipid	Cholesterol	Total lipid	Cholesterol
% of diet	*g*	*mg/100 g body wt*	*%*	*mg/g*	*mg/100 ml*	
		Experiment 1				
None	297±5 [4]	7.4±0.2	4.4±0.1	2.9±0.1	561±64	86±12
ST, 1%	287±3	7.3±0.3	4.2±0.2	3.0±0.1	590±36	80± 7
ST, 1% +DT, 0.1%	262±4	6.9±0.5	4.7±0.1	4.0±0.4	501±16	78± 7
C, 1%	293±4	7.4±0.4	5.6±0.4	8.5±1.7	602±30	69± 4
C, 1% +ST, 1%	297±4	7.3±0.1	7.1±0.9	12.5±1.7	709±48	84± 4
C, 1% +ST, 1% +DT, 0.1%	269±5	6.2±0.1	6.4±0.3	13.7±1.2	501±35	72± 6
		Experiment 2				
None	306±3 [4]	7.0±0.5	3.7±0.2	3.0±0.1	522±47	87± 4
SG, 1%	302±4	8.5±0.5	3.2±0.2	2.6±0.1	484±50	88± 9
SG, 1% +DT, 0.1%	270±6	4.7±0.3	4.2±0.1	3.6±0.1	543±29	81± 4
C, 1%	299±5	5.4±0.9	5.9±0.2	8.6±1.4	528±49	74± 8
C, 1% +SG, 1%	306±7	8.9±0.5	4.9±0.2	6.1±0.4	514±32	69± 7
C, 1% +SG, 1% +DT, 0.1%	266±5	4.9±0.2	5.0±0.1	5.4±0.1	543±80	62±10

[1] Male rats having an initial body weight of 156±1 and 182±2 g were used in experiments 1 and 2, respectively; the experimental diets were fed for 3 weeks.
[2] ST = succinylsulfathiazole, SG = sulfaguanidine, DT = desiccated thyroid, C = cholesterol.
[3] Liver values are expressed on a wet-weight basis.
[4] Mean for 8 rats ± SEM.

TABLE 2

Influence of dietary sulfaguanidine and desiccated thyroid on thyroid weight and plasma and liver lipids of rats fed cholesterol-free or cholesterol-supplemented diets [1]

Dietary supplements [2]	Final body wt	Thyroid wt	Liver [3]		Plasma	
			Total lipid	Cholesterol	Total lipid	Cholesterol
% of diet	*g*	*mg/100 g body wt*	*%*	*mg/g*	*mg/100 ml*	
None	223±4 [4]	6.4±0.2	3.3±0.1	2.8±0.1	358±42	96± 8
SG, 1%	223±3	9.0±0.3	3.1±0.1	2.6±0.2	329±28	92± 8
SG, 1% +DT, 0.1%	220±5	6.7±0.4	3.5±0.3	2.6±0.3	641±52	123±16
C, 1%	231±4	6.9±0.2	5.0±0.4	7.8±0.9	458±44	91± 9
C, 1% +SG, 1%	220±3	8.7±0.4	4.3±0.1	4.9±0.2	402±18	63± 3
C, 1% +SG, 1% +DT, 0.1%	217±4	6.5±0.2	4.3±0.2	3.9±0.2	651±31	94± 8

[1] Female rats having an initial body weight of 204±1 g were used; the experimental diets were fed for 4 weeks.
[2] SG = sulfaguanidine, DT = desiccated thyroid, C = cholesterol.
[3] Liver values are expressed on a wet-weight basis.
[4] Mean for 8 rats ± SEM.

statistically significant. In this case the lipid and cholesterol-lowering effect of sulfaguanidine was not overcome by the addition of desiccated thyroid to the diet. The depression of liver lipids by sulfaguanidine was unexpected in view of the lipid and cholesterol-elevating effect of succinylsulfathiazole.

The experiment summarized in table 2 was conducted to verify the liver lipid and cholesterol-lowering effect of sulfaguanidine. Female rats were used in this study. As previously noted, sulfaguanidine was goitrogenic. In this experiment sulfaguanidine did not influence plasma or liver lipids in the absence of dietary cholesterol. In rats fed the cholesterol-supplemented diet, sulfaguanidine did significantly depress liver and plasma cholesterol levels.

In an attempt to amplify the effects of sulfa drugs, rats were fed a cholesterol-cholic acid-supplemented diet known to be hypercholesterolemic. As shown in table 3, the supplementation of this diet with succinylsulfathiazole resulted in no significant changes in liver or plasma lipids,

TABLE 3

Plasma and liver lipids of rats fed diets supplemented with succinylsulfathiazole or sulfaguanidine in the presence of cholesterol and cholic acid [1]

Dietary supplement (2% of diet)	Final body wt	Liver [2]		Plasma	
		Total lipid	Cholesterol	Total lipid	Cholesterol
	g	*%*	*mg/g*	*mg/100 ml*	
None	213±6 [3]	9.3±0.2	12.7±0.3	346±21	110± 6
Succinylsulfathiazole	204±4	9.2±0.3	12.1±0.6	294±23	110±11
Sulfaguanidine	143±4	6.3±0.2	7.0±0.3	351±45	124±10

[1] Male rats having an initial body weight of 72±2 g were used; the experimental diets were fed for 3 weeks. The basal diet contained 1% cholesterol and 0.25% cholic acid.
[2] Liver values are expressed on a wet-weight basis.
[3] Mean for 10 rats ± SEM.

TABLE 4

Influence of dietary cholic acid, succinylsulfathiazole and sulfaguanidine on plasma and liver lipids of rats fed a cholesterol-supplemented diet [1]

Dietary supplements [2]	Final body wt	Liver [3]		Plasma	
		Total lipid	Cholesterol	Total lipid	Cholesterol
% of diet	*g*	*%*	*mg/g*	*mg/100 ml*	
None	278±6 [4]	5.7±0.1	8.5±0.5	522±23	99± 3
CA, 0.25%	282±4	12.8±0.6	39.6±2.3	773±60	192±17
ST, 1%	288±4	8.4±0.4	13.3±1.0	607±28	115± 3
ST, 1% +CA, 0.25%	279±5	10.8±0.7	34.5±2.3	750±37	185± 9
SG, 1%	273±5	5.4±0.2	6.1±0.4	460±28	92± 2
SG, 1% +CA 0.25%	272±5	10.5±0.4	31.2±1.8	636±32	174± 9

[1] Male rats having an initial body weight of 132±2 g were used; the experimental diets were fed for 4 weeks. The basal diet contained 1% cholesterol.
[2] CA = cholic acid, ST = succinylsulfathiazole, SG = sulfaguanidine.
[3] Liver values are expressed on a wet-weight basis.
[4] Mean for 10 rats ± SEM.

whereas rats fed the sulfaguanidine-supplemented diets had significantly lower liver total lipid and cholesterol levels than the control animals. The depression in weight gain observed in sulfaguanidine-fed animals is typical of the effect of this sulfonamide on growth of weanling rats fed a purified diet (20). The depression noted in this particular experiment(table 3), but not observed in previous experiments, can undoubtedly be attributed to the lower initial body weight of the animals used.

The results presented in table 4 demonstrate the interrelationships of dietary supplements of cholic acid with succinylsulfathiazole or sulfaguanidine in rats fed a cholesterol-containing diet. In the absence of cholic acid, succinylsulfathiazole significantly increased liver and plasma total lipid and cholesterol levels over the values observed in control animals. Cholic acid also increased plasma and liver total lipids and cholesterol. The addition of succinylsulfathiazole to the cholic acid-supplemented diet did not increase plasma or liver total lipid and cholesterol levels over those observed in animals fed diets supplemented with cholic acid alone. Actually, all parameters were lower in rats fed cholic acid and succinylsulfathiazole than in those fed cholic acid alone; however, only the liver total lipid difference attained significance. Thus, though dietary succinylsulfathiazole has a cholesterol-elevating effect in rats fed a cholesterol-supplemented diet, this effect is not observable in rats fed a cholesterol-cholic acid-containing diet. Sulfaguanidine, however, depressed liver cholesterol and plasma total lipid levels both in the absence and presence of dietary cholic acid. Also liver total lipid was lower in rats fed both sulfaguanidine and cholic acid than in animals fed cholic acid alone. Plasma cholesterol levels were also lower in sulfaguanidine-fed rats; however, these differences were not statistically significant.

DISCUSSION

The studies reported were initiated in an attempt to shed light on the mechanism by which succinylsulfathiazole, and presumably other sulfonamides, produce elevations in blood and liver cholesterol levels. These studies have yielded results, which although not clarifying the mechanism involved, do suggest a possible explanation for the cholesterolemic effects of sulfonamides. In general, the present investigations, in accord with previous reports (1–3), have shown that succinylsulfathiazole elevates liver, and in some cases plasma, cholesterol levels in rats fed a cholesterol-supplemented diet. Under similar conditions sulfaguanidine exerted a depressing effect on liver lipid and cholesterol levels. In rats fed cholesterol-free diets the effects of sulfaguanidine, like those of succinylsulfathiazole, were small and variable. Furthermore, the present studies have shown that the cholesterolemic effect of dietary succinylsulfathiazole, but not that of supplementary sulfaguanidine, is masked by a dietary supplement of cholic acid.

We originally felt that the effects of sulfonamides on cholesterol levels could be explained by 1) a metabolic effect of the drug at the cellular level, or 2) some influence of these drugs at the intestinal level. In the latter case the effects of the sulfonamides might result from a direct influence of these compounds on cholesterol or cholic acid absorption, or the effect might be due to the suppression of intestinal microorganisms by the sulfonamides. We set out to test the first mechanism, that sulfonamides exerted some metabolic effect which influenced cholesterol levels. The hypothesis was advanced that by virtue of the goitrogenic effect of orally administered sulfonamides (12, 13) and the known hypercholesterolemia associated with hypothyroid states (14), the cholesterol-elevating effect of succinylsulfathiazole was due to its suppression of thyroid activity. That this is not the case is amply documented by the data presented in tables 1 and 2. Succinylsulfathiazole which was hypercholesterolemic was not goitrogenic, whereas sulfaguanidine which did induce an increase in thyroid weight depressed liver cholesterol levels.

These results appear to point to the intestine as the site of action of the sulfonamides. Because of the known bacteriostatic action of these drugs, an indirect effect of the sulfonamides on cholesterol metabolism, mediated through an alteration of the intestinal microflora, appears to be reasonable. However, the evidence supporting such a concept is circumstantial. That the cholesterolemic effect of dietary antibodies can be dissociated from any effect of these compounds on the intestinal flora (7, 9) casts doubt on a mechanism involving alterations of the intestinal bacteria in the case of sulfonamides. The alternative explanation, however, that the sufonamides somehow interact with cholesterol or bile acids does not necessarily exclude a possible role of intestinal microorganisms. That the sulfonamides tested are most effective when added to cholesterol-supplemented diets might suggest that they influence cholesterol absorption. However, the sum of the data collected appears to be better-related to a possible effect on the excretion of bile acids.

Tissue cholesterol levels are the net result of rates of input and removal of this sterol. Input can be considered as cholesterol synthesized *de novo* and that supplied by the diet. Removal of cholesterol occurs primarily in the form of bile acid excretion (14). Cholesterol synthesis from acetate is under feedback control (21) and increasing the input of cholesterol from the diet has the effect of inhibiting the synthesis of cholesterol (21). Likewise cholesterol degradation is under feedback control whereby bile acids prevent the further degradation of cholesterol (22).

In view of these known regulatory pathways for the synthesis and degradation of cholesterol, the effects of the 2 sulfonamides studied might be explained on the basis of an alteration of bile acid excretion. Such an explanation would presuppose that sulfaguanidine facilitates the excretion of bile acids by somehow impairing the enterohepatic circulation of bile acids, whereas succinylsulfathiazole would have opposing effects. Thus in rats fed a cholesterol-free diet, cholesterol levels are the result of the balance between *de novo* synthesis of chol-

esterol and degradation to and excretion of bile acids. Under these conditions the rates of these 2 pathways can be regulated and neither sulfonamide has much of an effect. In animals fed cholesterol, synthesis of cholesterol would be inhibited (21) and bile acid formation increased (23). Under these conditions cholesterol input is constant and cannot be reduced; therefore a reduction in bile acid excretion (by succinylsulfathiazole) would increase the enterohepatic circulation, inhibit the conversion of cholesterol to bile acids and thereby increase cholesterol levels. A compound that would facilitate bile acid excretion (sulfaguanidine) would have opposing effects and would lower tissue cholesterol levels. When both cholesterol and cholic acid are fed, tissue cholesterol levels attain an equilibrium under conditions where both cholesterol synthesis and bile acid formation are severely inhibited (22). Under these conditions a further increase in bile acid absorption (by succinylsulfathiazole) would probably not result in further elevations in cholesterol levels since the conversion of cholesterol to bile acids is maximally inhibited. An increase in bile acid excretion (by sulfaguanidine) would partially overcome the inhibition of cholesterol conversion to bile acids and might be expected to result in lowered tissue cholesterol levels.

This proposed hypothesis is in accord with the results of this report. However, the data presented can be interpreted only as suggesting the possibility of such regulation. The validity of the proposed mechanism for the cholesterol effects of sulfaguanidine and succinylsulfathiazole must await further study.

ACKNOWLEDGMENTS

We thank W. R. Casebeer, C. A. Drescher and L. Nash for assistance in preparation of diets and care of the animals and Mrs. Gaye Castor for assistance in the preparation of the manuscript.

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Zirconium, Niobium, Antimony and Fluorine in Mice: Effects on growth, survival and tissue levels [1]

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ABSTRACT To evaluate innate effects of certain trace elements, 540 mice were fed a diet of rye, corn oil and dried skim milk containing moderate amounts of zirconium and niobium and no detectable antimony or fluorine, in an environment relatively free of trace contaminants. Groups of 108, divided as to sex, were given 5 ppm zirconium, niobate or antimony or 10 ppm fluoride in drinking water from weaning until natural death. Females given fluoride grew somewhat larger at older ages and both sexes survived as long as their controls. Inherent toxicity, manifest by decreased median life spans and longevity and by some suppression of growth of older animals, was observed in females given niobium and antimony. The feeding of niobium was associated with an increased incidence of hepatic fatty degeneration. No element was carcinogenic or tumorigenic. Fluoride did not accumulate in soft tissues, but increments of niobium and antimony were found. Zirconium occurred in both controls and experimental groups, and showed slight toxicity. Of 15 trace elements studied in this manner, chromium, fluorine and nickel showed no demonstrable innate toxicity, whereas tellurium, arsenic, tin and vanadium showed the most.

Innate biological effects of small doses of trace elements, given to mice and rats in drinking water from the time of weaning until death are being studied in an environment relatively free of contaminating trace elements. Effects of 9 elements given to mice have been reported: titanium, vanadium, chromium, nickel, germanium, arsenic, cadmium, tin, and lead (1, 2). The present study concerns zirconium, niobium, antimony and fluorine, all of which are present in food and in human tissues.

METHODS

The environment, diet and basal drinking water to which mice were exposed from the time of weaning for their lifetimes have been described in the first 3 papers of this series (1–3). They have not been altered, except for the addition of 1 μg/ml chromium as the acetate to the basal drinking water, not used in the first series of experiments (3).[3] Randombred mice of the Charles River CD strain were born from pregnant females purchased from the supplier.[4] At the time of weaning, groups of about 54 male and 54 female mice, six to a cage, were given the basal drinking water to which was added, at 5 ppm metal, either zirconium sulfate, sodium niobate, or antimony potassium tartrate, or at 10 ppm element, sodium fluoride. Equal numbers of control animals received only the basal water.

The diet contained 2.66 μg/g zirconium, and 1.62 μg/g niobium. Fluorine and antimony were not detected.

Animals were weighed weekly for 8 weeks and then at monthly intervals. Dead animals were dissected, grossly visible tumors and other lesions were noted and abnormal tissues sectioned and stained with hematoxylin and eosin for microscopic examination. Hearts, lungs, kidneys, livers and spleens were pooled in samples of 5 to 15 from various age groups and analyzed for the elements given.

Tissues were ashed at 450° in muffle furnaces and handled as reported (1, 2).

Received for publication November 4, 1968.

[1] Supported by Public Health Service Research Grant no. HE-05076 from the National Heart Institute, Contract DA 2595 from the U. S. Army, and CIBA Pharmaceutical Products, Inc.

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[3] The diet was composed of seed rye flour (60%), dry skim milk (30%), corn oil (9%), and sodium chloride (1%), to which were added ferrous sulfate and vitamins (3). The basic drinking water contained, as soluble salts: (ppm element) zinc, 50; manganese, 10; copper, 5; chromium, 1; cobalt, 1; and molybdenum, 1. The water was obtained from a spring and was doubly deionized.

[4] The Charles River Mouse Farms, Inc., North Wilmington, Massachusetts.

Those analyzed for antimony were ashed in a low-temperature asher.[5] Analyses for zirconium were made by the method of Thamer and Voight (4), modified by Oak Ridge National Laboratory (5), using chloranilic acid; for niobium by the method of Belcher et al. (6); for antimony by atomic absorption spectrophotometry;[6] and for fluoride by a fluoride specific electrode.[7] Sensitivities for zirconium and niobium have been reported (7, 8). The detection limit for fluorine was 0.1 μg/ml solution. For antimony, 0.25 μg/ml solution was the detection limit and the reproducibility was of the order of 24%. The cathode source was relatively insensitive, variability of results on the same sample was large, and the method was unsatisfactory except for semi-quantitative measurements. This lack of sensitivity was enhanced by the necessity of using small samples (< 1–2 g) in the low-temperature asher. About 50% of added antimony was lost at high temperatures (450°).

RESULTS

Growth rates. No element significantly suppressed the growth of male mice during the first year of life, except for occasional periods (table 1). At 18 months of age, losses of weight occurred in those fed zirconium, niobium and antimony. Females showed similar differences from the controls at this age. Those given niobium were smaller than their controls at 5 months of age and subsequently, and a similar trend was noted in the antimony-fed group. Enhanced growth occurred in females given fluorine, to the extent that at a year and 18 months of age their mean weights exceeded those of males by 2.1 and 5.2 g, respectively ($P < 0.05$).

Survival rates, life spans and longevity. The percentage of animals surviving at each 3-month period is shown in figures 1–4. Mice fed fluoride and antimony survived as well as their controls. Mice fed zirconium survived somewhat less well than their controls, although differences were significant only for females. Males in the niobium group had rates of survival similar to the controls, whereas females lived for shorter intervals after 12 months of age.

[5] Tracerlab 500-A, Richmond, California.
[6] The Perkin-Elmer Corporation, Norwalk, Connecticut.
[7] Orion Research Inc., Cambridge, Massachusetts.

TABLE 1

Mean weights of mice given trace elements at various ages

Age	Controls	Zirconium	Niobium	Antimony	Fluorine
days	*g*	*g*	*g*	*g*	*g*
			Males		
30	26.0 ± 0.68 [1]	26.6 ± 1.19	25.5 ± 1.29	24.6 ± 0.72	26.4 ± 0.54
60	39.1 ± 0.68	39.2 ± 0.93	37.8 ± 0.38	38.6 ± 0.59	39.8 ± 0.58
90	45.2 ± 0.75	42.8 ± 0.90 [2]	42.5 ± 0.41 [3]	43.0 ± 0.79 [2]	47.4 ± 0.63 [2]
120	49.3 ± 1.06	48.2 ± 0.93	48.4 ± 0.49	49.2 ± 0.72	49.8 ± 0.89
150	52.0 ± 1.42	51.0 ± 0.75	47.5 ± 0.68 [3]	49.2 ± 0.95 [4]	51.8 ± 0.59
180	51.6 ± 1.38	51.1 ± 1.06	51.1 ± 0.68	51.4 ± 0.89	53.6 ± 1.06
360	56.8 ± 2.16	54.7 ± 1.40	55.4 ± 1.25	56.1 ± 1.11	55.6 ± 1.48
540	58.0 ± 1.91	50.3 ± 2.59 [5]	53.1 ± 1.26 [2]	51.7 ± 2.50 [2]	54.5 ± 1.69
			Females		
30	22.1 ± 0.42	20.1 ± 0.49	20.8 ± 0.09	20.4 ± 0.34	21.7 ± 0.33
60	28.6 ± 0.54	30.4 ± 0.55 [2]	30.0 ± 0.07	28.8 ± 0.50	29.3 ± 0.10
90	35.1 ± 0.56	35.1 ± 1.29	34.5 ± 0.47	34.8 ± 1.06	36.4 ± 0.58
120	38.2 ± 0.89	39.0 ± 1.03	39.2 ± 0.75	39.1 ± 0.83	42.4 ± 0.92 [3]
150	44.0 ± 0.98	42.6 ± 1.52	39.3 ± 1.06 [3]	40.4 ± 0.69 [3]	46.2 ± 1.21
180	45.2 ± 0.80	43.8 ± 0.94	42.1 ± 1.17 [2]	43.4 ± 1.02	50.0 ± 1.09 [3]
360	54.3 ± 1.48	53.5 ± 1.12	44.7 ± 1.49 [3]	47.4 ± 1.67 [3]	57.7 ± 1.10 [4]
540	55.2 ± 1.45	50.7 ± 1.20 [5]	46.4 ± 0.70 [3]	50.5 ± 0.79 [3]	59.7 ± 2.06 [4]

[1] Mean ± SEM; 53 to 55 mice in each group.
[2] Differs from control, $P < 0.025$.
[3] Differs from control, $P < 0.005$.
[4] Differs from control, $P < 0.05$.
[5] Differs from control, $P < 0.01$.

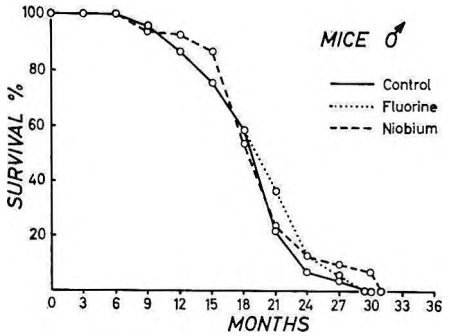

Fig. 1 Survival of 54 male mice given fluoride and 54 given niobium in water. No significant differences from the 54 controls appeared at any interval.

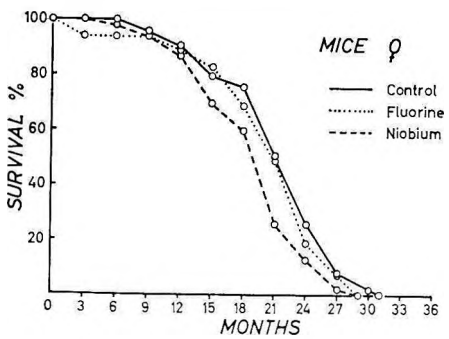

Fig. 2 Survival of 54 female mice given fluoride and 54 given niobium in water. Shorter survival times occurred for those fed niobium, with a significant difference from the 54 controls at 21 months of age ($P < 0.025$), by chi-square analysis. The half-life of the niobium group was 65 days less than that of the controls.

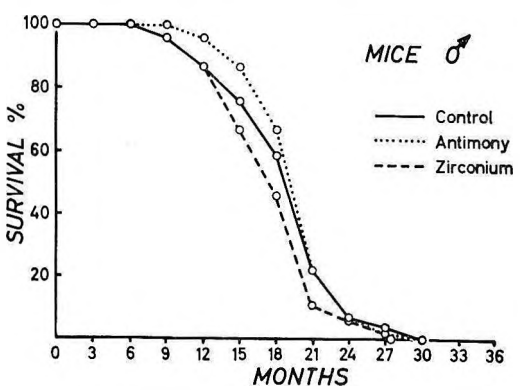

Fig. 3 Survival of 55 male mice given antimony and 53 given zirconium in drinking water. At no interval were the numbers significantly different from those for the 54 controls, although those fed zirconium had somewhat shorter survival times, their half-life being 27 days less.

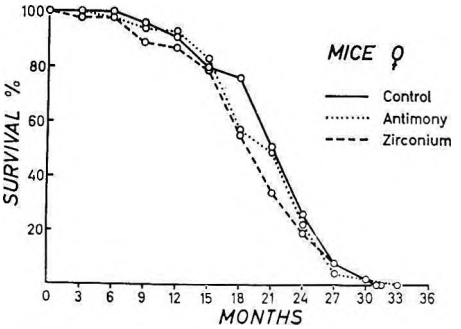

Fig. 4 Survival of 54 female mice given antimony and 53 given zirconium in water. The longest-lived mouse, aged 1,004 days, was fed antimony. There were 54 controls. At 18 months of age there was a difference in survival of mice fed zirconium ($P < 0.05$), and their half-life was 67 days less than that of the controls.

According to measured life spans (table 2), male mice fed fluorine lived longer than their controls by 29 to 60 days at 3 intervals, whereas females did not. However, the feeding of zirconium was associated with shortened life spans by 27 to 47 days in males and 67 to 85 days in females at 5 of 6 intervals. In females, niobium appeared to shorten median and 75% life spans by 65 to 101 days and antimony by 49 to 86 days, whereas males were little-affected. In the cases of the last 3 elements, the usually observed differences between the survival of males and females was lessened.

Longevity, defined as the mean age at death of the oldest 10% of animals, was significantly increased in males given niobium and reduced in no group. The greatest ages were attained by single animals in the male niobium and the female antimony groups, whereas a male in the zirconium group and females in the niobium and fluorine groups died 2 months or more before those in the other groups.

Accumulation of metals. No fluorine was detected in the tissues of mice fed this element, even when the mice were 2 years old, nor in their controls. Bone, however, which accumulates fluorine, was not analyzed.

Zirconium had a predilection for spleen and heart (table 3). Unexpectedly large amounts were found in all organs of control mice, probably reflecting the large amount in the diet. There appeared to be

TABLE 2

Life spans of mice fed trace elements

	No. mice	Mean	Median	75% Dead	90% Dead	Last	Longevity [1]
		days	*days*	*days*	*days*	*days*	*days*
				Males			
Controls	54	540	570	637	692	913	806 ± 34.3 [2]
Zirconium	54	520	543	599	645	832	760 ± 17.4
Niobium	54	560	563	603	787	946	910 ± 21.1 [3]
Antimony	55	542	582	626	651	910	786 ± 32.7
Fluorine	54	591	599	682	752	909	830 ± 28.3
				Females			
Controls	54	618	625	745	770	951	855 ± 29.3
Zirconium	53	580	558	660	800	955	901 ± 21.0
Niobium	54	536	560	644	752	886	803 ± 23.1
Antimony	54	569	576	659	742	1004	843 ± 47.8
Fluorine	54	629	630	707	789	885	838 ± 14.5

[1] See text.
[2] Mean ± SEM.
[3] Differs from controls, $P < 0.025$.

TABLE 3

Mean zirconium levels in the tissues of mice, wet weight [1,2]

	Control		Zirconium-fed	
	No. mice		No. mice	
		μg/g		*μg/g*
Liver	64	12.30	37	10.20
Kidney	52	21.38	42	32.60
Heart	57	46.03	28	25.93
Lung	61	19.78	34	15.20
Spleen	34	41.12	42	64.21
Tumor	2 [3]	13.37	2	0.0 [4]
Whole, newborn	1	9.45		
Total or mean	268	26.59	183	31.07

[1] Tissues were pooled in lots of 3 to 10. Zirconium was found in all but 6 samples.
[2] Control mice were 241 to 752 days of age; zirconium-fed mice were 300 to 614 days of age.
[3] Renal and hepatic tumors.
[4] Mammary tumors.

little, if any more, in those fed the metal. No age-linked accumulation was demonstrated.

Niobium, when fed, accumulated in spleen and heart in larger amounts than in the controls (table 4). Relatively little was found in liver, kidney and lung. Although the data on antimony are semiquantitative, this element was not detected in tissues of control mice, whereas considerable amounts were found in 17 to 60% of the tissues when it was fed. Lung and liver appeared to accumulate antimony; when not detected, the tissues were usually from younger animals (table 5).

Incidence of spontaneous tumors. Unlike arsenic and germanium (9), none of these elements appeared to influence the incidence or type of spontaneous tumors, either benign or malignant. Tumors were found in 34.8% of the controls, 22.7% of the zirconium, 23.6% of the niobium, 18.8% of the antimony and 30.3% of the fluorine groups. Four to eight tumors in each group were malignant. Those of the lung were the most prevalent, making up 58.5% of all tumors (range of 5 groups 50 to 62.5%).

Other pathological changes. Microscopic examination of tissues showed a high incidence of hepatic fatty degeneration in the niobium group; this lesion occurred in 41.2% of animals examined ($P < 0.02$). In table 6 are shown the incidences of this disturbance and its severity, graded from + to +++. Included for comparison

TABLE 4

Mean niobium levels in tissues of mice, wet weight [1]

	Control		Niobium-fed	
	No. mice		No. mice	
		μg/g		μg/g
Kidney	29	0.0	53	2.10
Liver	23	1.14	62	2.37
Heart	22	0.22	62	11.41
Lung	24	2.94	62	2.15
Spleen	19	1.74	62	15.61
Mean	117	1.51	301	6.87

[1] Control mice were 241 to 752 days of age; niobium-fed mice were 343 to 689 days of age; tissues were pooled in groups of 6 to 12 samples.

TABLE 5

Mean antimony levels in the tissues of mice, wet weight [1]

	Control		Antimony-fed		
	No. mice		No. mice	% found	
		μg/g			μg/g
Kidney	19	ND [2]	60	25.0	12.96
Liver	38	ND	48	51.3	6.38
Heart	19	ND	88	17.1	9.12
Lung	19	ND	61	60.5	11.09
Spleen	19	ND	78	19.2	13.78

[1] These values are approximate, ± 25% (see text); tissues were pooled in lots of 5 to 15; control mice were 574 to 636 days of age; antimony-fed mice were 381 to 751 days of age.
[2] Not detected.

TABLE 6

Fatty degeneration of the liver in mice fed trace elements [1]

	No. mice	+	++	+++	Total	
					no.	
						%
Control	99	4	12	6	22	22.2
Zirconium	60	8	8	6	22	36.7
Niobium	68	8	15	5	28	41.2 [2]
Antimony	67	2	8	1	11	16.4
Fluorine	56	4	6	3	13	23.2
Arsenic	55	2	5	0	7	12.7
Germanium	75	1	10	3	14	18.7
Tin	24	1	0	0	1	4.2
Vanadium	19	2	0	1	3	15.8

[1] Lesions were graded as to severity from 0 to +++.
[2] Differs from controls, $P < 0.02$, by chi-square analysis.

are the data on livers of mice from the previous series (2). Niobium appeared to be the only active element among these eight in this respect.

DISCUSSION

Recondite toxicity of a trace element given to small mammals for their lifetimes may become manifest by adverse effects a) on growth and body weight, b) on median life span, c) on longevity, d) on the incidence of spontaneous tumors, and e) on the microscopic appearance of tissues at death. We have used these criteria in an attempt to discover whether certain trace elements to which modern man is exposed may have adverse effects on health and life span. Furthermore, favorable effects from an element, according to these criteria, may appear, suggesting that the element may have some essential role, but such effects may depend on whether

or not the basic diet contains insufficient amounts to provide optimal function.

In this light, the feeding of fluorine as 10 ppm fluoride to mice given a diet deficient in fluorine caused no demonstrable adverse effects. In fact, female mice fed fluoride grew somewhat larger, males lived somewhat longer and both groups showed no significant pathological changes compared with their littermate controls. Nor did fluoride accumulate in their soft tissues. Therefore, fluoride at this level demonstrated no inherent toxicity. To our knowledge, life-term experiments on mice exposed to this level of sodium fluoride have not been conducted (10).

Niobium, on the other hand, may have exerted several signs suggestive of recondite toxicity. Exposed mice weighed somewhat less at older ages. Females had shortened life spans. Hepatic fatty lesions were found frequently. The element accumulated in tissues. Therefore, niobium, which is present in human foods and tissues (8), may have some adverse effects which have not been described, perhaps as an antimetabolite for another element.

The feeding of antimony also was associated with some decrease in the life spans of most females, although one mouse receiving it survived the longest by 2 months of any of the others. This element accumulated in tissues without definitive pathological lesions being found. There was also some suppression of the growth of older mice. Exposure to this element is largely a function of civilization, for its abundance on the earth's crust is low, 0.2 ppm (11).

Less obvious changes were found in zirconium-fed animals, tissues of which showed no characteristic pathology. Accumulation was evident, however, in both control and treated mice. It appeared to be slightly toxic. Human tissues have contained quite large concentrations of this element (7), but its biological role, if any, is unknown.

The daily intakes of these elements can be calculated on the assumption that mature mice ingest 7 g water and 6 g food/100 g body weight/day (1). Under certain circumstances, intakes can be larger. The relative intakes of the controls and experimental animals on this basis were, respectively (μg/100 g body weight): zirconium, 16 and 51; niobium, 9.7 and 44.7; antimony, < 5, and 35; fluorine, < 0.6 and 70. On a comparable weight basis, the daily intake of human beings would be 24.5 mg of the element (49 mg fluoride). These levels are many times larger than the measured or estimated intakes in human food (7, 8, 10). Furthermore, tissue accumulations in mice were somewhat larger than those of human beings in the case of zirconium (7), in the same order of magnitude in the case of niobium (8) and probably much larger in the case of antimony. Therefore, despite the high intakes of mice, the human situation was nearly duplicated for 2 of the 4 elements and probably exceeded for another.

To compare innate toxicities of these 4 elements with those of others, the mean ages at death of the mice in these studies, of the 9 groups previously reported (1, 2), and of 2 groups which will be reported, are shown in table 7. Mice of both sexes fed fluorine, chromium and nickel had the greatest mean ages, whereas those given tellurium, arsenic, tin and vanadium had the least. Sex differences, however, were apparent. Males fed chromium, fluorine, antimony and niobium survived longest, and those taking lead, germanium, vanadium, tellurium, arsenic and cadmium survived to lesser ages. Females fed nickel, lead, titanium, fluorine and chromium were relatively long-lived compared with those given tin, niobium, antimony, selenium, tellurium and zirconium.

Differences in mean ages of males and females fed the same element were apparent. Females of the three control groups lived 78, 69 and 60 days longer than males. However, females lived 117 to 224 days longer than males when both were taking vanadium, titanium, germanium, cadmium, lead and nickel, which suggests that these elements were toxic to males. Mice of both sexes given chromium, antimony, niobium, selenium, and tin showed minor differences in mean ages.

Therefore, according to the criterion of mean age at death, chromium was the least toxic element, whereas toxicity of niobium, zirconium and antimony lay midway among the 13 elements studied, with no demonstrable effect on males. The mean age of control groups given chro-

TABLE 7

Mean ages of mice fed various trace elements [1]

	Cr in water [2]	Both sexes	Males	Females	Difference
		days	*days*	*days*	*days*
Chromium	+	606	587	625	38
Fluorine	+	594	572	617	45
Nickel	0	591	479	703	224
Control III [3]	+	580	546	618	78
Titanium	0	570	511	629	118
Antimony	+	569	560	577	17
Lead	0	567	464	670	206
Zirconinm	+	550	520	580	60
Cadmium	0	549	474	624	150
Niobium	+	548	560	536	−24
Control II [4]	0	543	510	570	60
Control I [5]	0	530	496	565	69
Selenium	+	527	540	514	−26
Germanium	+	525	462	588	126
Vanadium	0	525	466	583	117
Tin	0	522	511	533	22
Arsenic	+	513	473	554	81
Tellurium	+	500	463	537	74

[1] There were 108 or more animals in each group, except in the case of vanadium, where there were 52. Fluoride was given in water at 10 ppm, selenite at 3 ppm, tellurite at 2 ppm and the other elements at 5 ppm.
[2] Chromium in water at 1 ppm.
[3] Present series.
[4] Reported previously for germanium, vanadium, tin and arsenic (2).
[5] Reported previously for titanium, lead, cadmium, nickel and chromium (1).

mium was 50 to 76 days greater than those of the other two control groups not fed this metal.

ACKNOWLEDGMENTS

The courtesy of Professor Kurt Benirschke in providing the microscopic sections and of Dr. D. V. Frost in making the analyses for fluoride, is greatly appreciated. We also thank Dr. Eric G. W. Barradale for his interest and efforts.

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Enzyme Activities in Tissues of Zinc-deficient Rats [1]

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ABSTRACT Activities of 1) alcohol and glutamic dehydrogenase in liver and kidney, 2) alkaline phosphatase in intestinal mucosa, kidney, and plasma, 3) catalase in liver, kidney, and blood, 4) xanthine oxidase in liver and kidney, and 5) aspartic and alanine aminotransferases in liver were measured in rats fed diets low in zinc or protein, or both, and in their pair-fed controls for 45 to 180 days. Deficient diets contained either 1 to 2 or 2 to 4 ppm of zinc; control diets, 20 or 30 ppm. Alkaline phosphatase activity decreased (together with zinc concentration) in the intestinal mucosa of rats fed 2 to 4 ppm but not in plasma or kidney. Addition of 0.5 mmoles of zinc increased activity to the same extent in mucosal homogenates of zinc-deficient and control rats. Alcohol dehydrogenase activity in liver decreased marginally in rats receiving 2 to 4 ppm of zinc and substantially in those receiving 1 to 2 ppm. A correlation existed between alcohol dehydrogenase activities and zinc concentrations in livers of the latter. Lowered glutamic dehydrogenase activities of marginal significance occurred in kidney when 2 to 4 ppm diets were fed with 14.8% protein. No changes occurred in activities of the other enzymes. Elevated concentrations of uric acid in plasma occurred infrequently. Only zinc-dependent enzymes suffered impairment of activity in zinc deficiency. The lowered activity is attributed to decreased enzyme concentrations. Lack of sufficient zinc to maintain the structural integrity of these enzymes is suggested as a cause.

An inadequate intake of zinc decreases growth rate and causes abnormalities of dermal, skeletal, gastrointestinal and male reproductive systems in all the avian and mammalian species that have been examined. If the zinc intake is markedly lowered, the changes become sufficiently severe to cause death within a few weeks. Numerous studies have had as their purpose the discovery of the nature of the biochemical disturbances at fault. Such studies have demonstrated that the activities of several enzymes requiring zinc as a cofactor are impaired (1–5). Others, however, are said to remain unaffected (3, 5–8). It has been claimed also that certain enzymes not dependent upon zinc, including intestinal amylase (1, 9), and catalases in blood, liver and kidney (10) have decreased activities in zinc-deficient states.

The main objective of the present study was the acquisition of additional information about the effects of zinc deficiency upon the behavior of both classes of enzymes, those requiring zinc for maintenance of the active state or as a cofactor and others not dependent upon zinc for their functions. The importance of the intracellular location of the enzyme also has been considered, as has the effect upon activity of zinc added to the incubation mixture.

EXPERIMENTAL

The plan and experimental procedure have been described previously (11). Most of the work here described was carried out concurrently with studies previously published on zinc and copper concentrations in visceral organs and hair of rats fed diets low in zinc (11). Rats of series 1 to 8 had been fed diets containing 2 to 4.3 ppm of zinc. The duration of the experimental periods of series 1 to 5 ranged from 90 to 150 days;[3] those of series 6 to 8, 61 to 73 days. Each series included 9 to 16 rats. In a more recent experiment, designated series 16, and not previously described, a

Received for publication October 31, 1967.

[1] Supported in part by Public Health Service Research Grant no. AM-09622 from the National Institute of Arthritis and Metabolic Diseases and by a grant from the Medical Research Committee of the American University of Beirut.

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[3] The diets fed rats of series 5 were listed as containing 1.73 ppm. However, additional experience with diets containing less than 2 ppm suggests that the zinc intake of this group was higher than this figure indicates.

further lowering of the zinc content of the diet to 1.8 ppm was accomplished by substitution of calcium phosphate prepared in the laboratory for that obtained by purchase. This diet, fed for a minimum of 49 and a maximum of 59 days, caused more severe symptoms in a shorter time. Two deaths believed to be due to zinc deficiency occurred after 45 days.

Diets of pair-fed control rats were the same as those low in zinc except that sufficient zinc carbonate was added to increase the zinc concentration to 20 or 30 ppm.

In 2 experiments (series 3 and 4) the combined effects of low protein and low zinc intakes were studied. With the exception of these, all diets contained 14.8% of a mixture consisting of two-thirds casein and one-third gelatin (designated NP). The diets low in protein (designated LP) contained 7.4% of the mixture. Sucrose replaced the protein calories in the LP diets. Food intake of the control rats was adjusted daily to that of the zinc-deficient rats.

At the end of the feeding period, the rats were fasted 24 hours. Laparotomy was performed under light ether anesthesia. Blood was aspirated either from the heart or from the pleural cavity after cutting the inferior vena cava before retrograde catheterization. When plasma was examined, heparin was used to fill the needle and dead space of a syringe of 2.5 ml capacity. The quantity of heparin used was shown by trial to be without effect on zinc concentration or phosphatase activity.

The liver was perfused with cold 0.14 M NaCl until nearly colorless to remove blood. Usually 50 ml of NaCl solution was required. The perfusion also removed blood almost completely from kidneys, intestines, and other abdominal organs. Liver and other tissues were reduced to a brei by means of a Potter-Elvehjem homogenizer with Teflon pestle. Ten to 20 volumes of cold 0.14 M NaCl or water were used as a diluent, and the homogenization was continued for 60 seconds with 6 strokes of the pestle.

In these experiments the small intestine was divided into a proximal segment of 10 cm, and the remainder divided into halves which are designated jejunal and ileal. The lumen was washed with cold 0.14 M NaCl until no further detritus was dislodged. The mucosa was then stripped, without opening the intestine, by means of a microscope slide. The mucosa was then weighed and homogenized.

Alcohol dehydrogenase activity was measured by the method of Bonnichsen (12) at pH 8.5 with pyrophosphate buffer; glutamic dehydrogenase by the method of Christie and Judah (13) with phosphate buffer at pH 7.8; alkaline phosphatase by the method of Bessey et al. (14) with *p*-nitrophenyl phosphate as substrate and a glycine-NaOH buffer at pH 9.25. The Mg^{++} concentration was maintained at 0.5 mM.

Xanthine oxidase activities were measured by the method of Al-Khalidi et al. (15) and uric acid by the methods of Brown (16) in series 3 and of Praetorius and Poulsen (17) in later series using uricase.[4] Analyses by the 2 methods did not differ. Aspartic and pyruvic aminotransferase activities were measured by the method of Karmen (18).

Catalase was measured in homogenates and in laked blood cells by the method of Feinstein (19) and of Tarlov and Kellermeyer (20), modified so that reaction times were shortened to exactly 15 and 30 seconds. Titers at the 2 intervals showed the theoretical relationship in most instances. This is accepted as evidence that destruction of enzyme by the perborate substrate was not serious. When the 2 titers failed to agree, calculation was based on the 15-second titration. Results are expressed as milliliters of 0.1 N $KMnO_4$ consumed/g of Hb or per milligram of tissue nitrogen. The specific activities were affected by sample size in that decreased amounts of blood or tissue yielded higher activities. However, the use of samples sufficiently small to overcome this effect impaired the precision to an unacceptable degree. The effects of error from this source were kept relatively constant by adjusting sample sizes to give titers that ranged between 0.50 and 1.00 ml of $KMnO_4$. Since control and deficient rats were always examined in pairs simultaneously, it is believed that differences between them would have been demonstrated by the method used.

[4] Purchased from Worthington Biochemicals, Freehold, New Jersey.

RESULTS

Alkaline phosphatase activities in plasma were unchanged in the moderately deficient rats. Moreover, addition of zinc failed to cause a significant rise in activity The quantity of zinc added was sufficient to give a concentration of 0.5 mmoles/liter which previous studies had shown to be optimal. In the presence of zinc, alkaline phosphatases of serum and intestine undergo a shift in pH optimum to higher values (21). Provision for this has been made in these experiments by adjustment to the new optimum.

Significantly decreased alkaline phosphatase activities occurred in mucosa throughout the small intestine in the rats fed diets low in zinc (table 1), including the ileal portion (for which results are not shown). The intestinal mucosa was one of the few tissues in which zinc concentrations were significantly lowered in these experiments (11), a finding that gains significance because of the association with lowered enzyme activities. Addition of zinc to the incubation mixture increased the phosphatase activity substantially. However, it is important to note that the increase expressed as percentile gain over the activity without added zinc was the same in the zinc-deficient as in the control rats. The response to added zinc by the enzyme of the distal segments closely resembled that of the proximal segment.

Zinc depletion at the level produced in these experiments failed to lower alkaline phosphatase activities in the kidney (table 1). This was not unexpected for there was no decrease in zinc concentration in kidneys of deficient rats (11). Lowered protein intake caused a substantial decrease in alkaline phosphatase activities of similar degree in kidneys of both deficient and control rats.

Catalase. Measurements of catalase activity in blood and liver are shown in table 2. Zinc depletion had no effect upon the activity in either tissue, or in that of kidneys of a smaller group (results not shown). Lowered activities occurred in the blood of rats fed the low protein diet with adequate zinc intake but not in those with low zinc intakes.

TABLE 1

Tissue alkaline phosphatase activities in zinc-deficient and control rats; effects of added zinc and of low protein intake

	Series	No. rats	Control	No. rats	Deficient	P value [1]
			mmoles pNP [2]/mg N/min		*mmoles pNP/mg N/min*	
Intestine, first 10 cm						
pH 9.25	7,8	19	9.51 ± 0.58 [3]	19	6.17 ± 0.43	< 0.01
pH 10 + Zn		19	13.88 ± 0.72	19	9.35 ± 0.66	< 0.001
P value [4]			< 0.001		< 0.001	
Intestine, next 20 cm						
pH 9.25	7,8	18	1.47 ± 0.102	18	1.19 ± 0.092	0.05
pH 10 + Zn		19	2.21 ± 0.124	19	1.84 ± 0.126	0.05
P value [4]			< 0.001		< 0.001	
Kidney						
NP	2,3	10	1.050 ± 0.101	11	1.085 ± 0.057	ns
LP		10	0.682 ± 0.095	11	0.727 ± 0.075	ns
P value [5]			< 0.01		< 0.01	

[1] Significance of the difference between the means of control and deficient rats.
[2] p-Nitrophenol.
[3] Mean ± SE of mean.
[4] Significance of the difference between mean activities in absence and presence of added zinc.
[5] Significance of the difference between kidney alkaline phosphatase activities of rats fed 14.8% protein (NP) and 7.4% protein (LP).

TABLE 2

Catalase in blood and liver in zinc-deficient and control rats (series 2, 3, 5, and 7)

	No. rats	Control	No. rats	Deficient
		units/g Hb [1]		*units/g Hb*
Blood				
NP diet	15	7.77 ±0.42 [2]	16	7.05 ±0.44
LP diet	12	6.36 ±0.36	12	6.90 ±0.69
P value [3]		0.02		ns
		units/mg N		
Liver	17	0.335±0.032	22	0.343±0.031

[1] Milliliters of 0.1 N $KMnO_4$/g of Hb.
[2] Mean ± SE of mean.
[3] Probability that the difference between means of rats fed LP (7.4%) and NP (14.8%) protein diets is significant.

Glutamic dehydrogenase. The zinc-deficient rats tended to have lower activities in both liver and kidney (table 3). However, the difference between means was significant only in the kidneys of those receiving the diets containing 14.8% protein.

Alcohol dehydrogenase. Assays of this enzyme were made both in moderately deficient (series 2 and 3) and severely deficient (series 16) rats (table 3). Lowered activities were observed in both groups. However, the response was much more pronounced in those fed the diets containing 1.8 ppm of zinc than in those receiving the higher intakes. In the latter, the differences between means failed significance at the 5% level by a small margin in the NP group. Many of the values in the zinc-deficient rats overlapped those of the control groups.

A substantial lowering of activity was brought about by the consumption of diets low in protein in both control and deficient rats. Lowered food intake may also explain the lower activities of the controls in series 16 in comparison with those of series 3. The more severely zinc-depleted rats of series 16 ate less than those fed diets of higher zinc content and the food consumption of the pair-fed controls decreased correspondingly.

Zinc concentrations of the livers of the rats of series 16 fed diets low in zinc were significantly lower than those of the controls. This is in contrast with findings reported previously (11) for livers of rats fed low zinc diets containing 2 to 4 ppm. Figure 1 shows the relationship of alcohol dehydrogenase activity to the concentration of zinc in liver. Calculation of the coefficient of correlation gave a value of $r = 0.505$ which is significant at the 0.05 level. However, no correlation was demonstrated when similar calculations were made for the controls. Neither was there a significant correlation between rate of gain in body weight and liver alcohol dehydrogenase activity.

Xanthine oxidase and uric acid. Xanthine oxidase activities in liver and kidney were frequently higher in the zinc-deficient rats than the controls (table 3). However, partly because of the large variations within some groups, the results were not statistically significant. Low protein intakes were associated with lowered activities, but again the differences were not significant.

Only 5 of 29 rats receiving the zinc-deficient diets had uric acid concentrations which significantly exceeded those of the control rats (table 4). Thus the elevation of uric acid concentrations reported by others in zinc deficiency did not occur under the conditions of these experiments.

Aminotransferase activity in liver. There is no evidence of a change in aspartate or alanine aminotransferase activities related to zinc depletion in the moderately deficient rats of series 2 (table 5).

DISCUSSION

Of the enzymes investigated in this study, the alkaline phosphatases (22, 23), alcohol dehydrogenase (24) and glutamic dehydrogenase (25) are known to be zinc-dependent. It was only among this group that changes in activity occurred in association with zinc deficiency.

The activity of alcohol dehydrogenase was significantly decreased in the livers of severely depleted rats. The decrease in

TABLE 3

Enzyme activities in liver and kidney in zinc-deficient and control rats

		Series	No. rats	Control	No. rats	Deficient	P value [1]
				units [2]/mg N/min		*units/mg N/min*	
Glutamic dehydrogenase							
Liver	NP diet [3]	1–3	16	0.86 ± 0.056 [4]	16	0.81 ± 0.030	ns
	LP diet	2–3	10	0.86 ± 0.059	10	0.75 ± 0.073	ns
Kidney	NP diet	1–3	16	0.85 ± 0.073	16	0.67 ± 0.041	< 0.05
	LP diet	2–3	10	0.02 ± 0.052	10	0.90 ± 0.090	ns
Alcohol dehydrogenase							
Liver	NP diet	3	6	1.14 ± 0.305	7	0.57 ± 0.126	< 0.10 > 0.05
	LP diet	2–3	9	0.35 ± 0.063	9	0.31 ± 0.084	ns
	P value [5]			< 0.01		< 0.01	
	NP diet	16	17	0.52 ± 0.065	15	0.27 ± 0.054	< 0.001
				μmoles [6]/mg N/min			
Xanthine oxidase							
Liver	NP diet	3–5,7,8	30	0.636 ± 0.115	32	0.796 ± 0.137	ns
	LP diet	3	6	0.560 ± 0.022	6	0.737 ± 0.103	ns
Kidney	NP diet	3	5	0.558 ± 0.138	5	0.818 ± 0.219	ns
	LP diet	3	6	0.475 ± 0.076	6	0.555 ± 0.140	ns

[1] Significance of difference between the means of control and deficient rats.
[2] One unit is a change of absorbance of 0.001.
[3] NP and LP refer to protein intakes of 14.8 and 7.4%, respectively.
[4] Mean ± SE of mean.
[5] Significance of difference between mean activities in absence and presence of added zinc.
[6] Xanthine oxidase activity is expressed as micromoles of xanthine converted to uric acid per minute.

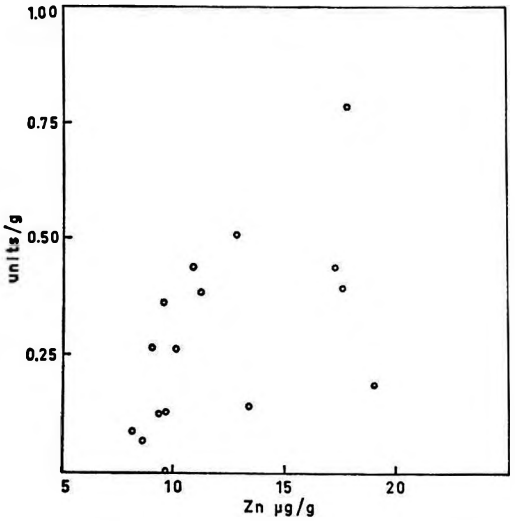

Fig. 1 Alcohol dehydrogenase activities of livers of zinc-deficient rats plotted against the concentration of zinc in the liver. A significant correlation exists (see text). Other signs of zinc deficiency such as lowered food consumption, growth rate, loss of hair, and development of skin lesions also tended to be more severe in rats with the lower zinc concentrations in liver.

activity gains significance because it was proportional to the decline in zinc concentration in liver. Marginally significant changes were found in the rats receiving diets containing zinc in the 2- to 4-ppm range. Prasad et al. (6) report alcohol dehydrogenase activity to be decreased in testis, esophagus and bone in zinc-deficient rats, but Hsu et al. (5) observed no change in the liver.

Hove et al. (1), in early studies of zinc deficiency in rats, described decreased alkaline phosphatase activity in the intestine. In the present studies intestinal alkaline phosphatase showed the largest and most consistent changes of the enzymes studied. Decreased activity was prevalent when other signs, such as growth rate, suggested no more than moderate deficiency of zinc. However, alkaline phosphatase activities in kidney remained unchanged contrary to findings of Prasad et al. (6) and Hsu et al. (26), and despite the decreased activity in the intestinal enzyme. The vulnerability of the latter may be explained in part by

TABLE 4

Uric acid in plasma

	No. rats	Control	No. rats	Deficient	No. elevated [1]
		Uric acid		Uric acid	
		mg/100 ml		*mg/100 ml*	
NP diet [2]	17	2.51 ± 0.257 [3]	18	2.57 ± 0.417	3
LP diet	11	2.56 ± 0.290	11	2.87 ± 0.500	2

[1] Frequency of concentrations higher than those of the control rats.
[2] NP and LP refer to protein intakes of 14.8% and 7.4%, respectively.
[3] Mean ± SE of mean.

TABLE 5

Aminotransferase activities in liver in zinc-deficient and control rats (series 2)

	No. rats	Control	No. rats	Deficient	P value [1]
		units/g × 1000		*units/g × 1000*	
Alanine aminotransferase					
NP diet [2]	4	218 ± 13.5 [3]	5	222 ± 13.6	ns
LP diet	4	151 ± 41.1	5	191 ± 30.5	ns
P value [4]		ns		ns	
Aspartate aminotransferase					
NP diet	4	180 ± 42.8	5	189 ± 32.3	ns
LP diet	4	133 ± 45.1	5	157 ± 58.7	ns
P value [4]		ns		ns	

[1] Significance of difference between the means of control and deficient rats.
[2] NP and LP refer to protein intakes of 14.8 and 7.4%, respectively.
[3] Mean ± SE of mean.
[4] Significance of difference between mean activities in rats fed 14.8 and 7.4% protein.

the rapid turnover of mucosal cells and consequent total loss of zinc from the tissue. It should be recalled that the decrease in zinc concentration in intestinal mucosa in these experiments was significant (11), although it was not large, whereas zinc concentrations in kidney remained unchanged.

Addition of zinc in vitro to homogenates of intestinal mucosa produced a substantial activation of alkaline phosphatase activity. However, the increase was identical when calculated as percentile increase in the mucosas of zinc-deficient and control rats. Thus, the lowered activities in the former are not accounted for by lack of sufficient zinc to support the activity of the enzyme. Instead, decreased production or increased losses of enzyme from the mucosa cells appear to be more acceptable explanations.[5]

In plasma, a slight decrease in alkaline phosphatase activity in the zinc-deficient rats was not significant. A similar tendency was observed by Macapinlac et al. (7). Morrison and Sarett (8) also failed to find a change in alkaline phosphatase activity of serum of chickens. Decreased activities in serum were reported by Day and McCollum (2) in rats, Miller et al. (3) in calves and Pond et al. (4) in pigs.[6]

The failure of glutamic dehydrogenase to undergo more than a marginally significant change in activity in the zinc-deficient rats is of interest. Possibly its location in the mitochondria (27) may be responsible. It has not, however, been examined in the more severely deficient rats.

Catalase, although it has no known content or requirement for zinc, nevertheless was reported by Day and Skidmore (10) to undergo a decrease in activity in liver, kidney and blood in zinc-deficient mice. This finding was not confirmed by our study. The work of these investigators was carried out before the lability of catalase in the presence of its substrate was recognized, and the relatively long reaction times used for the assay may have permitted destruction at rates which differed in tissues of zinc-deficient and control rats.

Xanthine oxidase activities were examined in the zinc-deficient rats in an attempt to learn more about the disturbance of uric acid metabolism which Wachtel et al. (28) described. Xanthine oxidase is a metalloenzyme containing iron and molybdenum but without known dependence upon zinc. It is located in the soluble cell fraction. No well-defined changes in its activity occurred in the zinc-deficient rats. Moreover, only a few sera showed elevations of uric acid in the several groups of rats in which it was measured.

In addition to the zinc-dependent enzymes which have been shown in this paper to undergo a loss of activity in zinc deficiency, Hsu et al. (5) have reported that carboxypeptidase A, but not carboxypeptidase B activities of pancreas, are decreased by zinc deprivation.

No decrease in activity which could be related to zinc deficiency was detected among the enzymes having no known dependence upon zinc. These included alanine and aspartate aminotransferases in liver, catalases of liver, kidney, and blood, and xanthine oxidases of liver and kidney. The enzyme of each of these tissues represents a distinct entity or a family of isozymes possibly under separate genetic control. The failure of any of the seven to undergo a loss of activity in the zinc-deficient rats suggests the absence of a general depression of protein synthesis and supports the existence of a specific requirement for maintenance of tissue levels of zinc by those enzymes requiring zinc as a cofactor. The finding by Mills et al. (29) that pancreatic carboxypeptidase activity decreased as a result of zinc deficiency whereas trypsin and chymotrypsin did not, is in agreement with this interpretation of our results. The first is zinc-dependent, the latter are not. However, conclusions concerning the general application of these findings must await the study of larger numbers of enzymes in tissues in zinc-deficient states.

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[5] Luecke, R. W., M. E. Olman and B. U. Baltzer (Federation Proc., 26: 523, 1967) found lowered serum and intestinal phosphatase activities in weanling rats fed diets containing 0.9 ppm of zinc for an unspecified time. In agreement with our findings, addition of zinc in several concentrations failed to restore the activity to that of the control rats. Although Hove et al. (1) claimed that addition of zinc enhanced the activity of intestinal alkaline phosphatase to a greater degree in zinc-deficient than in control rats, a statistical evaluation of their results fails to support this conclusion.

[6] See footnote 5.

Zinc has been shown to have structural or organizing functions for alcohol and other dehydrogenases, various alkaline phosphatases and other enzymes. Akeson (30) found that 4 atoms of zinc are essential for full activity of liver alcohol dehydrogenase and Drum et al. (31) have recently demonstrated the importance of zinc in maintaining quaternary structure in vitro. The progressive removal of zinc from liver alcohol dehydrogenase by dialysis in experiments described by Oppenheimer et al. (32) caused the activity to a decline to a level proportional to the concentration of zinc retained by the enzyme. The relationship resembles that shown in figure 1 where liver alcohol dehydrogenase activity is correlated with concentration of zinc.

It is possible that in the presence of subcritical concentrations of zinc, the cells are unable to maintain the polymeric states required for the enzymes to function. As a result, unorganized monomers may be lost from the cell, or, if retained, may repress synthesis of new monomer.

Several of the enzymes examined showed signficantly decreased activities in tissues of the rats fed the low protein diets. These included alkaline phosphatase in kidney, catalase in blood, and alcohol dehydrogenase in liver. However, no evidence was found that low protein intake modified the effects of low zinc intake upon the activities of the enzymes studied. In fact, the depressed activity of catalase in blood of the rats fed low protein diets took place only in the control rats. In the zinc-deficient rats no change occurred.

ACKNOWLEDGMENTS

The authors thank Aznive Sabundjian for technical assistance and Madeleine Basmadjian for help with the manuscript. Xanthine oxidase measurements were carried out under Dr. Usama Al-Khalidi's direction by Margaret Keverian and aminotransferase measurements by Souren Chekijian.

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Effect of Neonatal Food Restriction in Mice on Brain Growth, DNA and Cholesterol, and on Adult Delayed Response Learning [1]

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ABSTRACT This study was designed to examine the long-term effects of a limited period of nutritional restriction on ultimate brain size and functional capacity. Experimental mice were intermittently removed from their mothers during the period of rapid brain growth between 2 and 16 days of age, producing a 57% reduction in body weight compared with littermate controls. Thereafter they were fed ad libitum. At 9 months, body, cerebral and cerebellar weights were reduced in the males by 17, 7 and 14%, respectively, below control values. Total DNA was reduced 8% in the cerebrum and 22% in the cerebellum. Cerebral cholesterol was reduced slightly. Despite these brain changes, the restricted groups showed no lasting impairment in voluntary running, in learning a Lashley type III maze, or a visual discrimination with escape from water as a reward. The restricted males showed an unexpected improvement in learning a delayed response task. The restriction experience may have altered reaction patterns so that the restricted males were able to more than compensate for any possible handicap due to the nutritional deprivation. The restricted females did not manifest this improved performance, and their final body size reduction exceeded that of the males.

In experimental studies on the effects of nutritional factors on behavior, one may differentiate between deficits limited to certain developmental periods, and deficits present during the period of functional testing. Altered metabolic states associated with functional impairments may appear in experimental animals fed various inadequate diets (1); hence studies on learning behavior in the presence of a deficiency are difficult to interpret. However, if the deficit is produced during the development of the nervous system, and the animals are subsequently returned to full nutrition, although some metabolic alterations may persist, it may be possible to study the effects of irreversible loss or suppression of neurons or alteration of the pattern of neuronal interconnections in animals with competent neuromuscular function. In the case of the mouse, it has been shown that reduction of nursing time can produce an interference with net cerebral DNA synthesis during the first week of postnatal life (2). Similar findings have been reported for the rat, and extended to show a persistent deficit in brain DNA after neonatal food restriction followed by several months of feeding a normal diet (3). Although cell division of the neurons of most cerebral areas in the mouse is completed before birth, there is some cell division of neurons postnatally, especially in the cerebellum, as well as multiplication of glial cells, and extensive neuronal maturation (4–6). Hence it was of interest to examine the possibility of producing irreversible behavioral alterations in this species by means of a period of neonatal food restriction.

Previous work dealing with neonatal dietary restriction in rodents, and behavioral testing after a period of unrestricted feeding, includes 3 studies on rats in which alterations in body growth were produced by varying the numbers of young in the nursing groups (7–9). Seitz (7) observed a number of differences in behavioral traits, and Barnes et al. (9) found a significant increase in the number of errors made by the restricted rats in a water maze discrimination test.

Although nutritional restrictions imposed on the mother during pregnancy might be expected to produce a greater derangement of brain function than would be produced by postnatal restriction alone, the present study has been limited to the

Received for publication November 17, 1967.

[1] This investigation was supported in part by Public Health Service Research Grant no. AM-02679 from the National Institute of Arthritis and Metabolic Diseases, and by a grant from the Henry Foundation.

effects of restricted nursing time between 2 and 16 days of age in mice, with full renourishment before testing. Following the behavioral tests, the brains were taken at 9 months of age, weighed and analyzed for DNA as an index of cell numbers, and cholesterol as a representative of those lipids that increase in concentration during brain maturation.

METHODS

Albino mice from a Swiss strain maintained in this laboratory were used. At 2 days of age, all mice were removed from the nest at 4 PM and littermate pairs set up, matched for sex and nearest body weight, one member of each pair to have its nursing time restricted. The individuals were labeled by a small skin incision until the ears were large enough to receive ear notches. The mice were nursed in litters of six to nine. If the litter size was over eight, an odd mouse was retained during the nursing period, and thereafter discarded. After the initial handling, the controls were returned to the nest, and the experimental mice placed in an incubator overnight on a soft surface at 30° and approximately 50% humidity. Thereafter the food-restricted mice were in the incubator from 4 or 5 PM until 9 AM unless they were found to be losing weight, in which case the time with the mother was increased. During this period of food restriction 2 mice disappeared (presumably eaten by their mothers) from the group later used for behavioral testing. The removal of the experimental mice from the nest involved some daily disturbance of the mother and control siblings, but otherwise the controls were not handled except for the weekly cage cleaning and weighing, and ear notching at 8 days of age.

The overnight removal from the mother was terminated at 16 days of age. At this time some of the litters were killed to obtain brain weights, and the remainder were left with their mothers undisturbed until they were weaned at 22 days of age to an ad libitum diet of laboratory ration. After 35 days of age the males were housed individually to prevent fighting. The females were housed in pairs of one control and one restricted littermate until 7 months of age, when they were housed individually during the delayed response testing. The study includes a total of 104 animals.

At the time of killing, hypothermia was induced before removing the brains in order to reduce metabolic rates concomitantly with the slowing of blood flow and hence to minimize metabolic changes during dissection.[2] The cerebrum was isolated by transecting the brain stem between the anterior and posterior colliculi, and the olfactory lobes were not included. The cerebellum was isolated by transecting the peduncles, and the posterior colliculi were included with it.

Analytical methods were used as previously described (2) except that separation of the RNA fraction was omitted, and 2 modifications were introduced: namely, a change in the temperature of DNA extraction from 70° to 90° (10–12) and a change in the temperature of color development with diphenylamine from 35° to 10° (13, 14). In brief, the procedure was as follows: The frozen tissue was homogenized in a volume of acetone:ethanol (1:1) equal to 25 times its weight, then extracted in chloroform: methanol and ethanol (14). After washing twice in cold 5% TCA, the DNA was extracted from the residue by heating for 20 minutes at 90° in 5% TCA, centrifuging at room temperature, re-extracting for a second 20-minute period at 90° and washing the residue twice, combining supernatants for DNA estimation.[3] DNA was estimated by the diphenyl-

[2] Animals were sedated by Phenergan (promethazine hydrochloride) in a dose of 0.85 mg/10 g at 16 days or 1.5 mg/10 g for adults, and placed in a ventilated metal container at 5°. This induced gradual slowing of respiration and pulse as rectal temperature declined, with termination of activity at rectal temperatures of about 15°, achieved within 30 or 40 minutes. Although there was no clear-cut reduction in the total DNA content of brains obtained after mice were simply etherized and allowed to stand at 24° for 2 hours before removing the brains, the hypothermia procedure was retained as the chilled brains contained little blood and were convenient to dissect.

[3] To study the extraction procedure, 2 cerebri, from normal adult mice A and B, were sectioned in the midline, and the right half of A plus the left half of B were pooled for analysis and compared with the pool of the left half of A plus the right half of B. In comparisons of extractions of 8 such pairs of half cerebri, one extraction for 20 minutes at 70° plus 3 successive extractions of one hour each were required to remove all the diphenylamine-reacting material, whereas two 20-minute extractions at 90° were sufficient. When the diphenylamine reaction was read against DNA standards heated for 20 minutes in 5% TCA at the respective temperatures, the yields at 70° were 98 $\pm$ 2% (range) of the yields at 90°. The indole reaction for DNA estimation (15) gave values 85 to 100% of those from the diphenylamine reaction developed at 10°, but the values were less reproducible with the indole procedure.

amine reaction, using the Burton procedure, with the modification that the color was developed for 48 hours at 10° as recommended (13) to eliminate a contribution to the color from sialic acid. Color development at 10° decreased the apparent DNA in extracts from adult brains by 5% compared with color development at 35°, and also increased the sensitivity of the reaction by about 25%. Extracts were read against standards made from sperm DNA.[4] Cholesterol was determined on the lipid fraction after digitonin precipitation (2).

Behavioral tests included measurements of spontaneous running in activity wheels (16), exploratory activity in an "open field," learning in a Lashley type III maze modified for use in water (17) and visual discrimination and delayed response in a water Y-maze. Animals were given access to the activity wheels for 60-minute periods on six separate days. The mouse had a choice between running in the wheel or resting in the adjoining small cage during this period. No food or water were available during the test hour. Paired controls and food-restricted littermates were always tested simultaneously. Wheels and connecting cages were dismantled and washed meticulously following each test to minimize effects of odors, and control and restricted mice were assigned alternately to each wheel.

The "open field" consisted of a 61 cm × 61 cm square with a metallic screen floor divided by lines into 30 equal squares. The solid metal walls were 11.5 cm high, and it had a clear plastic cover. Metal barriers were arranged in the field to divide it into a series of alleys and cul-de-sacs. The investigator sat within view of the mouse and recorded the number of squares the mouse entered, and other activities, for 8-minute periods, on three consecutive days.

The Lashley maze was filled with water at 22° to a depth of 8 cm, requiring the animals to swim. Finding the escape route was the reward for learning. When the mice were 50 days old they were given three preliminary trials in a straight alley filled with water at 24–25°, with a vertical rubber escape cord at one end. On the following day testing in the Lashley maze began. Two trials a day, spaced by 4 to 6 hours, 6 days a week, were conducted until the mouse completed 7 of 8 trials without an error, or had reached the seventy-fifth trial. The time taken to escape from the water was recorded, as well as the errors, consisting of the entries into cul-de-sacs and retraces within an alley.

Visual discrimination was tested in a Y-shaped swimming maze similar to that used for rats by Barnes et al. (9) but modified to permit a study of delayed response, after the mice had learned the visual discrimination. Each limb of the Y measured 4 × 38 cm. A shielded 6-volt light was turned on in the arm of the maze containing the escape cord. The escape cord (black, thick-walled rubber tubing 1.2 cm in diameter) was placed in the right or left limb according to a Gellerman schedule of randomization. A similar cord was placed just outside the end of the unlighted limb to equalize any possible visual cues after the signal light was off. Room illumination was such that the observer required some dark adaptation to be able to see the white mouse in the maze. A shielded red light was used to record data. The maze was filled with water at 22° to a depth of 8 cm. The mouse learned to swim to a clear plastic door in the starting limb of the Y through which the light could be seen. After a 2- to 3-second wait the plastic door was raised and the mouse permitted to swim out and make its choice. (A black gate was then lowered to prevent retracing.) If the choice was the limb that contained the escape cord, the mouse was returned to its cage, via a towel. If the mouse swam into the arm that lacked the escape cord, an error was recorded and it was again placed in the starting arm and permitted another choice. A maximum of 4 choices was given for each trial, but for the purposes of scoring only the first choice was counted, the others being considered as training.

One trial was conducted each day. The first 18 trials constituted a simple visual discrimination test, as the light was left on after the plastic door was raised. The next 50 trials incorporated a small but significant delay in that the light was turned off automatically by a mechanical switch before the door left the water, re-

[4] Nutritional Biochemicals Corporation, Cleveland.

quiring the mouse to make an adaptive response to a visual cue that had already been removed.

RESULTS

Growth. The effects of food restriction produced by intermittent removal of the young mice from their mothers, between 2 and 16 days of age, are shown in figure 1 and table 1. By 16 days the body weights of the food-restricted mice were only 43% of the control weights, and the animals appeared so emaciated and feeble that it was felt they would probably not survive further food restriction of this degree. Although they were left with their mothers from 16 days of age until weaning at 22 days, they were at 16 days at a disadvantage in competing with the controls for the maternal milk supply, which had begun to decline by this time (18), and they appeared to be less ready to make the transition to eating pellets. Hence they probably suffered some degree of deprivation for a few days beyond the 16-day age. However, between this time and 6 weeks of age their appearance improved and their growth rate increased so that the percentage reduction of body weight was considerably less than it had been. At 225 days, when body weights were approximately maximal, some degree of stunting was present, the restricted males being 17% below their littermate controls, and the restricted females even lower.

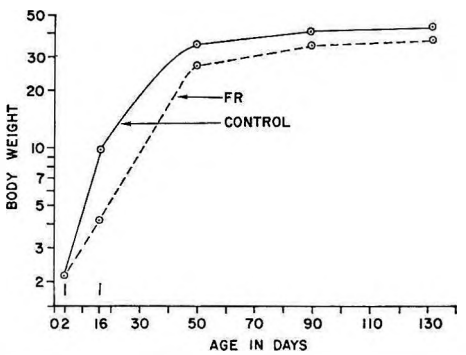

Fig. 1 The effect of restricting nursing time between 2 and 16 days of age on body growth in mice. Abscissae, age in days, ordinates, body weight in grams on a logarithmic scale. Males only are presented, the data for the females being similar. The circles indicate the size of the standard errors of the means. The weight differences at all ages are significant, with $P < 0.001$ ($N = 26$).

The brain weight changes were similar in direction to the body weight changes. At 16 days of age there was a 20% reduction of cerebral weights, whereas in the group carried to 270 days the cerebral weight reduction was only 7 to 8%. It is of interest that in the adults the percentage cerebellar weight reductions were significantly greater than the percentage cerebral reductions: for males $P < 0.02$; females,

TABLE 1

Effect of food restriction from 2 to 16 days of age on body and brain weight [1]

Group [2]	Body wt [3]		Cerebrum wt		Cerebellum wt	
		Difference		Difference		Difference
	g	%	*mg*	%	*mg*	%
2 days:						
Untreated (10)	2.15 ± 0.07	—	95.5 ± 2.7	—	17.6 ± 1.8	—
16 days:						
Controls (21)	9.83 ± 0.33		305 ± 2.8		55.9 ± 0.8	
Restricted (21)	4.21 ± 0.13	−57.2 ± 1.4	244 ± 4.6	−20.2 ± 1.6	41.1 ± 1.4	−26.5 ± 2.9
270 ± 15 days:						
Males						
Controls (14)	45.5 ± 1.2		337 ± 2.3		69.9 ± 0.8	
Restricted (14)	37.7 ± 1.0	−17.2 ± 2.2	313 ± 3.6	−7.2 ± 1.1	59.9 ± 1.6	−14.4 ± 2.3
Females						
Controls (12)	39.9 ± 1.1		350 ± 3.7		71.6 ± 1.0	
Restricted (12)	30.2 ± 0.6	−24.4 ± 1.6	321 ± 4.3	−8.4 ± 0.9	59.4 ± 1.2	−17.0 ± 1.1

[1] Values are mean ± SE of mean. All weight differences between controls and restricted mice are significant within their respective groups with P less than 0.001. The standard errors of the percentage differences were calculated according to formula (6.8) given by Cochran (19) for the case where N = infinity.

[2] Numbers of mice in parentheses. The sexes were combined at 2 and 16 days of age since there were no significant differences between male and female body or brain weights at these ages.

[3] Body weights for the adults are given at 225 days of age, when they were approximately maximal.

< 0.001. In other words, there was relatively more recovery of weight in the cerebrum than in the cerebellum.

Brain composition. There was an 8% reduction in total cerebral DNA ($P < 0.001$) (table 2), which paralleled the weight reduction, so that the ratio of the weight of tissue to total DNA showed no significant change in the cerebrum. In the cerebellum, however, the 22 to 24% reduction in DNA exceeded the percentage weight reduction. This is interpreted as indicating that in the cerebellum there had been a selective suppression of cells with smaller cell territories. The amount of tissue per milligram of DNA is much lower in the normal cerebellum than in the normal cerebrum (table 2). If the DNA per cell were constant, this would indicate that the cells of the cerebellum have, on the average, smaller cell territories than cerebral cells. But a preliminary report of tetraploidy in Purkinje cells of the human cerebellum [5] indicates that actual calculation of cell numbers from cerebellar DNA is subject to uncertainty.

In addition to the reduction in total DNA, there was a small but significant reduction in the cholesterol content ($P < 0.04$, table 2). This suggests some reduction in the size of the structures depositing cholesterol, which might reflect some irreversible interference with the growth of myelinating fibers, or some reduction in the extent of total cell membrane surface area. A similar reduction in the cholesterol of mouse cerebrum was found after neonatal treatment with corticosterone (2), and this has now been found to persist into late adult life.[6] A 12% reduction in cholesterol was reported in brains of malnourished rats, taken at 21 days of age, without refeeding (20).

Behavior. Certain behavioral tests were carried out to learn whether the period of neonatal food restriction had resulted in detectable functional alteration remaining after nutritional rehabilitation. Tests of voluntary running, when the animals were given access to exercise wheels for one hour a day, revealed that the food-restricted group showed significantly less activity between 35 and 40 days of age (on each of 3 trials $P < 0.02$). However, when the tests

[5] Lapham, L. W. 1966 The tetraploid DNA content of normal human Purkinje cells. J. Neuropathol. Exp. Neurol., 25: 131 (abstract); see also Science, 159: 310, 1968.

[6] Howard, E., manuscript in preparation.

TABLE 2

Effects of food restriction from 2 to 16 days of age on DNA and cholesterol in brains of adult mice [1]

Group [2]	Total DNA	Difference	Tissue wt / DNA	Cholesterol	Difference
	μg	%	*mg/mg*	*mg/g*	*mg/g*
Cerebrum					
Males					
Controls (12)	499 ± 6.8		676 ± 7.6	15.55	
Restricted (11)	456 ± 5.6 [3]	−8.4 ± 1.8	687 ± 9.9	15.10	−0.45 ± 0.19 [4]
Females					
Controls (11)	501 ± 3.4		701 ± 5.1	16.09	
Restricted (12)	460 ± 3.3 [3]	−8.2 ± 1.0	697 ± 7.5	15.37	−0.72 ± 0.23 [4]
Cerebellum					
Males					
Controls (14)	540 ± 11		129.8 ± 1.7		
Restricted (13)	424 ± 12 [3]	−21.5 ± 2.4	140.4 ± 1.5 [3]		
Females					
Controls (12)	548 ± 9		130.7 ± 1.0		
Restricted (12)	412 ± 9 [3]	−24.8 ± 1.6	144.2 ± 1.1 [3]		

[1] Values are mean ± SE of mean. Brains were taken at 270 ± 15 days of age.

[2] Numbers of mice in parentheses. Due to loss of an occasional analysis, n varies slightly from the values in table 1, where brain weights for the groups are given.

[3] $P \leq 0.001$.

[4] $P \leq 0.04$.

were repeated at 3 months of age, this difference was no longer present (fig. 2). Since voluntary running increases during puberty (16) the early inactivity may reflect some retardation of development; or, it may reflect some incomplete recovery in some other sense. The observation emphasizes the importance of allowing adequate time for recovery before concluding that a particular effect is irreversible.

Spontaneous exploratory activity was studied in an open-field test conducted when the mice were approximately 9 months of age. The data are summarized in table 3, and show no significant differences in the number of squares entered, or the defecation frequency between control and restricted mice. The restricted males, however, had a significantly higher defecation frequency than the restricted females. Under the conditions of the test, this trait has been regarded as being associated with a greater emotional response to the strange situation (21, 16). The number of times the animals reared up on their hind legs was also counted, but showed no significant differences between groups.

Performance in the Lashley maze is illustrated in figure 3. Both groups were able to swim and to learn the maze with similar facility. Comparisons of the performance on the basis of either the total number of errors, the trials to reach a criterion of 7 out of 8 trials without an error, or the time to reach the criterion revealed no differences in learning ability. However, during the first 10 days (20 trials) when the mice were 51 to 61 days old, the food-restricted group achieved fewer errorless trials than their control littermates ($P \leq 0.02$). One cannot be certain whether this early difference signifies that the treated mice would have a persistent slight impairment in the rate of acquisition of a maze pattern, or, on the other hand, whether the difference simply reflects an incomplete recovery from the effects of the neonatal food deprivation.

The test of visual discrimination learning in the Y-maze was begun when the mice were 6 to 7 months of age. Data for males are summarized in fig. 4. Initially both groups learned to make the visual discrimination within 18 trials, swimming to the lighted arm of the Y to find the escape cord. At trial 19 the light was turned off automatically as the plastic door was raised, requiring the mouse to perceive the light through the plastic door, and later to swim in that direction in near

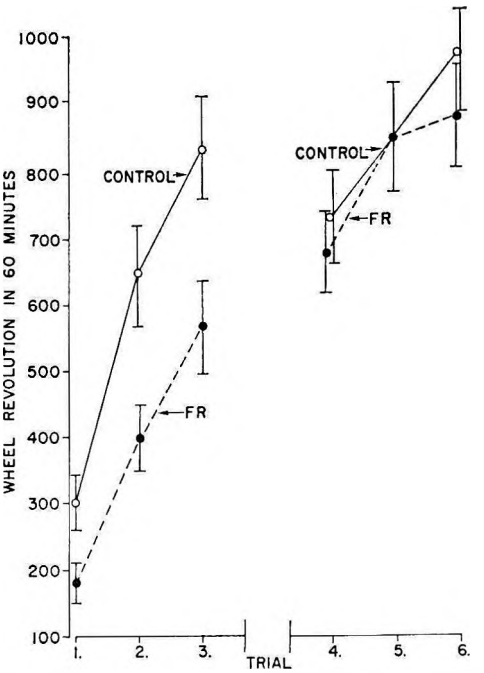

Fig. 2 Voluntary running in activity wheels after neonatal food restriction. Ordinates are wheel revolutions in 60 minutes: 1000 revolutions equaled 0.21 kilometers. Three tests were carried out between 35 and 40 days and tests 4, 5 and 6 were carried out between 90 and 110 days of age. Data for males and females are combined. The differences between controls and restricted groups tended to be greater for the females than for the males but not significantly. Bars indicate standard errors of means (N = 26).

TABLE 3

Activity in an open field after neonatal food restriction [1]

Group	No. squares explored [2]	Fecal boli [3]
Males		
Controls (9)	343 ± 25	1.7 ± 0.5
Restricted (11)	288 ± 21	4.1 ± 1.2 [4]
Females		
Controls (9)	276 ± 26	0.78 ± 0.44
Restricted (11)	273 ± 20	0.45 ± 0.31

[1] Tests were run at 9 months of age, on 3 consecutive days, shortly after the conclusion of the delayed response testing. Values are mean ± SE of mean.
[2] Means of 3 trials.
[3] Total deposited during 3 trials.
[4] $P < 0.02$ with respect to difference from food restricted females.

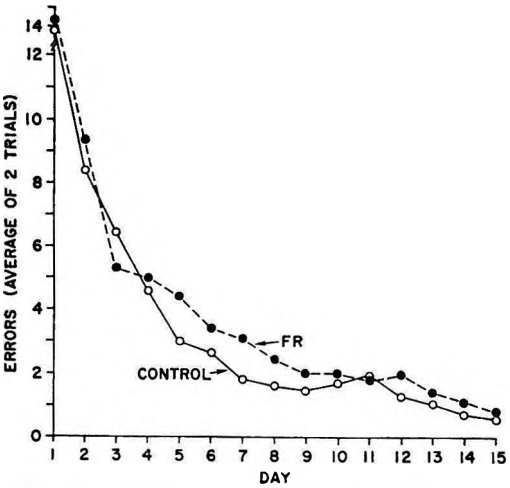

Fig. 3 Learning a Lashley type III maze adapted for use as a water maze, after neonatal food restriction. Ordinates, the mean number of errors per day, 2 trials daily; abscissae, days of testing. Controls, open circles and continuous line; restricted, solid circles and broken line. Although the controls showed slightly more rapid learning, the difference in performance was not sustained. Testing was begun at 51 days of age. Data for males and females showed no significant differences, and were combined ($N = 26$).

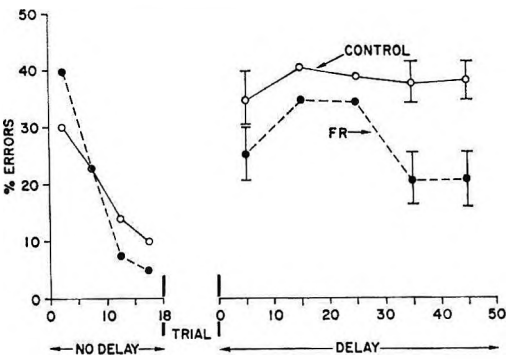

Fig. 4 Learning of a visual discrimination in a Y-shaped water maze by adult males after neonatal food restriction. Ordinates, errors as percentage of total trials for blocks of 5 or 10 trials; abscissae, number of trials. Controls, open circles and continuous lines; restricted, solid circles and broken lines. Testing was begun at 6 to 7 months of age. After the first 18 trials without delay, the signal light was turned off before the mouse was permitted to swim forward to make a choice between the right and left arms of the Y. This enforced delay between signal and response increased the errors made by both groups, but after 30 trials the restricted group showed a decrease in errors, whereas the controls failed to demonstrate significant improvement during the delayed response testing. Bars indicate standard errors of the mean, and were omitted where there would have been considerable overlap ($N = 14$).

darkness, thus making the correct response in the absence of the cue.[7] With the introduction of the delay, the errors increased toward the chance score of 50% for both groups; but, after 30 trials with delay, the unexpected result emerged that the food-restricted group of males performed significantly better than the littermate controls. The restricted mice made fewer errors than the controls during trials 31 to 40 ($P \leq 0.04$) and during trials 41 to 50 ($P \leq 0.02$). In addition, when the 2 groups were compared on the basis of reaching the criterion of 13 correct choices in 15 trials (fig. 5), the more rapid learning on the part of the food-restricted mice was again evident ($P \leq 0.02$), Fisher test (22)).

The female food-restricted and control groups both learned the visual discrimination in the Y with facility, but did not demonstrate statistically significant differences in performance of delayed response, although the trend was in the same direction as with the males.

DISCUSSION

The curve of brain weight increase in normally growing mice inflects sharply to a near plateau at 15 days of age (6). Similarly, histological and electroencephalographic studies show that between birth and 16 to 17 days of age the mouse cortex changes from a structurally very underdeveloped state with no spontaneous electrical activity to a cortex with the adult type of axonal and dendritic proliferation, and the mature type of spontaneous electrocorticogram (6). Actual cell division of neurons in the cerebrum is probably completed before birth in the mouse, with the exception of certain special areas,

[7] In a pilot study of the ability of mice to learn delayed response, another group of 16 animals was started with the delay procedure, without previous training on the simple discrimination. Under these conditions, they gave no evidence of learning the correct response in 25 trials. After the present group had completed the testing shown in figure 4, the 8 mice with the fewest errors were tested with a longer delay of one second elapsing between turning off the light and starting to raise the plastic door. In 20 trials there were 50.6% errors. This is considered evidence that the mice had not solved the problem previously by using some extraneous cue. They were able to wait at the door without the light and apparently retain some orientation if the door had begun to rise before the light went out, but with the door motionless and without the light they appeared to be lost.

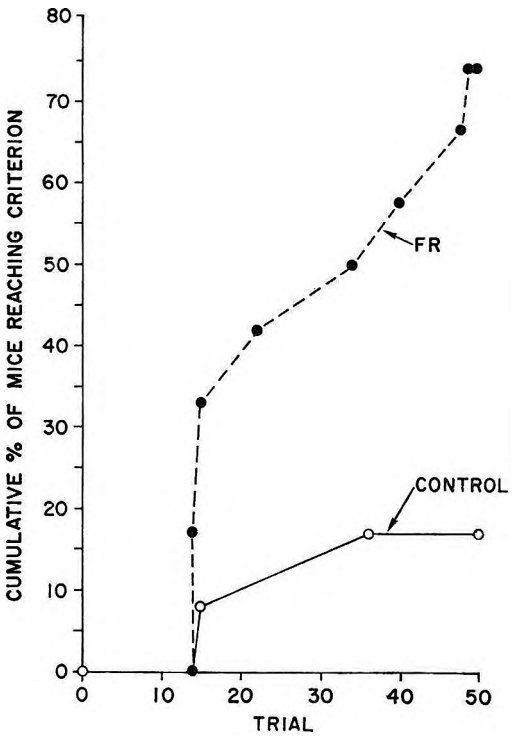

Fig. 5 Progress in learning a delayed response by adult males after neonatal food restriction. Ordinates, the cumulative percentage of mice reaching a criterion of 13 correct responses in 15 trials; abscissae, number of trials. At trial 50, the difference is significant with $P < 0.02$; Fisher test (22).

notably the area dentata of the hippocampus (4). Cell division of glia, however, presumably continues postnatally in the cortex, concomitantly with dendritic growth.

Food restriction from 2 to 16 days of age, during the period of rapid growth and maturation, produced a 7 to 8% reduction in the weight of the cerebrum in mice at 9 months, an age that approximates the end of the period of female fertility in the colony. This reduction in weight was associated with a similar reduction in total cerebral DNA, which indicated there had been an irreversible decrease in cell numbers. The findings are compatible with observations of a persistent reduction in brain DNA after preweaning food restriction in rats reported by Winick and Noble (3), although the data of these authors were based on only 5 animals in each group, and statistical comparisons were not made. A lasting reduction in brain size and DNA content has also been observed after neonatal treatment with corticosterone which may interfere with cell division in the dose used.[8] Presumably there is some loss of developmental potential in the brain sufficiently early that interference with cellular metabolism by a variety of agents during the period of rapid growth can lead to suppression of mitosis with an irreversible net reduction in numbers of cells. Further study will be required to determine what cell types are involved in this reduction of cerebral DNA.

The percentage reductions of weight and DNA content in the cerebellum were considerably greater than in the cerebrum. It is known that there is a great deal of mitosis in the cerebellum postnatally in mice (5). Although the Purkinje neurons, the large neurons of the roof nuclei, and the Golgi II neurons of the granule layer have completed mitosis before day 16 in the embryo, the granule cell neurons, the cells of the molecular layer and the cells clustered around the Purkinje perikarya continue cell division during the postnatal period, in some cases up to 15 days, and the glia continue to 20 days (5). The greater reduction of cerebellar DNA following postnatal food restriction accords well with the histological evidence of the greater amount of cell division in cerebellum as compared with cerebrum during the period in question (4, 5). The percentage of reduction of cerebellar DNA was similar to that following neonatal treatment with corticosterone.[9]

Despite the considerable DNA deficit in the cerebellum in the food-restricted mice, and a smaller deficit in the cerebrum, there was no permanent impairment of voluntary running in revolving wheels, or in the ability to learn a Lashley type III maze with escape from water as the reward. Similarly, both groups learned a simple visual discrimination in a Y-water maze with equal facility. Performance in a delayed response test in the Y-maze revealed an unexpected superiority of performance on the part of the restricted group.

[8] See footnote 6.
[9] See footnote 6.

The delayed response problem is considered to be a behavioral test sensitive to a wide range of experimental treatments (23). Delayed response has not, to our knowledge, previously been adapted to the study of mouse behavior. Frontal lobe damage has been found to give impaired delayed response performance in monkeys (23). However, monkeys with radiation damage have been found to give better delayed response performance than the controls, which has been attributed to reduced distractibility (23).

A general improvement in brain mechanisms as a result of food restriction of the degree used in this study seems unlikely. It is conceivable that the better performance of the food-restricted group might be due to a reduced distractibility, although no specific tests were made. The spontaneous activity in the open field (table 3) showed only a tendency toward a reduction in exploratory activity by the food-restricted males ($P \leq 0.12$). Since the fecal index of emotionality showed a tendency in the opposite direction ($P \leq 0.10$) the data suggest the possibility of a greater emotional reactivity among the food-restricted males. Nevertheless, there were no evident differences in emotional responses between the groups in the swimming situation. The animals were tractable and appeared equally motivated to get out of the water, as shown by the results with both the Lashley maze and the initial visual discrimination in the Y-maze.

The procedure of reducing the milk intake by intermittent removal of the nursing mice from the mother has the disadvantage that it subjects the young to intermittent deprivation of physical contact with the mother, as well as nutritional restriction.[10] This may have been mitigated by sibling contact, since the mice were always in groups of three or four when away from the mother. However, after 2 to 4 hours in the incubator, even at 2 days of age, the young mice were highly active, kicking and squirming around. In the morning, they were at times found to be active, at other times sleeping, but became very active on return to the nest, and sought the nipple aggressively. It is known that hunger increases motor activity (16) and, although quantitative comparisons were not made, it is probable that the experimental mice were stimulated to much greater activity than the controls during the period of feeding restriction. During the period up to 14 days, the controls remained in the nest and were not seen to do anything other than suckle and sleep. It is possible that the experience of the alternation of periods of deprivation with periods of satisfaction, with the early stimulation of motor activity, may have developed certain patterns of behavior that contributed to the success of the experimental group in the delayed response test. Conceivably the altered behavior could involve differences in effective motivation or goal-directed activity. In the case of the males, the pattern of experience may have more than compensated for any possible handicap due to nutritional effects on the size and DNA content of the cerebrum and cerebellum.

The finding that a significant difference in delayed response learning between restricted and control groups appeared only among the males could not be accounted for by any marked difference in the scatter of the data between male and female groups, the standard errors being approximately the same in all groups. The body weight reduction at 9 months in the food-restricted females was 24% of the control weights, significantly greater than the male reduction of 17% (table 1), with ($P \leq 0.05$). Cerebellar weight and DNA, as well as cerebral cholesterol, all showed a tendency toward greater reductions in the females, although the differences from the male reductions were not statistically significant. The data as a whole suggest

[10] The procedure of intermittent removal from the mother not only reduces the total possible nursing time, but imposes an abnormal pattern of feeding. It is known that near-adult rats given their entire food intake in a limited period each day deposit more fat and less protein than rats fed ad libitum to the same body weight gain, and exhibit various metabolic changes (24). While such factors may contribute to our results, in our experimental situation there was such a severe limitation of body growth that a conspicuous feature appears to be the limitation of total milk intake. Mice can be weaned at 18 days, but do not appear to take significant amounts of solid food before 16 days (18). If, in addition to the limitation of milk intake, there was operative a tendency to deposit less protein and more fat, this would make the utilization of the available food relatively unfavorable for true cellular growth. Whether the metabolic changes described in rats (24) would occur in neonates, and whether the changes would persist after a period of ad libitum feeding, are open questions.

that the disability produced in the females by the food restriction may have been appreciably greater than in the males, so that whatever alteration in behavior patterns may have occurred, it did not compensate for the deleterious effects of the restricted nutrition. Another suggestion of a sex difference is that the restricted females showed a significantly lower fecal index of emotionality than the restricted males ($P \leq 0.02$), although there was no significant difference between the female and male controls (table 3).

The degree of food restriction used in the present study produced, during the period of rapid brain growth, a somewhat greater reduction in body weight than has been reported by others for the postnatal period in question. However, our observation of the lack of an effect on simple visual discrimination learning agrees with the finding of D'Amato (8). Comparing litters of six with litters of twelve, Seitz (7) reported that large-litter rats hoarded more food and were more successful in competition for food, showed less activity and more defecation in the open field, were more active sexually and more vicious, and reacted with greater "startle" to opening of the cage door. Without further experimental study, one cannot say whether these differences are determined by nutritional level per se, or by other aspects of the experience of being suckled in large litters. The traits described by Seitz seem quite compatible with the present findings of the greater success in the delayed response task by the restricted male group, although the species and the conditions of restriction were different. However, Barnes et al. (9) found that restricted rats made significantly more errors on a visual discrimination test in a water maze, but noted emotional behavioral differences among the groups and therefore could not conclude as to the relative contribution of "drive" and "capacity" in the altered learning behavior.

It would be expected that there would be some degree of functional brain damage from nutritional interference, if the nutritional restriction is begun early at a critical period of development, and lasts long enough. Although we produced a 20% reduction in cerebral weight by 16 days of age, there was a considerable resumption in brain growth after termination of the feeding restriction, the experimental brains increasing by 28% in the males (69 mg) while the control brains increased by 10.5% (32 mg). Thus the experimental brains exhibited considerably more growth potential after 16 days than those of controls. Since there was a lasting reduction in cerebral weight of 7 to 8% in the food-restricted groups, there was some degree of irreversible interference with brain growth. Although no functional impairment was detected, a longer period of food restriction might be expected to produce a greater degree of lasting reduction in brain size and an appreciable functional impairment. Furthermore, other types of tests might be more suitable for the analysis of possible functional alterations. The finding of a particularly large reduction in cerebellar DNA suggests the desirability of further study directed to an analysis of cerebellar function after food restriction in early life.

ACKNOWLEDGMENTS

The authors thank Dr. Eliot Stellar and Dr. Ernesto Pollitt for advice regarding the testing of rodent learning; Dr. John Money for helpful discussions; Dr. Helen Abbey for statistical consultations; and Mrs. Piroska Bujnovszky and I. Jeffry Deitz for technical assistance.

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Dietary Factors Affecting Utilization of Urea Nitrogen by Sheep in Purified Diets [1]

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ABSTRACT A series of experiments was conducted over a period of 3 years investigating the effect of several factors on the utilization of urea in purified diets for the growth of lambs. The factors were supplements of sodium and potassium bicarbonates (cations), alfalfa and ad libitum vs. limited feeding regimens. The overall effect of cations was to improve the growth of lambs whether the dietary intake was limited or the animals were fed ad libitum. In general, the inclusion of 5% alfalfa also improved growth. An ancillary measurement which correlated with the gains was the level of lactate in the rumen; that is, in general, high levels of ruminal lactate were correlated with lower gains and vice versa. Under limited feeding conditions, both cations and alfalfa were required to decrease ruminal lactate to low levels. Evidence was obtained that the "ammonia-producing mechanism" for the excretion of anions by the kidney was stimulated when supplemental cations were omitted from the diet.

In previous studies (1), it was shown that sodium and potassium bicarbonates added to roughage-free purified diets containing casein as the source of protein improved the efficiency of the diet as measured by growth of lambs. Subsequent studies indicated that the cations were involved in the buffering capacity of the rumen [2] and in the activation of certain enzyme systems (2, 3). In the next phase of this investigation, urea was substituted for casein in these purified diets. Since ammonium ions from urea could act as a buffering agent, an experiment was designed to test this possibility. Another variable which was investigated was the effect of 5% alfalfa in these diets.

The results reported herein are based on a series of experiments designed to study the effects of supplemental sodium and potassium bicarbonates on animals fed purified diets with urea as the sole source of nitrogen.

EXPERIMENTAL

The details of the biological procedures used in the growth study have been described previously (1, 4). In 1961 (exp. 1) 12 lambs approximately 2.5 months of age were divided into 6 pairs based on weight, and then each pair was assigned at random to the 2 experimental diets 26B and 26C (table 1). This study was carried out over a period of 25 weeks. In the second experiment, conducted in 1964, lambs of the same age and history as those in experiment 1 were used. They were divided into groups of six, based on weight and assigned at random to each of 4 experimental diets (table 1). The 4 replications of animals were maintained with the experimental diets over a period of 35 weeks.

In 1965, the same 4 diets used in experiment 2 (table 1) were fed by 2 different feeding procedures. Based on weight, 24 lambs were divided into 8 groups. The animals in each weight group were then assigned at random to the 8 treatments to form a replication. These 3 replications also continued to use the diets over a period of 35 weeks. A total of 60 lambs was used in these 3 experiments. All experimental animals received vitamin A and D capsules (table 1).

The diets used in these experiments were similar to those used previously (5) except that urea was the sole source of nitro-

Received for publication October 11, 1967.

[1] Contribution from the Departments of Biochemistry and Animal Science, School of Agriculture and Life Sciences, and School of Physical Sciences and Applied Mathematics. Paper no. 2499 of the Journal Series of the North Carolina State University Agricultural Experiment Station, Raleigh, North Carolina. Supported in part by a grant from the Herman Frasch Foundation.

[2] Slyter, L. L. 1963 Sodium and Potassium of the Rumen as Related to Volatile Fatty Acid Metabolism. Ph.D. Thesis, North Carolina State University, Raleigh, North Carolina.

TABLE 1

Composition of experimental diets

	Diet 26B	Diet 26C	Diet 27B	Diet 27C
	%	%	%	%
Urea	4.6	4.6	4.2	4.2
Glucose [1]	45.5	35.5	40.9	30.9
Starch	30.6	30.6	30.6	30.6
Hydrogenated vegetable fat [2]	4.0	4.0	4.0	4.0
Cellulose	3.0	3.0	3.0	3.0
Alfalfa leaf meal [3]	—	—	5.0	5.0
Vitamin mix [4]	5.0 [5]	5.0 [5]	5.0 [5]	5.0 [5]
$NaHCO_3$	—	6.0	—	6.0
$KHCO_3$	—	4.0	—	4.0
$Ca_3(PO_4)_2$	1.8	1.8	1.8	1.8
Mineral mix [6]	3.0	3.0	3.0	3.0
Methionine mix [7]	2.5	2.5	2.5	2.5

[1] Crystalline glucose, Corn Products Sales Company, Norfolk, Virginia.

[2] Grateful acknowledgment is made to Procter & Gamble Company, Cincinnati, Ohio, for Primex B and C (pure vegetable shortening).

[3] Alfalfa leaf meal (20% protein) is generously contributed by National Alfalfa Dehydrating and Milling Company, Kansas City, Missouri.

[4] Vitamin mixture/45.36 kg diet: thiamine·HCl, 400 mg; riboflavin, 850 mg; nicotinic acid, 1.13 g; Ca pantothenate, 1.42 g; pyridoxine·HCl, 570 mg; folic acid, 57 mg; *p*-aminobenzoic acid, 1.13 g; inositol, 11.35 g; biotin, 11.4 mg; choline chloride, 113.45 g; menadione (2 methyl-naphthoquinone), 115 mg; 0.1% vitamin B_{12} (with mannitol), 4.66 g; α-tocopheryl acetate, 570 mg; and glucose, 2132 g. Acknowledgment is gratefully made to Hoffmann-La Roche, Inc., Nutley, New Jersey, for the biotin and to Merck and Company, Rahway, New Jersey for contributing all other vitamins.

[5] In addition, 4000 IU of vitamin A (from fish liver oil) and 400 IU of vitamin D (irradiated ergosterol)/day/45.36 kg body weight were administered by capsules, which were contributed by R. P. Scherer Corporation, Detroit.

[6] Mineral mixture/45.36 kg diet: KCl, 273 g; NaCl, 239 g; $MgSO_4$, 204 g; Na_2SO_4, 90 g; $CuSO_4 \cdot 5H_2O$, 893 mg; $FeSO_4 \cdot 2H_2O$, 7648 mg; $MnSO_4 \cdot H_2O$, 1399 mg; ZnO, 2263 mg; $CoCO_3$, 9 mg; KI, 6 mg; $Na_2SeO_4 \cdot 5H_2O$, 4.5 mg; $Na_2MoO_4 \cdot 2H_2O$, 4.5 mg; and glucose, 633 g.

[7] Methionine mixture/45.36 kg diet: DL-methionine, 91 g; glucose monohydrate (Cerelose, Corn Products Company, Argo, Ill.), 1043 g.

gen, with the exception of 1% protein furnished by the alfalfa supplement used in experiments 2 and 3. The only other dietary variable was the presence or absence of sodium and potassium bicarbonates. Diets 26B and 26C contained no alfalfa, while the series 27 diets contained 5% alfalfa supplement. Those diets labeled *C* indicate the presence of Na and K bicarbonates and *B* indicates the absence of supplements of these cations. The symbol (x) designates a controlled feeding regimen, i.e., experiment 1, two times daily, 90-minute feeding period; experiment 3, two times daily, 45-minute feeding period (table 2).

The animals were housed in individual cages described previously (5), and were fed twice daily by the particular regimen specified. The diets were mixed twice weekly in a food mixer.[3] Records of daily feed intakes and weekly weights were kept during the experiment. At the end of the experiment all animals were deprived of feed for a period of 24 hours, after which blood, rumen fluid and urine samples were taken. Immediately thereafter, the ewes were catheterized, and all the animals were force-fed 227 g of diet in one liter of water by stomach pump. Subsequent blood and rumen fluid samples were taken at 0.5, 0.75, 1.5 and 3.0 hours after feeding. A total urine collection was made on the ewes over this 3-hour period. Measurements of ruminal volatile fatty acids were made in all experiments, and total and L(+)-lactic acid measurements in experiment 3. Blood ammonia and urea N measurements were made in experiment 1, and urine ammonia measurements in experiments 1 and 3. Sodium and potassium were determined on the urine in experiments 1 and 3 and on the rumen fluid in experiment 3 by means of a flame photometer.[4] Volatile fatty acid analyses of the rumen contents were made by gas chromatography [5] by the procedure of Erwin et al. (6), L(+)-lactic acid was measured enzymatically,[6] and total lactate was determined by the procedure of Pennington and Sutherland (7). The results are reported as percentages of total volatile fatty acids (5). Immediately after the rumen samples were collected, pH was determined.[7] The free ammonia content of the blood, urine and rumen fluid was determined by the method of Conway (8), and urea ammonia determinations were made according to Conway and O'Malley (9).

Data were subjected to analysis of variance; the 0.05 level of significance represents odds of 1:19, and the 0.01 level, odds of 1:99 (10).

[3] Hobart Manufacturing Company, Troy, Ohio.

[4] Baird Atomic Flame Photometer, Model Ky-1, Baird Atomic, Inc., Cambridge, Massachusetts.

[5] Perkin-Elmer Vapor Fractometer, Model 154C, modified for flame ionization operation, Perkin-Elmer Corporation, Norwalk, Connecticut.

[6] Sigma Chemical Company Tech. Bull 825 (1963). The determination of L(+)-lactic acid in blood, plasma and other fluids at 340 mμ. Sigma Chemical Company, St. Louis.

[7] Beckman Zeromatic pH Meter, Beckman Instruments, Inc., Fullerton, California.

RESULTS

The gains and feed efficiency data are shown in table 2. The results of the growth trial in experiment 1 show that the animals receiving the diets with the cations gained significantly ($P \leq 0.01$) more weight than those receiving the diets without cations. Feed efficiency was also markedly higher for the group receiving cations in the diet. Since these animals were fed twice a day, it was of interest to observe the effects when the animals were fed ad libitum. The ad libitum study, experiment 2, was conducted in 1964, and the gains and feed efficiency data are shown in table 2. Significant differences between cation and no cation supplementation were observed neither in the gains nor the feed efficiency data. This strengthened the notion that the differences observed in 1961 were associated with the controlled feed intake. The animals fed the urea diets supplemented with alfalfa (table 2, diets 27B and 27C) gained significantly more than those receiving the urea diets without alfalfa, confirming results reported previously (5). Subsequently, another experiment was designed in which both controlled and ad libitum-type feeding were studied (exp. 3, table 2).

In experiment 3, feed was kept before the control-fed animals for 45 minutes twice daily. In addition to an ad libitum feeding, the alfalfa variable was also studied in this experiment. In all, 8 diets were used in which cations and alfalfa were studied in all combinations in a 2 × 2 factorial design.

An analysis of these data from the factorial experiment indicated that the ad libitum-fed animals gained significantly more per week ($P \leq 0.01$) than those which were control-fed (772 g vs. 171 g). Overall, the animals fed the alfalfa supplement gained significantly more ($P \leq 0.05$) than those without an alfalfa supplement (558 g vs. 385 g). The overall effect of supplemental cations was to increase gains significantly ($P \leq 0.01$) (C, 533 g vs. B, 410 g). The greatest difference between cations vs. no cations, both in terms of gains and feed efficiency, was obtained in the control-fed groups not receiving the alfalfa supplement. In fact, the small

TABLE 2

Gains and feed efficiency of experimental lambs

	No. animals	Avg initial wt	Avg weekly gain	Feed/gain
		kg	*g*	*kg/kg*
Experiment 1 [1]				
Diet 26BX [2]	6	17.6	32.4	37.3
Diet 26CX	6	17.5	567.0	11.2
Experiment 2				
Diet 26B	6	17.9	539.8	11.5
Diet 26C	6	18.0	548.8	12.5
Diet 27B	6	17.2	748.4	10.4
Diet 27C	6	18.5	703.1	11.6
Experiment 3				
Diet 26B	3	18.5	544.3	12.1
Diet 26C	3	16.9	730.3	11.0
Diet 27B	3	17.1	830.1	9.8
Diet 27C	3	17.0	984.3	9.5
Diet 26BX	3	18.8	68.0	27.0
Diet 26CX	3	16.3	198.2	19.9
Diet 27BX	3	16.9	196.9	15.1
Diet 27CX	3	15.7	220.4	13.5

[1] Experiment 1 was run 25 weeks (3 replications with females, 3 with wethers) and experiments 2 (3 replications with females, 3 with wethers) and 3 (2 replications with females, 1 with wethers) were run 35 weeks.

[2] The symbol (X) is used to represent the controlled feeding regimen; the number 27 is used to represent the alfalfa supplement; the letter C represents the cation diet.

gains and high feed efficiency of the control-fed group without cations were similar to those obtained with these same diets in experiment 1.

The data which follow were obtained from samples taken from force-fed animals at the end of the growth trial.

The ruminal volatile fatty acid and lactic acid results are shown in table 3. Examination of the volatile fatty acid values obtained for the ad libitum animals reveals a higher percentage of butyrate and a lower percentage of acetate for the animals receiving cations. This trend was not observed for the animals subjected to the controlled feeding regimen. The most striking difference in the lactic acid data is the higher values obtained for the animals that were control-fed. Another effect discernable particularly in the total lactic acid values is the effect of alfalfa on decreasing the lactic acid accumulation in the rumen fluid. Since it is reasonable to assume that the difference between total lactic acid values and L(+)-lactate represents D(−)-lactate, it becomes apparent that the lactate which accumulated was largely in the latter form and that much more D(−)-lactate was present when the animals were control-fed. In reference to this point, it is of interest that the presence of both alfalfa and cations decrease the concentration of D(−)-lactic acid in rumen fluid. Ruminal pH values followed similar trends, i.e., both cations and alfalfa increased ruminal pH.

The results of an in vitro study comparing the buffering capacity of sodium and potassium bicarbonates vs. ammonium hydroxide are shown in figure 1. In this study 10 ml of 0.2 M acetic acid was titrated with a 0.133 M solution of NH_4OH and a 0.133 M solution of a mixture of sodium and potassium bicarbonates (7:5). As can be observed from the graph, between the pH of 4 and 6, equivalent amounts of the bicarbonates resulted in a higher pH. Above 6, the pH of the solution titrated with NH_4OH rose sharply indicating a poor buffering capacity in this region. Presumably, this would offer an explanation for the lower pH values observed in the rumen of sheep fed urea without cations (table 3).

TABLE 3

Ruminal volatile fatty acid, pH and lactic acid data

Diet	Data from exps. 1, 2 and 3 [1]						Lactic acid data, exp. 3 [2]		
	Acetic	Propionic	Butyric	Isovaleric	Valeric	pH	L(+) −	Total	D(−) − [4]
	%	%	%	%	%		*μmoles/ml*	*μmoles/ml*	*μmoles/ml*
Ad libitum									
26B [3]	28.4	31.7	28.8	4.8	3.6	5.7	1.35	40.7	39.4
26C	47.0	34.9	12.0	0.5	2.1	6.6	0.25	8.3	8.1
27B	30.8	33.8	25.6	3.5	4.8	6.1	0.76	26.9	26.1
27C	40.3	30.5	9.7	2.0	0.6	6.7	3.07	11.4	8.3
Controlled									
26BX	47.0	37.4	8.9	1.2	2.3	5.9	7.60	> 120.0	> 112.4
26CX	40.3	37.3	14.4	2.4	3.2	6.8	9.73	62.2	52.5
27BX	48.0	37.6	10.9	1.3	1.7	6.7	6.08	55.0	48.9
27CX	49.1	40.1	5.5	2.0	0.5	7.1	2.60	10.8	8.2

[1] Each value is a mean of 15 animals; 4 samples were taken from each animal over a period of 3 hours.
[2] Each value is a mean of 5 values on one animal.
[3] The symbol (X) is used to represent the controlled feeding regimen; the number 27 is used to represent the alfalfa supplement; the letter C represents the cation diet.
[4] Difference.

Additional measurements were made on samples taken from animals in experiments 1 and 3 with the idea of following the metabolism of NH_4^+, Na^+ and K^+ more in detail. As shown in table 4, ruminal NH_3 was highest in those animals subjected to the controlled feeding regimen and lowest for the animals fed the diets without cations. However, as indicated by blood urea and ammonia values from experiment 1, differences between cations and no cations were not significant. As shown in table 4, urinary ammonia was lower for the animals receiving cations. The differences between cations and no cations was most marked for the animals subjected to the controlled feeding regimen. The trends of sodium and potassium concentration in the urine were opposite to those obtained for ammonia. The animals receiving the cation-supplemented diets had higher levels of sodium and potassium in the urine than those not receiving cations. Sodium and potassium analyses on sequential rumen samples were made in experiment 3. There was little change in the sodium concentration of the rumen fluid in the first 1.5 hours of the sampling period, regardless of diet. The potassium values, however, not only showed a trend downward with time during the 1.5-hour interval, but, in addition, there was a significant difference in the rate of decrease in potassium concentration between animals receiving cations in the diet vs. those not receiving cations. The average rate of decrease of potassium concentration in the rumen for the animals receiving the cation diet was 0.835 mg K/ml/hour, whereas those receiving diets without a supplement of cations decreased at a rate of 0.281 mg K/ml/hour.

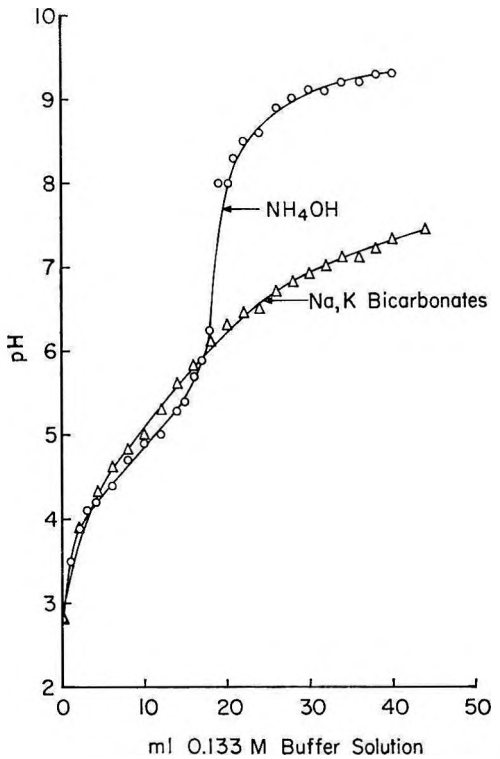

Fig. 1 Ten milliliters of 0.2 M acetic acid titrated with 0.133 M NH_4OH and 0.133 M Na and K bicarbonates (7/5).

TABLE 4

Rumen blood and urine measurements

	Rumen	Urine				Blood	
	NH_3-N	Na	K	NH_3-N	Urea-N	NH_3-N	Urea-N
	mg/100 ml	*mEq/liter*		*mg/100 ml*		*mg/100 ml*	
Experiment 1							
Diet 26BX	—	44	69	209	251	5.2	10.6
Diet 26CX	—	213	489	3	17	5.4	9.3
Experiment 3							
Diet 26B	—	24	60	—			
Diet 26C	—	146	300	25			
Diet 27B	33	18	24	89			
Diet 27C	108	160	480	36			
Diet 26BX	178	8	180	483			
Diet 26CX	439	85	100	30			
Diet 27BX	247	15	150	1298			
Diet 27CX	482	214	224	28			

DISCUSSION

In the diets studied, the primary source of nitrogen was supplied as urea. It was found that supplementing this basal diet with either sodium and potassium bicarbonate or 5% alfalfa increased the growth of lambs. The highest gains were obtained with the combination of alfalfa and cation supplements. These effects were noted with both ad libitum and control-fed animals. The hypothesis proposed to explain these results is as follows: Presumably ammonium ions substitute poorly for Na^+ and K^+ in their role as buffering agents in the rumen. Some evidence in support of this idea is reflected in the lower pH values obtained for the rumen fluid of animals fed the urea diet without supplements of cations. Additional supporting evidence is revealed in the in vitro study showing that ammonium hydroxide is a less efficient buffer at the critical ruminal pH values than are equimolar concentrations of sodium and potassium bicarbonates. It is suggested that the buffering capacity of the diet influences feed intake (1). Thus, this hypothesis would explain, in part, the better growth and feed intake of the animals fed the cation-supplemented diets. It would reconcile the differences obtained in growth between ad libitum-fed (nibblers) and control-fed (meal-eaters) animals. When the feeding is controlled, the animal eats only the amount it can buffer, thus restricting feed intake. However, if the animals are fed ad libitum, they become nibblers, managing with less buffering capacity.

In view of the fact that the concentration of lactic acid in the rumen appears to be inversely correlated with feed intake and gains of lambs, the factors affecting the level of lactic acid found in the rumen may be significant. Ruminal fermentation of diets high in carbohydrates produces large quantities of lactic acid. Under these conditions, a large portion of the lactate is metabolized to propionate via the acrylate pathway (11). It has been shown that under conditions which result in diminution of the acrylate pathway activity, lactate accumulates in the rumen and that much of the lactate is in the D(−) form (12). It appears, therefore, that addition of alfalfa or cations, or both, to the basal urea diet enhanced the acrylate pathway or the metabolism of lactate by some other pathway, as evidenced by lower levels of ruminal lactic acid obtained from sheep fed these dietary constituents. Conversely, the controlled feeding regimen resulted in diminished acrylate pathway activity. Presumably, all these effects could have been mediated by changes in ruminal microflora. The cations or alfalfa could furnish nutrients or cofactors favoring an increase in the microorganisms which operate the acrylate pathway. Under a meal-eating type of regimen, the amount of carbohydrate converted to lactate per unit of time is greater than under a nibbling regimen. Under these conditions, limitation of cofactors is more critical, and indeed the combination of alfalfa and cations was required to lower ruminal lactic acid concentration to that of the nibblers.

The lower ammonia concentration in the rumen fluid of the animals fed the basal urea diet as compared with those fed a cation supplement might be explained by either a greater absorption of a slower rate of production of ammonia. The greater concentration of potassium in the cation-supplemented diet, and the greater rate of disappearance of potassium from the rumen with these diets suggest that the net absorption of either cation is dependent upon the overall absorption of both. An explanation for the greater level of ammonia found in the urine of the animals fed the diet without supplemental cations as compared with those receiving cations (table 3), is a difference brought about by the stimulation of the ammonia mechanisms for sparing mineral cations (13). Ammonia functions to facilitate the excretions of anions by the kidney with a minimal excretion of mineral cations by the kidney. Conversely, when adequate mineral cations are present in the diet, the operation of the ammonia mechanism is diminished and the mineral cations are found in the urine (table 3).

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Effect of Diet on Serum Protein Fractions of Hong Kong Chinese Children [1,2,3]

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ABSTRACT The effect on the blood serum protein fractions of substituting wheat for approximately one-half the rice in the ordinary diet of 288 Chinese orphanage children was investigated over a 12-month period. The children ranged in age from 8 to 16 years at the beginning of the experiment. Blood samples taken before initiation of controlled diets, and after the children had received the experimental diets for 12 months were analyzed for total serum proteins and for five serum protein fractions. After 12 months, serum albumin levels were significantly higher for all age groups of children fed a combination of rice and wheat than for those fed rice alone. Total serum protein values tended to be lower for children fed the rice-wheat diet than for those fed the rice diet. All globulin fractions tended to be lower is response to the rice-wheat than to the rice diet. The greatest differences in various serum protein fractions between dietary treatments occurred at 10 to 11 years, suggesting that this was a critical age in the nutritional development of these children. The superior protein status of children fed the rice-wheat diet was indicated by higher concentrations of serum albumin and lower concentrations of serum globulins.

World population growth has exceeded growth in world rice production, yet rice remains the staple cereal for more than one-half the people in the world. Not only are supplies of rice insufficient, but the low protein content of rice suggests that it may be nutritionally inadequate as the primary food for children. Wheat, more abundant and higher in protein content than rice, has been suggested as a supplementary food for children in rice-eating countries.

Simple effective methods of measuring changes in protein nutriture are needed to evaluate the effect of dietary modifications such as substituting wheat for rice. Some investigators have shown that levels of albumin and total serum protein respond positively to increased intake of protein from either animal or vegetable sources, suggesting that serum protein fractions may be used as an index of protein nutriture (1–4).

The present paper reports serum protein fractions of Chinese children living in Hong Kong fed either rice or a combination of rice and wheat as the primary dietary component for a period of 12 months.

EXPERIMENTAL

Data are reported for 288 children, all residents of an orphanage in Wu Kwai Sha, New Territories, Hong Kong. The sample included 185 boys and 103 girls, ranging in age from 8 to 16 years. The experimental group had the same percentage sex distribution as the larger orphanage population of over 800. The mean age of the two experimental groups was between 12 and 13 years at the beginning of the experiment (table 1). All children were of Chinese extraction, except one girl who was part East Indian.

Two basal diets were used: the usual institutional diet providing protein primarily from rice (diet 1) and a diet providing approximately equal quantities of rice and wheat (diet 2). Each of the basal diets was modified as follows: 1) unsupplemented diet; 2) basal diet supplemented with a multivitamin and mineral preparation; and 3) basal diet supplemented with a

Received for publication November 27, 1967.

[1] This investigation was supported in part by the Foreign Agricultural Service of the U. S. Department of Agriculture through a contract between the University of Hong Kong and Wheat Associates, U.S.A. The project was supervised by the Agricultural Research Service, USDA.

[2] Published with the approval of the Director as paper no. 2255, Journal Series, Nebraska Agricultural Experiment Station.

[3] This paper is based on a dissertation presented by the senior author as partial fulfillment of requirements for the Ph.D. degree to the Graduate College of the University of Nebraska.

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TABLE 1

Ages, heights and weights of children fed 2 experimental diets

Age	No. of children			Height	Weight	Sex
	Diet plan 1	Diet plan 2	Total			
years				*cm*	*kg*	
8–9	13	9	22	120.34 ± 4.46 [1]	22.12 ± 2.62	M
	5	8	13	122.14 ± 6.95	22.28 ± 3.44	F
10–11	21	21	42	132.28 ± 7.20	26.68 ± 3.85	M
	8	9	17	128.77 ± 7.43	25.38 ± 4.82	F
12–13	33	31	64	140.00 ± 7.94	31.04 ± 5.27	M
	18	13	31	142.46 ± 6.40	32.86 ± 4.98	F
14–15	28	26	54	152.93 ± 9.65	39.31 ± 7.10	M
	18	19	37	151.50 ± 6.84	40.73 ± 4.84	F
16–17	1	2	3	161.13 ± 3.72	45.53 ± 4.27	M
	2	3	5	151.92 ± 4.20	38.76 ± 3.44	F
Total	147	141	288			
Mean age, years	12.3	12.4				

[1] Mean ± SD.

TABLE 2

Dietary treatment plans

Major cereals	Diet plan [1]	Supplements
	Rice diets	
Rice, polished	1	None
Rice, polished	2	Multivitamin product, including iron,[2] + dicalcium phosphate [3]
Rice, polished	3	Multivitamin product, including iron, + dicalcium phosphate, + 10 g protein from soy [4]
	Rice-wheat diets	
Rice, bulgar, noodles [5]	4	None
Rice, bulgar, noodles	5	Multivitamin product, including iron, + dicalcium phosphate
Rice, bulgar, noodles	6	Multivitamin product, including iron, + dicalcium phosphate, + 10 g protein from soy [6]

[1] The diet also included a variety of indigenous vegetables, fruits, small amounts of fish and meat, fat and sugar as available and purchased by the cottage mothers.

[2] Composition: vitamin A, 4,000 USP units; vitamin D_2, 400 USP units; vitamin C, 75 mg; thiamine, 1.2 mg; riboflavin, 1.5 mg; niacinamide, 15 mg; pyridoxine, 1.2 mg; iron, 10 mg; 1 capsule/child per day. Same for diet plans 3, 5 and 6.

[3] Dicalcium phosphate 0.5 g/capsule; 3 capsules/day to supply 375 mg calcium per child. Same for diet plans 3, 5 and 6.

[4] Actual amount used supplied an average of 6.3 g protein daily during last 8 months of study.

[5] Contained rice, 50%; bulgar, 25%, and noodles, 25%; the noodles were made from enriched white flour, water and salt; no other ingredients.

[6] Actual amount used supplied an average of 8.4 g protein daily during last 8 months of study.

multivitamin, mineral preparation, and 10 g soy protein. Table 2 presents the dietary treatment plans. Values for albumin and total protein did not vary significantly among the sub-groups fed each of the basal diets. Albumin concentrations were 47.22, 45.34 and 51.28% for rice diet modifications 1, 2 and 3, respectively, and total protein values were 7.12, 6.54 and 7.10 g/100 ml. Comparable values for sub-groups fed the rice-wheat diet were 51.72, 56.94 and 58.10% for albumin and 6.45, 6.37 and 6.58 g/100 ml for total protein. Since supplementation of the basal cereal-containing diets did not alter the serum protein components, results for sub-groups

were combined and data are presented only on the basis of the two basal diets.

Wheat was provided from enriched flour made into noodles and from bulgar. The average caloric intake of children fed the 2 diets was not significantly different (2231 and 2279 kcal daily), but the protein intake was slightly higher for those fed rice-wheat (62 g daily) than for those fed rice (56 g daily). A previous report (5) showed that 45 to 68% of the protein consumed by Hong Kong children came from cereal sources. The rice-wheat diets provided larger amounts of all essential amino acids than the rice diets. The rice-wheat diets provided all amino acids in excess of the minima suggested by Rose et al. (6), and the rice diets provided 90% of the minima for the sulfur-containing amino acids and all others in recommended quantities. In addition to the cereals, the diet provided a variety of indigenous vegetables and fruits, as well as small amounts of fish, meat, fat and sugar. Detailed information concerning the diet, living conditions and heights and weights of the children are reported elsewhere (5).

Blood was taken by venipuncture at the beginning of the experiment and after 12 months of experimental treatment. Total serum proteins were estimated by refractometry, using an American Optical Company total solids meter. Individual serum protein fractions were determined by paper electrophoresis by use of a modification of the method of Block et al. (7). The intensity of color of each of the bands on the paper strips served as a quantitative index of the concentration of each protein fraction. The paper strips were placed in a Spinco Analytrol densitometer to obtain a graphic representation of the colored band. The amount of total area contained under each component was calculated, and values converted to a percentage of the whole area.

For purposes of comparison, blood samples were obtained from 30 boys and girls living in an orphanage in Nebraska. These samples were treated and analyzed in precisely the same manner as those from Hong Kong children. Serum protein data from a study by Oberman et al. (8) of 24 Eastern United States children are also available for further comparisons.

RESULTS

Total serum protein values and the concentration of the different serum protein fractions at each age level, measured at the initiation of the Hong Kong study, are shown in table 3. The total serum protein levels were appreciably lower for Hong Kong children of all ages than for the 2 groups of American children, the Nebraska children and children from Eastern United States (8). Albumin and γ-globulin levels were lower in Chinese than in American children, whereas the α_1-, α_2- and β-globulin components were higher in Chinese children. Values for α_1- and β-globulins were approximately twice as high for Hong Kong as for Nebraska children. Total protein was considered "deficient" and albumin "low" when the initial serum protein levels of Hong Kong subjects were compared with tentative standards suggested by the Interdepartmental Committee on Nutrition for National Defense (ICNND) (9). A modified biuret technique for total serum protein and separation of serum proteins by paper electrophoresis are the analytical methods recommended by ICNND.

Data from the present study may be examined by comparing data from the children fed the rice-wheat diet with that from children fed rice at the end of the 12-month period (latitudinal evaluation). Alternatively, the data may be examined by comparing the changes occurring within each basal diet group (longitudinal evaluation).

Table 4 presents the latitudinal evaluation. Total serum protein values and concentrations of the various serum protein fractions measured at the conclusion of the one-year dietary study are presented. When the age groups were combined, albumin concentration was significantly higher ($P < 0.0005$) in children fed rice and wheat than in those fed rice, whereas the concentration of all four globulin components and total serum protein level were significantly higher ($P < 0.0005$) for the children fed rice. A comparison of the final albumin and total serum protein values with ICNND suggested levels of nutritional adequacy indicated that total serum proteins were "acceptable" in both groups but that al-

TABLE 3

Serum protein values of Chinese orphanage children at beginning of dietary study

Age	No. children		Albumin	Globulins				Total protein
				α_1-	α_2-	β-	γ-	
			%	%	%	%	%	*g/100 ml*
8–9 years	35	mean	52.13	6.45	10.86	18.96	11.59	6.24
		SD	7.12	2.07	3.80	5.30	3.21	1.02
10–11 years	59	mean	48.14	6.90	10.59	21.10	13.26	5.94
		SD	6.85	2.72	3.25	5.55	6.27	0.94
12–13 years	95	mean	49.44	6.79	10.45	20.98	12.30	5.84
		SD	7.67	2.61	3.84	6.40	6.22	0.88
14–15 years	91	mean	49.99	6.94	10.66	18.90	13.56	5.95
		SD	8.00	2.38	3.39	5.98	8.01	0.87
16 years	8	mean	48.25	6.59	11.15	22.69	11.32	5.76
		SD	5.08	1.63	4.10	4.48	3.46	0.70
All ages	288	mean	49.58	6.82	10.61	20.13	12.79	5.93
		SD	7.53	2.48	3.56	5.99	6.57	0.90
(Mean age = 12.35 yr)								
ICNND "acceptable" range			54–61	4–7	9–11	11–15	12–16	6.5–6.9
Nebraska children, all ages	30	mean	59.66	3.27	8.71	10.83	17.53	6.93
		SD	10.64	0.86	2.68	2.71	5.57	0.31
(Mean age = 11.37 yr)								
Eastern U.S. children, 6–13 yr [1]	24	mean	54.90	4.74	10.97	13.07	16.31	7.35
		SE	0.97	0.24	0.43	0.41	0.61	0.09

[1] Oberman (8).

bumin was "acceptable" only in children fed the rice-wheat diets.

Table 5 presents the longitudinal evaluation, that is, the change in serum protein values achieved within each of the two major experimental groups in one year's time. Percentage changes in total serum proteins showed significant increases for children fed both diets, but the increase was greater for children fed rice than for those fed rice and wheat. Albumin as a percentage of total protein tended to increase and globulin to decrease in children fed rice and wheat, whereas the opposite tendency predominated in children fed rice diets. Comparison of the globulin concentrations indicated that the overall change in total globulins was due to an increase in the γ-fraction and to decreases in α_1-, α_2- and β-fractions.

DISCUSSION

Data on blood constituents in adult Southern Chinese provide grounds for interpreting the present findings from children of Southern Chinese origin. Ma (10) found lower albumin and higher γ-globulin concentrations in Chinese adults than in Europeans living in Hong Kong. He reported no significant differences for total proteins, α_1-, α_2- or β-globulins. The findings of Chen and Ting (11) on 139 adult Formosan subjects corroborate Ma's report of low albumin and high γ-globulin levels in Orientals.

The serum protein values in the Hong Kong study (table 4) were classified as "low" with respect to albumin for children fed the rice diet and "acceptable" for those fed the rice-wheat diet, and children fed both diets were classified as "high" with respect to γ-globulin. These findings are consistent with reports from other Asian countries. The "acceptable" serum albumin rating attained by children fed the rice-wheat diets indicated a more favorable status than is general for persons living in this part of the world.

Scrimshaw et al. (12) described the serum protein pattern of kwashiorkor patients, noting low serum albumin levels, high levels of α- and γ-globulins and normal levels of β-globulin. When the total

TABLE 4

Mean serum protein values of children fed rice and rice-wheat diets for one year

Age and diet	No. children		Albumin	Globulins				Total protein
				α_1-	α_2-	β-	γ-	
			%	%	%	%	%	g/100 ml
8–9 years:								
Rice diet	15	mean	48.31	3.15	10.47	11.69	26.28	6.37
		SD	7.68	0.61	2.78	2.54	6.72	1.01
Rice-wheat diet	12	mean	56.98** [1]	2.92	9.26	9.58*	21.26**	5.59**
		SD	8.72	0.77	2.72	2.85	4.94	0.60
10–11 years:								
Rice diet	21	mean	44.64	3.68	11.98	10.92	28.79	6.95
		SD	6.32	1.88	2.45	2.47	5.33	1.27
Rice-wheat diet	25	mean	55.41**	2.80*	8.52**	9.14	24.14*	6.42
		SD	11.06	0.79	3.09	2.85	6.80	0.99
12–13 years:								
Rice diet	38	mean	47.06	3.59	9.97	12.36	27.02	6.83
		SD	8.68	1.57	2.38	3.31	6.00	1.01
Rice-wheat diet	41	mean	53.86**	3.10	8.37**	9.44**	25.09	6.58
		SD	11.38	1.36	2.82	2.79	7.95	1.00
14–15 years:								
Rice diet	50	mean	48.90	3.60	9.78	11.84	25.62	7.05
		SD	9.46	2.01	2.70	3.53	5.99	1.16
Rice-wheat diet	34	mean	54.69**	2.70*	7.95**	10.59	24.10	6.76
		SD	9.47	0.52	2.84	2.74	6.19	1.14
16–17 years:								
Rice diet	23	mean	49.99	3.00	9.77	10.89	26.33	7.09
		SD	11.86	0.70	2.96	3.38	8.49	1.49
Rice-wheat diet	29	mean	56.85*	2.53*	7.86*	9.62	23.17	6.43*
		SD	13.47	0.81	3.14	2.77	8.23	0.92
All ages:								
Rice diet	147	mean	47.90	3.45	10.20	11.75	26.62	6.91
		SD	9.15	1.63	2.71	3.23	6.43	1.18
Rice-wheat diet	141	mean	55.39**	2.81**	8.26**	9.72**	23.78**	6.48**
		SD	11.09	0.95	2.92	2.80	7.18	1.02
Comparison with ICNND:								
Rice diet			low	low	acceptable	acceptable	high	acceptable
Rice-wheat diet			acceptable	low	low	low	high	acceptable

[1] * indicates significant at 5% level; **, significant at 1% level.

proteins and amounts of the various protein fractions of the Hong Kong children fed rice or rice-wheat diets are compared with values for the Nebraska children (tables 3 and 4), α_1-, α_2- and β-globulin levels are comparable in all 3 groups. The greatest differences occurred in the γ-globulin fraction, with Nebraska children having the lowest level and children fed the rice diet, the highest. The opposite trend was observed in serum albumin values with children fed the rice diet having the lowest concentration and the Nebraska children, the highest. For both serum albumin and γ-globulin, the values of children fed the rice-wheat diet were intermediate between the values of the other 2 groups. Therefore, the trend in albumin and γ-globulin levels of Hong Kong children follows that described by Scrimshaw et al. (12), but the α-globulins do not fit their description. In each case, children fed the rice diet were in a less favorable position than those fed the rice-wheat diet.

Albumin was consistently higher and the globulin component and total serum protein levels lower in children fed rice and wheat than in those fed rice alone. The greatest discrepancy in serum protein values of children fed the 2 diets occurred at 10 to 11 years. Since children fed rice as the major cereal displayed serum protein patterns similar to those described by Scrimshaw et al. (12) for children with

TABLE 5

Differences between final and initial serum protein values for children fed rice and rice-wheat diets for one year

Age and diet	Albumin	Globulins				Total protein
		α_1-	α_2-	β-	γ-	
	%	%	%	%	%	*g/100 ml*
8–9 years:						
Rice diet	−3.82	−3.30** [1]	−0.39	−7.27**	14.69**	2.08
Rice-wheat diet	4.85	−3.53**	−1.60	−9.38**	9.67**	−10.26**
10–11 years:						
Rice diet	−3.50	−3.22**	1.39*	−10.18**	15.53**	17.00**
Rice-wheat diet	7.27**	−4.10**	−2.07*	−11.96**	10.88**	8.08
12–13 years:						
Rice diet	−2.38	−3.20**	−0.48	−8.62**	14.72**	16.95**
Rice-wheat diet	4.42*	−3.69**	−2.08*	−11.54**	12.79**	12.67**
14–15 years:						
Rice diet	−1.09	−3.34**	−0.88	−7.06**	12.06**	18.48**
Rice-wheat diet	4.70*	−4.24**	−2.71**	−8.31**	10.54**	13.61**
16–17 years:						
Rice diet	1.74	−3.59**	−1.38	−11.80**	15.01**	23.09**
Rice-wheat diet	8.60**	−4.06**	−3.29*	−13.07**	11.85*	11.63

[1] * indicates significant at 5% level; **, significant at 1% level.

kwashiorkor, it might reasonably be inferred that the protein status of these children is inferior to that of children fed rice and wheat as a cereal source.

Mack (13) reported that albumin values increased when children in United States orphanages were given higher meat diets, whereas globulin values increased when the children were fed low meat and high vegetable protein diets. More recently, similar differences in the γ-globulin fraction were reported between vegetarian and non-vegetarian Indian adults (14). Research workers in New Guinea (15), Africa (16) and Formosa (11) have shown that qualitative or quantitative inadequacy in protein supply results in lower concentrations of serum albumin.

ACKNOWLEDGMENTS

Appreciation is expressed to Dr. Connell Marsh, Clifford Lasnetske, Suna Göksu and Donald R. Schmidt for their technical assistance.

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counts per minute	count/min
cubic centimeter(s)	cm^3
cubic millimeter	mm^3
degree(s)	°
degrees of freedom	df (*in tables*)
gram(s)	g
international unit(s)	IU (*to be used only when weight can not be given*)
kilogram(s)	kg
liter(s)	(spell out)
meter(s)	m
microgram(s)	μg (not γ)
micromicrogram(s)	$\mu\mu$g
microcurie(s)	μCi
micron(s)	μ
micromicron(s)	$\mu\mu$
micromolar (unit of concn)	μM
micromole (unit of mass)	μmole
milligram(s)	mg
milligrams %	(*never use*)
milliliter(s)	ml
millimeter(s)	mm
millimicrogram(s)	mμg
millimicron(s)	mμ
millimole(s)	mmole
molar (mole per liter)	M
parts per million	ppm
per cent	%
probability (in statistics)	*P*
square centimeter	cm^2

[1] Style Manual for Biological Journals 1960 American Institute of Biological Sciences, 2000 P street, N. W., Washington 6, D. C.

square meter	m^2
square millimeter	mm^2
standard deviation	SD
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t (Fisher's test)	*t*
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