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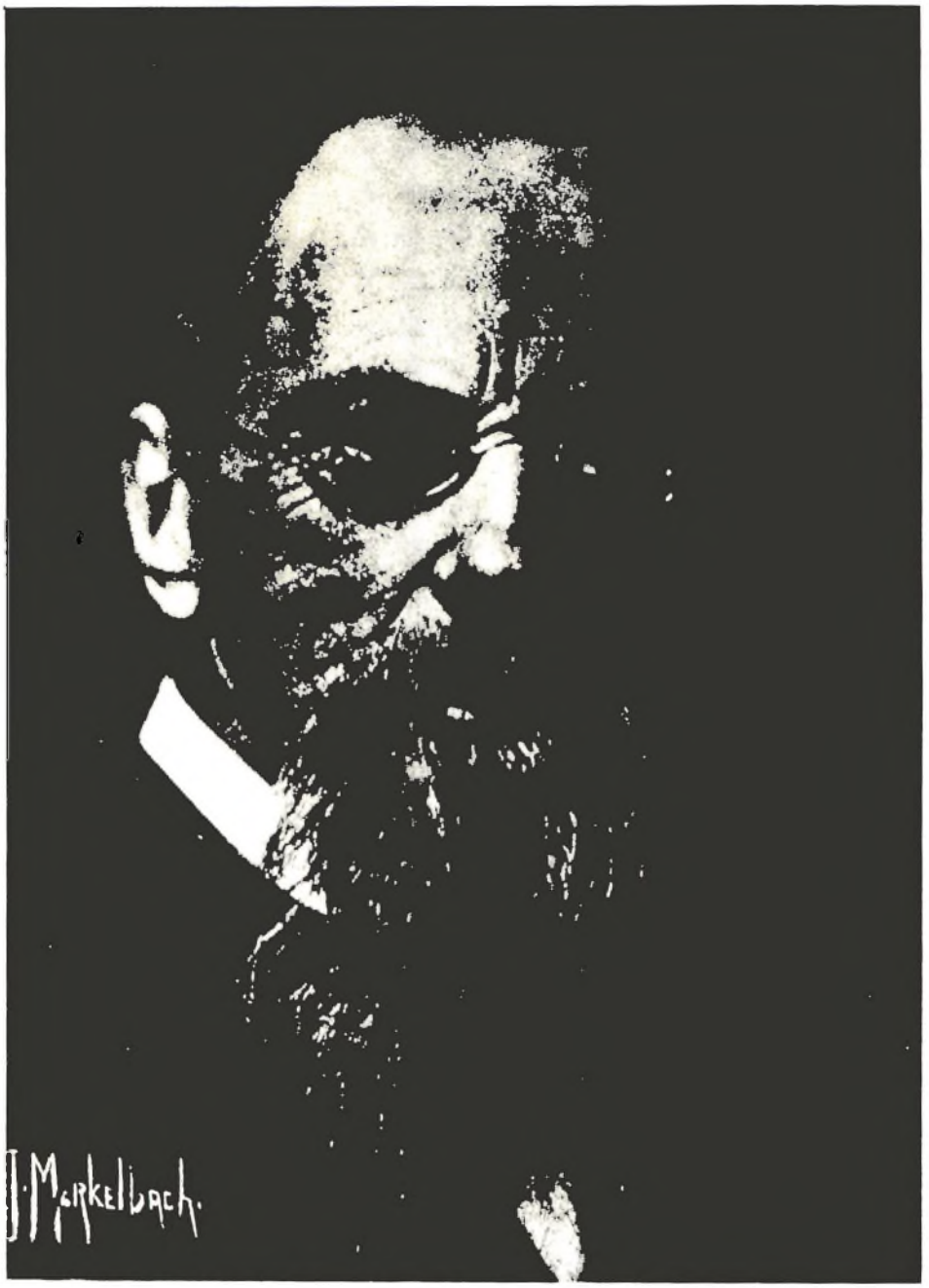

Cornelis Adrianus Pekelharing

Cornelis Adrianus Pekelharing — A Biographical Sketch

(July 19, 1848 – September 18, 1922)

Cornelis Adrianus Pekelharing was a true son of his time and nation. He was born as the sixth child of Cornelis Pekelharing, M.D. and Johanna van Ree in the small industrial town of Zaandam in western Holland.

His childhood was a happy one. His father, son of a country doctor, was a busy physician and a scholar, devoted to his patients. Unlike many of his colleagues, he kept abreast of new medical developments through scientific journals and close contact with the medical school at the nearby University of Amsterdam. Cornelis' mother also came from a doctor's family. They were married in 1840. Her life centered around her family of seven children and a husband. They worked hard and lived simply to enable all six sons to obtain a university education. Vacation was unknown to them. The children were brought up in the stern tradition of duty, integrity, industry and frugality. These characteristics remained Pekelharing's most outstanding ones all through his life.

The parents were Baptists but they let their children make their own decisions as to the denomination they wanted to select. When Cornelis Adrianus reached the age of reason, he decided not to join any church. His entire life, however, showed that ethical principles were all-important to him. He was devoted to his country and because of his work there, he had a great love for the Dutch East Indies. He served higher education in the Netherlands for forty-one years.

The physiologist, Heynsius, developed Pekelharing's interest in laboratory research while he was a student at the University of Leiden. Throughout his lifetime he retained his love for physiological chemistry, although he branched out into other major areas of biology and pathology. Even though he practiced medicine for only a few years, he remained a doctor at heart. He was always interested in the medical profession and on several occasions served on committees to study medical education in his native Holland.

The well-known physiologist, F. C. Donders, expected much of the young doctor and was instrumental in obtaining Pekelharing's services for the University of Utrecht. For his part, Pekelharing admired Donders and experienced one of his happiest moments when, mainly through his efforts, a statue of Professor Donders was erected in Utrecht. At the occasion of the unveiling in 1921, Pekelharing delivered the main address.

When, at the age of seventy-four, Pekelharing died, the scientific world lost a devoted worker who knew of no compromise and looked upon hard work as the only basis for the discovery of the truth.

Pekelharing received his early education in Zaandam. The elementary and secondary schools in that city were quite good and a number of other well-known scientists received their basic education in the same schools. In 1865 he was admitted to the University of Leiden, the oldest in the nation, after passing examinations in mathematics, classics and modern languages. His father, however, decided that Cornelis, at the age of seventeen, was too young to enroll at the University. So Cornelis stayed home for another year and studied physics, chemistry and biology independently.

During this period his character also developed through contact and conversations with his older brothers and friends. One of his brothers had already joined the ministry; another studied political science at the University of Leiden. Political, social and religious questions were discussed extensively. During this period probably the foundation was laid for Pekelharing's later interest in many social problems of of his time.

Sports and athletics were considered unimportant and physical exercises were limited to work on equipment in the large back yard, walking and rowing on the many rivers and canals surrounding Zaandam. Pekelharing was not a sturdy young man, although he was healthy. His eyes were weak and at one time an eye ailment interfered with his studies. After recovery, however, his eyes served him well as his later microscopic studies show.

Cornelis enrolled at the University of Leiden in September, 1866. There he developed a special interest in nature, literature and music. He also enjoyed the freedom of student life and was appreciated by his fellow students for his good humor and friendliness. He pursued his studies with great diligence and interest. His most outstanding teacher was probably A. Heynsius, who taught physiology. Heynsius had studied with Gerrit Jan Mulder and was especially interested in the chemical aspects of his field. His laboratory was one of the best-equipped ones in the country and his students received excellent training in instrumentation and laboratory techniques. Much time was also devoted to histology, introduced by Professor Donders.

Pekelharing and his classmates were very appreciative of these new developments in their education and requested an additional course in physiological chemistry. Pekelharing's interest in the exact methods of observation and experimentation used in Heynsius' laboratory led to his appointment as an assistant to the famous scientist. Here he could pursue his own interests. But he was also a great help to students who were sometimes fearful of the stern professor.

After four years he passed his examinations for candidate in medicine with highest honors. In those days the study of medicine was rather simple and two years later he was licensed to practice medicine after successful completion of a week-long series of examinations. During the summer of 1872, the newly licensed Doctor Pekelharing replaced his father in medical practice to allow the latter to take his first vacation abroad. It turned out to be his last one; he died in January, 1873.

After this brief but satisfying experience with patients, young Pekelharing decided to enter the practice of medicine. Since he also wanted to continue his work as Heynsius' assistant, he opened a medical practice in Leiden. The combined incomes enabled him to start a family. On July 14, 1873, he married Willemine Geertruida Campert, the sister of one of his best friends. He soon made a name for himself as a doctor and his practice grew rapidly. His name as a scientist became known after the successful defense of his dissertation entitled, "On the determination of urea in blood and tissues" on May 18, 1874.

In 1876 it became apparent that he could no longer hold both of his positions and reluctantly he resigned from his assistantship. Although he enjoyed his work in the laboratory and wanted to continue, on the meager salary it paid he could not maintain his growing family. His patients had great confidence in him, but Pekelharing gradually became dissatisfied with his medical work. He was constantly confronted with a lack of knowledge of the body's processes. He felt frustrated in diagnosing and prescribing therapy. His methodical mind demanded that he devote his time to finding solutions to the many problems of medicine.

When, in October, 1877 he was approached about a teaching position at the State School of Veterinary Medicine in Utrecht, he accepted. On January 1, 1878, Pekelharing was appointed instructor in physiology, histology and pathological anatomy. All this on a very modest salary. The availability of laboratory space was the great attraction for the young scientist. The scientific community of this university town welcomed him with open arms and soon he was an active participant in meetings and discussion groups. Here he met the outstanding physiologists of his days, Donders and Snellen. Although no longer a practicing physician, he was active in the local medical association and in 1880 was elected its president.

Pekelharing spent as much time as possible in his laboratory at the Veterinary School but he never neglected his teaching duties for the sake of his research. Two topics particularly held his attention. One

was protein digestion and its "end product" which he called "pepton." This pepton was the subject of much controversy in the scientific world of those days. Pekelharing, only thirty years old, was in the thick of the battle. He considered pepton a "modified" protein which passes through the intestinal wall and is absorbed by the bloodstream. His other interest was the occurrence of anthrax, a disease that caused many deaths in cattle. In this connection he had to take up bacteriology. To become better-informed about this new science, he traveled to Leipzig to visit J. Cohnheim who had studied with Virchow. There he learned new techniques and ways to prevent contamination. All his life Pekelharing was very fortunate in that he never contracted any infections in his long career of dissecting animal and human cadavers in the days when gloves were not yet used.

In the summer of 1881, the instructor, Pekelharing, was appointed professor of pathology and pathological anatomy at the University of Utrecht. His inaugural address was entitled "The Importance of the Study of Physiology for Medicine." He was extremely proud to be a colleague of Donders, whom he greatly admired. Donders was especially known in Europe as an ophthalmologist and director of a famous clinic. Also on the faculty was W. Koster, who taught anatomy and medical criminology. Fortunately, adequate funds were available for the laboratories and Pekelharing enthusiastically continued his distinguished career as a teacher and researcher.

The number of medical students at the University was increasing and they were eager to learn of the new developments in the rapidly growing field of medicine. Laboratory experimentation, comparatively new as a regular phase in medical education, was carried out with great interest. When in 1884 Pekelharing expanded his laboratory facilities, young Dr. C. Winkler joined him. He wished to investigate tissue changes in nervous disorders and in psychiatric patients. The two men became close friends.

With Professor Talma, Pekelharing studied the role of leucocytes during inflammation. Diapedesis was investigated extensively by both. Two of Pekelharing's students wrote their dissertation on subjects related to inflammation processes. Metschnikoff's theory of phagocytosis brought about his investigations in later years on the behavior of leucocytes towards anthrax bacilli and spores.

During the summer of 1884, Pekelharing became seriously ill on his way to a medical congress in Copenhagen. The continued hard work had undermined his health and serious pleurisy developed. After a long rest, however, he returned to his work completely recovered. Over these years his family grew satisfactorily; he now had three sons and two daughters.

In 1886 he was elected a member of the Royal Academy of Sciences. At the meetings he met Holland's most outstanding natural scientists such as M. N. Beyerinck, the renowned bacteriologist. In the same year the Dutch government decided to send a committee to the Dutch East Indies to report on the increasing incidence of beriberi among the military. Pekelharing was asked to travel to Java and start an investigation. He consented on the condition that Winkler would accompany him. The latter had studied nervous disorders extensively and nerve deterioration was known as one of the most prominent symptoms in patients with beriberi.

In those days a great many hypotheses had been published regarding the cause of beriberi. The two most generally accepted ones laid the blame on bacterial infection and a toxic poisoning, respectively. Although some workers, including a Dutch Navy surgeon, had mentioned dietary imperfections as a possible cause as early as 1859, most workers, including Pekelharing, were inclined to regard the disease as a bacterial infection. Therefore, in preparation for their investigation, Pekelharing and Winkler traveled to Berlin to consult with Robert Koch. There they met a young Dutch military surgeon from the Colonial Army by the name of Dr. Christiaan Eykman, who was working in Koch's laboratory. Eykman promised to request a leave from the military service as soon as he had returned to the East Indies in order to join the two scientists.

Accompanied by a laboratory assistant, the committee left Holland on October 22

and arrived in Batavia on November 23, 1886. Eykman soon joined them there. They got the use of a large laboratory in the military hospital in that city. Several beriberi patients were in the hospital, brought there from Sumatra where the Armed Forces were involved in a war against rebellious natives. Realizing that the scheduled nine months were not enough to complete their assignment, the men immediately went to work. Winkler examined the nerve deterioration in the patients; Pekelharing set out to isolate a microorganism from the patients' blood. After several failures to isolate and culture a microorganism which would cause the beriberi symptoms when injected into a monkey, Pekelharing decided to go to Sumatra to see more new patients. On January 29, 1887, they left.

In Sumatra, continuing along the same lines of investigation, Pekelharing found several types of bacteria in patients' blood. Culturing these organisms, however, was not always successful. Furthermore, experimental animals were hard to come by in the remote outposts. Finally, after he had found a micrococcus in a patient's blood which, upon injection caused nerve deterioration in a rabbit, Pekelharing decided to go back to Batavia with its better-equipped laboratory. On May 3 they left Sumatra.

Upon return, Pekelharing was able to duplicate his earlier results several times using dogs as well as rabbits. (Chickens are nowhere mentioned as experimental animals.) Finally Pekelharing cultured an organism obtained from hospital air and injected it into a rabbit. Later he isolated from the blood of this rabbit an organism with the same pathogenic properties as the one found in beriberi patients. Time was pressing and Pekelharing felt that he had found at least one of the causes of beriberi. He recommended thorough disinfection of barracks, houses and ships. Strangely enough the incidence of beriberi decreased significantly in the first year after Pekelharing's stay in the East Indies. Later, however, it increased again. Even so, he did realize the imperfection of his evidence and before leaving, Pekelharing convinced the authorities of the need for permanent research facilities. Soon after his departure, the Netherlands Indies Research Laboratory for Bacteriology and Pathological Anatomy was established in Batavia under the direction of Eykman. Later the name was changed to Eykman Institute.

Shortly after Pekelharing's return home, the Dutch government awarded him one of its highest orders. Although generally acclaimed as the man who discovered the cause of beriberi, Pekelharing himself was not satisfied and continued his investigations. He and Winkler soon published valuable information on the effects of the dreaded disease based on some sixty-four autopsies. Needless to say that he remained in close contact with Eykman. What Pekelharing's reaction was to the later findings is not known. It seems probable that he, always interested in the fight against disease, was only grateful for the discovery of the cause of this rapidly increasing disease.

In 1888 Pekelharing was appointed professor of physiological chemistry and histology. He had always stressed the importance of both these disciplines to his students and with great enthusiasm he continued his research on the transmittance of anthrax as well as on blood coagulation, protein digestion and the functions of lymphatic tissues. A continuous stream of publications from his laboratory attests to the activities going on.

After many years of experimentation, Pekelharing determined the protein nature of pepsin. An international argument was raging in Europe about the nature of the milk-curdling enzyme in the mammal's stomach. Pavlov claimed that pepsin was responsible for this activity whereas Hammarsten and Bang were convinced of the presence of a different enzyme, chymosine, with this distinct function. Pekelharing carried out numerous experiments designed to solve this problem. He continued this work and publications until 1917. He sided with Pavlov in the controversy. Finally, when Northrop crystallized pepsin and Tauber and Kleiner produced very pure chymosine, it was shown that both will curdle milk protein but that the latter is much more powerful. It was not until 1943 that Hankinson and Berridge independently produced crystalline

chymosine. Although proven wrong, Pekelharing's carefully carried out experiments contributed greatly to the solution of the problem.

But Pekelharing was not just a laboratory scientist. He was interested in many problems of his day and participated in national and international disputes on scientific and social problems. He always carefully documented his opinion and spoke with dignity and integrity. One of his many social activities involved the fight against alcoholism. Although not an abolitionist, Pekelharing was quite convinced of the harmful effect of over-indulgence. For many years he was a board member and president of The National Society Against Alcoholism. As a true scientist he started laboratory investigations on the effects of alcohol on various body functions and tissues using experimental animals. Frequently he had to design his own techniques. As a result of his fight against alcoholism he became interested in improving the diets of low-income families. He worked in close cooperation with the first schools of home economics in The Netherlands in teaching the selection and preparation of adequate diets to the women. Pekelharing was also on the board of several orphanages and hospitals and, at the request of the Medical Association, studied medical insurance programs. In 1913 he was elected chairman of a group which concerned itself with a sociological and cultural study of the Dutch people.

Pekelharing was generally liked by his students, although he could be quite harsh with those who were not prepared to devote their best efforts to the study of medicine. For those who demonstrated a real interest in the study he was a true friend and advisor. He imbued in his students an appreciation for carefully planned and executed experiments. On several occasions he condemned theories based on assumptions and he taught his students to be extremely critical of their own work and that of others. He prepared his lectures with great care and was a good speaker. Before the famous professor began his classes, a custodian always came into the room to straighten the chairs, close windows and doors and announce to the students that they had better sit down for "the professor will be here presently." When everyone was seated he would notify Professor Pekelharing, who then entered immediately and started his lecture. Class attendance was not compulsory but his lectures were always well attended. And the professor noticed very well who was present and who was not. Pekelharing had a talent for making the most complex material crystal clear. His information was generally not available in any textbook. On several occasions he contributed articles on topics of national interest or of scientific significance to popular magazines.

In his laboratory work he stressed to his students the need to keep the cost of experimentation down. He emphasized to them the great expense of medical research and did not tolerate wastefulness. The equipment they used was simple and carefully maintained. If, on the other hand, a rare reagent, book or instrument was essential, Pekelharing was always able to secure it. His close contact and friendship with many scientists all over Europe were often helpful on such occasions.

In addition to his work with students and his research activities, Pekelharing was deeply involved in the medical problems of his days. In 1888 and again in 1896 he was elected president of The Netherlands Medical Association. In 1889 he was elected an honorary member of the Dutch Association for Psychiatry and Neurology and in 1897 he became a member of the Dutch Society of Sciences. From 1896 to 1897, Pekelharing was Rector Magnificus of the University of Utrecht, a function which rotates on a yearly basis. He enjoyed the many official functions connected with this position without neglecting his scientific duties.

Quite unexpectedly in November, 1897, he lost his wife. It was a severe blow for Pekelharing. Her cheerfulness and loving care had been a great help to him in his busy life. His sense of duty towards his students and his work enabled him to adjust to the changed conditions at home. But he never really recovered from this loss.

In 1902, Pekelharing published a brochure on the importance of sugar as a food in an effort to ward off the higher taxes on this commodity. In it he stated, "In many efforts we have never succeeded in keeping animals alive on a diet of protein, carbohydrates, fat and mineral salts in proportions as they occur in natural foods. Consequently, there must be other substances in our foods that are essential for us." For this work he had used white mice fed a kind of bread made of casein, albumin, rice flour, lard and mineral salts and water. When milk or whey replaced the water, the mice stayed alive "although the protein, lactose and fat from the milk are negligible in comparison to the amounts supplied by the bread." At a meeting of The Netherlands Medical Association in July, 1905, Pekelharing stated, "I intend to show that milk contains still another unknown substance which is of the greatest importance even in minute amounts. When this substance is absent, the organism loses the facility to utilize the main known nutrients. The appetite is lost and amidst apparent abundance the animals starve to death." This rather casually made statement did not receive much attention and Pekelharing did not want to announce his opinion to the world until he had discovered and isolated "this substance."

Pekelharing's lifelong interest in protein digestion partly resulted from his concern with the rationale of dietary prescriptions. In 1908 he published a brochure entitled "Proteins as Food." In it he stated, "There is probably no part of Physiology that is applied in medical practice more frequently than the science of nutrition. Unfortunately it becomes often obvious how far the physiology lags behind the demands of medical practice." Pekelharing himself had greatly contributed to this kind of knowledge. He stated that proteins are of different value as a nutrient and he investigated different proteins. In 1908, writing on the use of protein by the animal body, he discussed a subject of great interest to him. "Do we have a chemical method to determine variations in protein breakdown in the cells of various organs under different conditions?" he asked. In this connection he studied urinary creatine and creatinine excretion in relation to muscle tone. Two of his students wrote their dissertations on this subject; one on the excretion in normal persons, the other on the conditions in patients.

In 1906, an entire issue of the *Journal of the Netherlands Medical Association* was devoted to Pekelharing's 25 years as a professor of medicine. It was filled with contributions by his many friends and former students. Starting in 1908, two young scientists helped Pekelharing pursue his ever-growing research interests. Dr. W. E. Ringer, a chemist, and Dr. M. A. van Herwerden, who assisted with histology and cytology, were of great help when Pekelharing's age made itself felt.

In 1914 he undertook a second trip to Java at the invitation of one of his sons who worked there as a chemist. The outbreak of World War I, however, forced his return soon after his arrival.

When food became scarce in Holland during World War I, Pekelharing wrote several articles on feeding the low-income groups. He was involved in a study of the comparative economic advantages of whole wheat and white bread. He was instrumental in the establishment of The Netherlands Institute for Nutrition in Amsterdam. Its first director was Professor E. C. van Leersum. Later Dr. B. C. P. Jansen became its director. In 1916 Pekelharing investigated the nutritional value of margarine which was increasingly used to replace the scarce butter. He pointed out the lack of vitamins in margarine. His great concern was how to avoid harmful effects of the diminishing food supply.

But Pekelharing's interests still ranged widely. The history of medicine and natural science was his hobby and he contributed many biographies to a number of publications. Pekelharing was gifted with a great power of concentration. He could work amidst the liveliest conversation. As a rule he did not attend meetings outside The Netherlands, but he was in close contact with his many friends and colleagues abroad. A memorable event to him was a visit by Dr. Benedict after World War I.

In 1918 Pekelharing reached retirement age. On June 14 of that year he gave his last lecture as a professor of medicine.

Again he emphasized the importance of the study of chemistry and histology for medicine. In July, 1918, he was presented with a beautifully bound volume compiled in his honor. Its title was "Livre jubilaire-Archives Neerlandaises de Physiologie de l'homme et des animaux en l'honneur de C. A. Pekelharing." It contained contributions by Halliburton, Bayliss, Kossel, Hammarsten and many others. In that same year the Utrecht section of the Netherlands Medical Association made him an honorary member.

But retirement did not mean rest for the indefatigable worker. He continued to study and write on his many interests. In September, 1918, he contributed the introduction to the compiled works of C. Winkler at the occasion of his friend's 25 years professorship. Soon, however, his health began to fail him. His last public appearance was at the unveiling of Donders' statue on June 22, 1921. For eight years he had worked on this project and it was mainly through his efforts that it came into being.

His condition deteriorated and on September 18, 1922, Cornelis Adrianus Pekelharing died at his home in Utrecht. He was buried on September 21 in that same city. Innumerable letters and necrologies appeared in papers and professional journals mouring the loss of a man of irreproachable character, a scientist of great repute and integrity. He had published some 160 papers and a textbook of histology.

Pekelharing's work has frequently been quoted and appeared in periodicals such as the *Biochemische Zeitschrift, Hoppe-Seylers Zeitschrift für physiologische Chemie, Pflüger's Archiv für die gesamte Physiologie des Menschen und der Tiere, Deutsche Medische Wochenschrift, Archives neerlandaises des sciences exactes et naturelles, Chemical Weekly* (Dutch), *Netherlands Medical Journal* and several others. Twenty-six students completed their doctoral work under his direction.

Although he had made no world-renowned discoveries, his work has laid the foundation for many of the new developments in biochemistry and nutrition.

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Placental Transfer and Fetal Tissue Iron Utilization in Sheep

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ABSTRACT Radioiron and total iron data from blood-balance and tissue distribution studies with 24 ewes indicated fetal sheep organs and tissues to possess greater relative turnover rates during the second trimester than at later stages of gestation. Pattern of fetal hemapoiesis showed liver and spleen contribution early in fetal development, whereas red bone marrow (sternum) became increasingly more important during the second trimester, and all tissues involved in hemapoiesis achieved apparent equilibrium during the final stages of fetal life. Data implied that iron traversed the placenta of sheep at all stages of gestation. This transfer was apparently related directly to the amount of iron actually required for incorporation into growing tissues. Iron traversing the ovine placenta was probably of plasma origin, but some maternal tissues appeared to have initial priority over the developing fetus for iron storage. Calculated daily additional dietary iron requirement to meet the needs of pregnancy in sheep approached 5.0 mg.

Fetal nutrition studies dealing with iron utilization and placental transmission in domestic species have been confined largely to monogastric animals. Swine, particularly, have been of interest because of the high incidence of baby pig anemia. Although anemias are a rare occurence in ruminants, relationships of iron to parasitic and prevalent disease conditions make this an economic problem for cattle and sheep producers.

Among the first efforts to study placental transfer of iron were those reported in 1927 by Brunshwig (1). He investigated the placental transport of iron salts in the white rat by measuring their accumulation at sites of placental attachment. The reports of Flexnor and Pohl (2–4) and of Pecher and Pecher (5) were classical investigations of placental transmission with the advent and development of radioisotope procedures. Pommerenke and associates (6) administered Fe^{59} orally to pregnant women and reported rapid passage from mother to fetus. In 1958, Bothwell (7) and co-workers investigated iron transport across the placental membranes of the rabbit. This problem has been studied only recently in farm animals. Pond and associates (8) reported on the placental and milk transfer of stable iron in swine during late gestation and early lactation. Hoskins and Hansard (9) have reported the placental transfer of iron in swine to increase progressively with increased gestation age.

The paucity of information on iron metabolism and assimilation in the ruminant animal and the need for more information on prenatal nutrition prompted the initiation of this study on the placental transfer and tissue distribution of iron in sheep as a function of gestation age.

METHODS

Twenty pregnant and 4 control native 2-year-old ewes were maintained with adequate corn-soybean oil meal-cotton seed hull rations, supplying an average of 29 mg iron daily. Animals were hand-bred, and 19 of the ewes were divided into 3 lots according to breeding date. At the periods indicated each ewe was placed in an individual metabolism unit, equipped for the quantitative separate collection of urine and feces (10). Following a brief adjustment period, all animals were administered intravenously a single tracer dose (40 to 60 μc) of $Fe^{59}Cl$ (citrated) for 48-hour blood-balance studies previous to killing at 47, 94 and 141 days' gestation. The other 5 ewes were handled similarily except that after 141 days gestation they were killed at 12, 24, 48 and 144 hours

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after dosing with Fe^{59}. Selected maternal and fetal organs and tissues were weighed, ashed and analyzed radiochemically by conventional procedures. Radioiron was measured in sample aliquots, using a well-type scintillation detector attached to a scaler unit. Total iron determinations were made on plasma by the method of Barkan and Walker (11), and on feces and tissue samples by a modification of the method of Wong (12). Periodic blood samples were withdrawn from the jugular vein of dams, and by heart puncture in fetuses, for hematocrit and hemoglobin determinations, using the cyanomethemoglobin procedure (13).

RESULTS AND DISCUSSION

Blood and excretory iron. Plasma clearance and red blood cell Fe^{59} uptake by ewes followed the characteristic behavior patterns and were not significantly influenced by gestation. For brevity, therefore, these data are omitted. Maternal homoglobin averaged 10.9, and the mean hematocrit value was 38.6 ± 2. Fetal hemoglobin and packed cell volume increased with advanced gestation, averaging 6.0, 7.4 and 10.4 g hemoglobin/100 ml at 47, 94 and 141 days, respectively. The combined 48-hour urinary and fecal excretion of intravenously injected Fe^{59} for the 19 ewes was less than 2% of the dose, and did not vary with stage of pregnancy.

Iron in fetal tissues. A summary of radioiron, total iron and the specific activity of fetal sheep organs and tissues, as a function of gestation age, is presented in table 1.

Fetal blood radioiron concentration decreased significantly ($P < 0.01$) between the first and second trimesters, whereas total Fe^{59} increased, indicating a possible dilution effect to occur with increased fetal blood volume, as pregnancy advanced. Total iron concentration was lowest during the first trimester, but mean values were maintained essentially constant during the last 2 trimesters. Total circulating iron, calculated from fetal blood volume (10%) and hemoglobin levels, increased significantly ($P < 0.01$) with each progressive period of gestation, reflecting the large increase in total blood volume occurring with fetal growth.

TABLE 1

Summary of radioiron, total iron and specific activity of fetal sheep organs and tissues as a function of gestation age

Tissue	Dose [1]		Iron		Specific activity [2]
		Total		Total	
	%/g	%	mg/g	mg	
47 Days' gestation (2 fetuses)					
Blood [3]	24.0 ± 13.9 [4]	241.3 ± 178.0	0.060	0.602	400 ± 195
Liver	74.4 ± 25.9	26.0 ± 6.9	0.080 ± 0.008	0.280 ± 0.044	930 ± 512
Spleen	52.3 ± 21.0	3.1 ± 0.7	0.155 ± 0.019	0.009 ± 0.001	337 ± 4
94 Days' gestation (5 fetuses)					
Blood	8.1 ± 4.9**	807.0 ± 506.1	0.028 ± 0.007	2.8 ± 1.4	289 ± 82*
Liver	4.5 ± 1.3	230.0 ± 110.0	0.089 ± 0.043	4.5 ± 2.3***	50 ± 38**
Spleen	21.4 ± 9.4**	40.0 ± 26.5***	0.168 ± 0.043	0.319 ± 0.37***	127 ± 87
Kidney	1.8 ± 1.7	6.0 ± 3.1	0.036 ± 0.014	0.118 ± 0.054	50 ± 16
Sternum	17.3 ± 14.6	—	0.089 ± 0.092	—	194 ± 196
141 Days' gestation (5 fetuses)					
Blood	10.2 ± 0.4	4590.0 ± 178.7	0.035 ± 0.001**	15.8 ± 5.3***	291 ± 11***
Liver	4.7 ± 4.7	453.3 ± 432.5	0.077 ± 0.036	7.4 ± 2.8***	61 ± 13
Spleen	12.1 ± 0.7	63.8 ± 5.2	0.181 ± 0.028	0.96 ± 0.38	67 ± 4***
Kidney	0.7 ± 0.3	6.3 ± 0.6	0.045 ± 0.003	0.38 ± 0.28	16 ± 4***
Sternum	4.6 ± 2.2**	—	0.073 ± 0.025	—	63 ± 43**

[1] Per cent dose, Fe^{59}, corrected to absorbed radioiron, $\times 10^{-4}$ per g after 48 hours.
[2] Calculated as per cent dose/g divided by mg iron/g, $\times 10^{-4}$.
[3] Total circulating blood iron calculated from hemoglobin values assuming 10% total blood volume and 0.34 g Fe/100 ml.
* Denotes significance at $P < 0.10$; **, $P < 0.05$, and ***, $P < 0.01$ level, between trimesters.
[4] Mean ± SE of mean.

Red blood cell Fe^{59} values affirmed the increased fetal hemoglobin incorporation and current iron behavior at the 3 trimesters of pregnancy, and indicated rapid iron utilization during early prenatal development. Observed decrease in specific activity with advanced gestation implied a decrease in hemapoietic function, as well as an iron dilution effect, as pregnancy advanced and as stored iron levels increased.

Liver Fe^{59} concentration decreased significantly ($P < 0.01$) from 47 to 94 days, whereas radioiron in the total liver increased progressively during gestation. Concentration of total iron did not change during gestation; however, total liver iron increased with fetal growth and age. These observations were interpreted to mean that fetal liver storage of iron begins early and continues rather uniformly with growth throughout the prenatal period. Relative turnover rates were highest at 47 days, implying that liver contribution to hemapoiesis began early in fetal life and assumed decreasing importance as pregnancy advanced.

The Fe^{59} concentration decreased and total radioiron content increased significantly ($P < 0.05$) in the spleen during the gestation period. Total iron concentration, however, was not changed with advancing pregnancy, reflecting the large increases in growth occurring with advanced fetal age. Specific activity values decreased with time until 141 days. These observations were indicative of the importance of the spleen to hemapoiesis during the earlier stages of development, with this function becoming somewhat depressed at later stages of fetal growth.

Fetal kidney iron values denoted storage and assimilation to be most active at the earlier stages of gestation and to decrease with increased age. These data showed greater storage and turnover rate to occur early in fetal development, with dilution and decreased excretion resulting as growth increased.

Radioiron concentration in fetal sternum decreased significantly ($P < 0.05$) during the final stage of gestation, whereas total iron concentration remained essentially constant. Relative iron turnover rate for the sternum exhibited a significant decrease at 141 days as illustarted by specific activity values, and affirmed that the sternum, as representative of red bone marrow, contributed early to hemapoiesis in the prenatal life of the sheep.

Whole fetus. Summary information on the whole sheep fetuses in this study, as a function of gestation age are shown in table 2. Physical measurements showed that fetal length doubled between day 47 and 94, and fetal weight increased 10 times. Between the second and third trimesters, fetal length again doubled, and weight increased 4.5 times.

Total radioiron in whole fetuses from ewes, dosed intravenously 48 hours previous to killing, increased with advancing pregnancy, reaching a peak of 0.66% of the *absorbed* dose at 141 days' gestation. Total iron content increased 14 times from day 47 to 94, and 4 times between the second and final trimesters. A part of this increase in iron content was attributable to simple increase in fetal weight, although some was due to the increased total iron transfer with advancing fetal development. Fetal sheep, at 141 days' gestation, were shown to contain an average of 123 mg elemental iron. The observed increase in total iron and of radioiron content in these fetuses, with increased fetal weight, was clearly indicative of iron transfer according to body needs. This fact was verified by the iron values in the single in-

TABLE 2

Average measurements of whole sheep fetuses as a function of gestation age

Days of gestation	No. fetuses	Length	Weight	Total	
				Fe^{59}	Iron
		cm	*g*		*mg*
47	2	13.3	103	874 ± 56 [2]	2.27 ± 1.5
94	5	29.0	1014	3173 ± 128	28.70 ± 36.9
141	5	55.2	4564	6585 ± 463	123.00 ± 29.0

[1] Per cent dose, Fe^{59}, corrected to absorbed radioiron, $\times 10^{-4}$.
[2] Mean ± SE of mean.

stance where twin lambs were born to one ewe killed, during the third trimester, 24 hours after radioiron administration. Total fetal iron was double that of single lambs and total Fe^{59} transfer was higher than anticipated for this time interval. Unfortunately the radioiron values were not directly comparable because of limited samples at this period.

Fetal partition of iron and radioiron. The partition of radioiron and of total iron in the fetal organs and tissues of sheep, 48 hours after dose administration, is shown in table 3.

These data demonstrate blood to be the principal pool for both total iron and radioiron in the sheep fetus at all stages of gestation. Fetal liver, spleen and kidney followed, in that order, in relative contribution of iron and Fe^{59} to total fetal content.

It was calculated, that during the first trimester, the fetus itself represented 4.9% of the Fe^{59} and 14.1% of the total iron present in the whole fetal complex, including fetus, fetal fluid and the placental membranes. During the second trimester, these percentages increased to 37.6 and 42.3%, respectively. However, in the final stage of gestation, 60.8% of the Fe^{59} and 68.3% of the total iron in the fetal complex was represented by the fetus. These values further indicate that, 48 hours following maternal dose administration, iron-Fe^{59} equilibrium has been essentially established, at 141 days of gestation, but occurs less rapidly during the earlier periods.

Tissue Fe^{59} movement with time. Shown in table 4 is a summary of radioiron in fetal organs and tissues of sheep as a function of time after maternal intravenous Fe^{59} dosing during the third trimester. Between 24 and 48 hours following dose administration, placental transport appeared optimum. Since plasma Fe^{59} levels were minimum at this time, data indicate that iron was transported directly from plasma to the placenta. Values listed at 48 hours compare favorably with those reported for other 141-day fetuses taken at the same time period after dosing (table 1).

Blood Fe^{59} levels exhibited a tendency for increased uptake with time to 144 hours, when slightly more than 1% of the absorbed dose was measured in the circulating fetal blood. Liver and spleen values likewise increased with time, implying continuous iron storage for this organ during the last trimester. Fetal kidney levels also increased with time, indicating active iron passage. The sternum showed progressive increases in Fe^{59} concentration, reflecting sternal red bone marrow contribution to hemapoiesis during this final trimester of pregnancy. Comparisons with values in table 3 indicate that the incorporation of radioiron in bone marrow was less rapid than in liver or spleen. Whole fetal Fe^{59} values were shown to increase with time after dose administration. Total fetal radioiron levels increased threefold between 12 and 24 hours and 24 times from 24 to 48 hours, with only a twofold increase noted between 48 and 144 hours post-dosing.

These data therefore, indicate placental transfer of iron in sheep to be an active process, increasing progressively during the last trimester and with time after dose administration. Radioiron traversing the

TABLE 3

Partition of radioiron and total iron in fetal organs and tissues of sheep 48 hours after maternal dose administration [1]

	Days of gestation					
	47		94		141	
	Fe^{59}	Iron	Fe^{59}	Iron	Fe^{59}	Iron
	%	*mg*	%	*mg*	%	*mg*
Fetus, whole	874	2.27	3173	28.7	6585	123.0
Blood, total [2]	241	0.6	807	2.8	4590	15.7
Liver	26	0.3	230	4.5	453	7.4
Spleen	3	0.009	40	0.3	64	0.9
Kidney	—	—	6	0.1	6	0.4

[1] Total per cent dose, $Fe^{59} \times 10^{-4}$ and total mg iron.
[2] Total circulating blood iron calculated from the blood volume (10%) and hemoglobin values.

TABLE 4

Summary of radioiron [1] in fetal organs and tissues of sheep as a function of time after dosing (3rd trimester) intravenously with radioiron

No. fetuses	(1)		(2) [2]		(2)		(1)	
	12 Hours		24 Hours		48 Hours		144 Hours	
	Dose	Total	Dose	Total	Dose	Total	Dose	Total
	% /g	%	% /g	%	% /g	%	% /g	%
Fetus	—	85	—	280	—	6743	—	11,428
Blood	0.17	51	0.61	183	11.58	3474	14.80	4,440
Liver	0.68	24	0.68	25	3.12	265	4.78	406
Spleen	—	—	1.24	4	7.10	97	9.41	197
Kidney	0.19	1	0.43	3	0.76	6	2.19	12
Sternum	1.48	—	4.67	—	5.48	—	37.71	—

[1] Per cent dose, Fe^{59}, corrected to absorbed radioiron, $\times 10^{-4}$.
[2] These figures are average values for twin female lambs.

placenta before 24 hours was probably of plasma origin, but increased transfer at later times denotes major transfer to occur from that iron which may have been deposited initially as maternal body stores. This could imply that certain maternal organs and tissues may have initial priority over the fetus for iron storage or for preparation for subsequent membrane transfer, or for both. The fact is not overlooked that more time was apparently required for maternal-fetal equilibirum and subsequently greater fetal iron transfer, with increasing time after dose administration. However, 48 hours after maternal dose administration was selected as the killing time primarily to insure minimal sampling error due to presence of maternal and fetal Fe^{59}-labeled red blood cells themselves.

In this study the sheep fetus contained 123 mg elemental iron after 141 days' gestation. The conventional balance data showed these 20 ewes to absorb an average of 7.3 mg iron/day, with no apparent effect of gestation on retention. During the final trimester (141 days), 180 mg of iron were accreted by the fetal complex. Of this amount, 123 mg (68.3%) were retained by the fetus itself. It was calculated, therefore, that these ewes would require an additional 5.0 mg of iron/day from dietary sources to meet the average iron requirements of the developing fetal complex in sheep.

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Effects of Dietary Magnesium and Fluoride on the Magnesium Content of Tissues from Growing Chicks[1,2]

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ABSTRACT Two factorial experiments were conducted in which the effect of supplemental magnesium and fluoride on the magnesuim content of plasma (total and diffusible), bone, muscle, heart, liver and kidney were determined. Bone, liver and total and diffusible plasma magnesium were greater in birds fed diets containing high levels of both magnesium and fluoride than in birds fed diets high in either magnesium or fluoride alone. Muscle magnesium did not vary greatly with the dietary treatments and the magnesium content of the heart was remarkably constant.

Previous work in this laboratory has shown that the addition of high levels of both magnesium and fluoride to the diets of growing chicks caused a greater reduction in growth rate than the addition of high levels of either magnesium or fluoride alone (1, 2). Leg weakness and reduced bone ash were observed in the birds fed high magnesium-high fluoride diets but not in birds fed diets high in either element alone. The lack of a rapid and accurate method of analysis for magnesium limited the amount of information obtained on magnesium distribution in the earlier studies. The acquisition of an atomic absorption spectrophotometer which facilitates rapid, precise and accurate analysis made it possible to determine the influence of dietary magnesium and fluoride levels on the distribution of magnesium in various tissues.

Two experiments were conducted to determine: 1), the normal magnesium content of chick plasma, bone, liver, kidney, muscle and heart; 2), the effects of excess dietary magnesium and fluoride on the magnesium content of the above tissues; and 3), the effects of excess dietary magnesium and fluoride on the distribution of total and diffusible magnesium in the blood plasma.

EXPERIMENTAL

The first experiment was conducted to determine the magnesium content of the various tissues. One-day-old male and female White Cross chicks were randomly assigned to 9 groups of 10 birds each, banded, weighed and placed in electrically heated, wire-floor starting batteries. The percentage composition of the basal diet was as follows: ground yellow corn, 47.30; dehulled soybean meal, 36.00; fish solubles, 4.00; NaCl (iodized), 0.25; $MnSO_4$, 0.05; dicalcium phosphate, 1.80; ground limestone, 1.10; soybean oil, 5.00; vitamin premix, 1.25.[4] This diet, which contained 1800 ppm magnesium, was supplemented with 2 levels of magnesium ($(MgCO_3)_4 Mg(OH)_2 \cdot 4H_2O$) and 2 levels of fluoride (NaF) in a factorial design as shown in table 1. Dietary sodium was held constant by adding sodium carbonate to the low fluoride diets. Feed and deionized water were provided ad libitum.

After two weeks, blood samples were taken by cardiac puncture with heparin as an anticoagulant from each of the 10 birds in each group. The plasma was separated by centrifugation, sealed in

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[4] The vitamin premix supplied the following: (per 100 g of diet) vitamin A, 1000 IU; vitamin D_3, 150 ICU; vitamin E acetate, 0.56 IU; choline chloride, 150 mg; niacin, 3.17 mg; D-calcium pantothenate, 1.41 mg; riboflavin, 0.71 mg; menadione sodium bisulfite, 0.07 mg; procaine penicillin, 0.44 mg; vitamin B_{12}, 0.66 μg.

TABLE 1

Effect of supplemental magnesium and fluoride on the magnesium content of various tissues

Sample	Supplemental F	% Supplemental Mg		
		0.00	0.15	0.25
	%	*ppm*	*ppm*	*ppm*
Plasma	0.00	23.4 ± 0.8 [1]	27.4 ± 1.8	24.7 ± 1.0
	0.05	22.1 ± 0.6	30.9 ± 3.1	34.5 ± 2.0
	0.08	19.0 ± 0.6	29.5 ± 2.1	39.0 ± 2.7
Bone [2]	0.00	3931 ± 114	4933 ± 238	4635 ± 536
	0.05	4483 ± 99	5326 ± 208	5653 ± 185
	0.08	5208 ± 257	5700 ± 274	5284 ± 385
Muscle [3]	0.00	303 ± 11	309 ± 12	316 ± 34
	0.05	304 ± 16	324 ± 14	258 ± 10
	0.08	321 ± 8	243 ± 23	363 ± 45
Heart [3]	0.00	262 ± 11	265 ± 9	257 ± 12
	0.05	273 ± 11	271 ± 12	256 ± 18
	0.08	261 ± 12	269 ± 24	272 ± 16
Liver [3]	0.00	239 ± 11	212 ± 13	296 ± 17
	0.05	261 ± 17	252 ± 18	295 ± 19
	0.08	228 ± 13	289 ± 12	309 ± 10
Kidney [3]	0.00	221 ± 16	289 ± 30	220 ± 17
	0.05	165 ± 13	218 ± 22	209 ± 25
	0.08	198 ± 16	248 ± 19	238 ± 13

[1] Average of 10 samples ± SE.
[2] Based on dry, fat-free bone.
[3] Based on fresh weight of tissue.

plastic vials and frozen immediately. Samples of bone (femur), muscle (thigh), heart, liver and kidney were collected from each bird, weighed, sealed in individual plastic vials and frozen immediately. All samples were stored at −15°C until analyzed. Blood plasma was prepared for analysis with an atomic absorption spectrophotometer by direct dilution similar to the method described by Willis (3).

The diluent used consisted of deionized water and sufficient strontium chloride to give the final solutions (containing 0.1 to 1.0 ppm Mg) a strontium content of 329 ppm (0.1% $SrCl_2 \cdot 6H_2O$). The strontium was added to prevent phosphate interference with magnesium analysis. The interference, which was observed using a brass burner with 1-mm holes,[5] was prevented completely by the addition of strontium. A stainless steel burner with a 0.5-mm slot was acquired after the present work was completed and phosphate interference with magnesium absorption was not observed with the slotted burner. The percentage of absorption observed in the samples was compared with that of standard solutions prepared by dissolving pure magnesium metal with a minimum of nitric acid and diluting with deionized water and a $SrCl_2$ solution to give working standards containing 0.1 to 1.0 ppm magnesium and 329 ppm strontium.

The bones were extracted with ethanol and then with ether in a Soxhlet apparatus, dried and weighed. The soft tissue and bone samples were then digested in approximately 2 volumes (w/v) of concentrated nitric acid and diluted. The final solutions contained 329 ppm strontium and the acid strength of standards and samples were adjusted to 0.1 N with nitric acid.

The second experiment was conducted to determine the total and diffusible plasma magnesium in normal birds as well as the effect of high dietary magnesium and fluoride on these values. One-day-old White Cross cockerels were randomly separated into 4 groups of 10 birds each, banded and placed in batteries. The basal diet was the same as that used in the first experiment except dehulled soybean

[5] This was the original burner supplied with the Model 214 Atomic Absorption Spectrophotometer, Perkin Elmer Corporation, Norwalk, Connecticut.

meal constituted 40% of the diet and no fish solubles product was included. This diet, which was found to contain 1688 ppm magnesium, was supplemented with one level of magnesium and one level of fluoride as shown in table 2. Feed and water were provided ad libitum.

At the end of 2 weeks, plasma samples were obtained and stored as previously described. An aliquot was used to determine total plasma magnesium and the remainder was pressure-filtered through a cellulose ester membrane[6,7] using the apparatus shown in figure 1. The filtrate, which contained the diffusible magnesium, was diluted to a suitable volume and analyzed by atomic absorption. One milliliter of plasma was sufficient for filtration and analysis.

Statistical analyses were made according to the methods of Snedecor (4).

RESULTS AND DISCUSSION

The growth rate data followed the same pattern as that observed in previous work (1, 2). Growth rate was depressed more

[6] Protein enrichment membrane with pore sizes of 0.004 μ, Gelman Instrument Company, Chelsea, Michigan.

[7] Duro-Plastic E-Pox-E Glue, The Woodhill Chemical Corporation, Cleveland 14.

TABLE 2

Effect of dietary magnesium and fluoride on total and diffusible plasma magnesium

Sample	Supplemental F	% Supplemental Mg	
		0.00	0.25
	%	*ppm*	*ppm*
Total plasma Mg	0.00	17.5 ± 1.0 [1]	23.7 ± 1.1
Total plasma Mg	0.08	17.9 ± 1.1	31.4 ± 1.9
Diffusible plasma Mg	0.00	12.3 ± 0.8	15.3 ± 0.5
Diffusible plasma Mg	0.08	10.1 ± 0.5	19.2 ± 1.7

[1] Average of 10 samples ± SE.

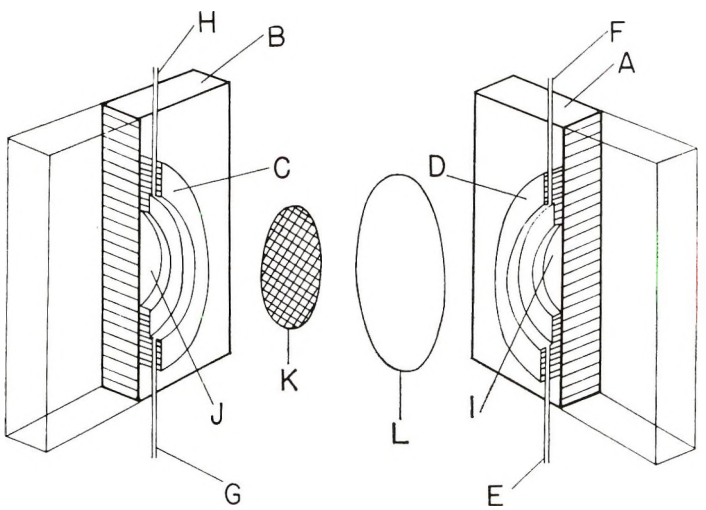

Fig. 1 Diagram of micro-filtration apparatus. The apparatus is constructed from two 7.6 × 7.6 × 1.3 mm plexiglas blocks (A and B) to which Teflon rings C and D, milled as shown, are attached with epoxy glue. Twenty-gauge hypodermic needles (E, F, G, H) extend through the Teflon rings into chambers I and J. A stainless steel screen K supports membrane L. The apparatus is fastened together with 4 bolts through the corners of the plexiglas blocks and plasma is forced through needle E into chamber I under nitrogen pressure. Needle F, serving as an air vent, is clamped off after the chamber fills with plasma and about 40 psi pressure is applied. The filtrate is recovered from chamber J through needle G by applying pressure on needle H.

by the addition of both magnesium and fluoride to the diet than by the addition of either element alone and the characteristic leg weakness developed only when the diet was supplemented with both magnesium and fluoride. The affects of supplemental magnesium and fluoride on the magnesium content of various tissues are shown in table 1. No differences due to sex were observed. The magnesium content of plasma increased with each increase in dietary magnesium at all levels of dietary fluoride except the zero fluoride — 0.25% Mg treatment. Plasma magnesium also increased as dietary fluoride was increased at the highest level of dietary magnesium, but the reverse was true at the lowest magnesium level and no significant change occurred at the intermediate level. It should be recalled that a practical-type diet was used and that the dietary ingredients supplied 1800 ppm magnesium, some 4 to 8 times the minimum requirement for the nutrient (5, 6). Analysis of variance showed that the magnesium-fluoride interaction was significant ($P < 0.01$).

At each level of dietary magnesium, fluoride supplementation of the diet resulted in an increase in the magnesium concentration in bones ($P < 0.01$). The addition of 0.15% magnesium to the diets caused a significant increase in bone magnesium ($P < 0.01$) but further magnesium supplementation caused no changes.

Muscle magnesium content was rather constant across treatments except for 2 values which were lower than the others. No explanation can be offered for this apparent discrepancy.

The magnesium content of the hearts was remarkably constant and was not influenced by dietary magnesium or fluoride levels. Magnesium content of liver was increased by fluoride only at the 0.15% dietary magnesium level. Increased dietary magnesium was accompanied by increased liver magnesium and there was a significant magnesium-fluoride interaction.

Kidney magnesium tended to be high in the treatments supplemented with 0.15% magnesium and low in the treatments supplemented with 0.05% fluoride.

The results of the second experiment are shown in table 2. Total plasma magnesium, although lower than in the previous experiment, followed the same pattern. Increased dietary magnesium was accompanied by moderate increases in total and diffusible plasma magnesium; the addition of fluoride to the basal diet had no effect. Supplementing the basal diet with both magnesium and fluoride, however, resulted in greatly increased total and diffusible plasma magnesium, indicating a magnesium-fluoride interaction in each case ($P < 0.01$ and $P < 0.05$, respectively).

The fact that maintenance with high magnesium-high fluoride diets was accompanied by increased magnesium content of the plasma, bone and liver suggests the possibility that these 2 elements formed a stable complex such as the one described by Warburg and Christian (7). These workers showed that the inhibition of enolase by fluoride could not be due to a reduction in the concentration of free magnesium since the fluoride concentration needed to inhibit enolase decreases as the magnesium concentration increases. Instead Warburg and Christian suggested the formation of a magnesium-fluorophosphate-enzyme complex. It is suggested that a complex similar to that mentioned by Warburg and Christian may have inhibited an enzyme system involved in bone or muscle metabolism. Previous work (2) indicated that enolase activity in muscle was not altered by increasing the dietary levels of magnesium and fluoride. The inhibition of some other enzyme system could be the cause of the reduced bone ash and leg weakness observed in chicks fed high magnesium-high fluoride diet. This possibility exists although the total magnesium content of the tissues and the total and diffusible magnesium content of the plasma were increased in the high magnesium-high fluoride diets.

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Possible Goitrogenic Effects of Selected Japanese Foods

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ABSTRACT Goiters frequently develop among Japanese living along the seacoast despite high daily iodine intake. To evaluate possible goitrogenic effects of certain Japanese foods, groups of Wistar albino rats were fed, for 5 months, a low iodine basal diet (Remington ration) supplemented with Chinese cabbage, turnip, buckwheat noodle, soybean, or seaweed. Some of these supplemental foods already were known to be goitrogenic. Of 7 groups of rats one group was fed the Remington ration alone; another group received Remington ration supplemented with 2 μg iodide (as 2.4 μg sodium iodide) per rat per day. These 2 groups served as controls for the 5 test groups. Goiters developed in the rats of all groups except those fed seaweed. Goiter was not completely prevented by addition of 2 μg iodide to the Remington ration, apparently indicating that Remington ration increases iodine requirement. Foods supplementing the Remington ration did not produce goiters significantly larger than those observed in the controls. Histologically, goitrous thyroid tissue revealed hyperplastic follicles with scanty colloid, dissimilar to colloid goiter commonly occurring in man. Thyroid tissue of rats given seaweed or supplemental iodine was virtually indistinguishable histologically from normal rat thyroid. The experiment indicated goitrogenicity of the Remington ration but failed to demonstrate positively the goitrogenicity of supplemental foods. Response to the seaweed diet showed that ingestion of large amounts of iodine can prevent development of goiter.

In view of the frequent occurrence of goiter among the Japanese people in areas near the sea where the dietary iodine intake is large (1), an experimental study was designed to evaluate the role of dietary goitrogens as possible etiologic agents. The experiment consisted of feeding 5 foodstuffs to groups of rats for 5 months. All selected foods were staples of the daily Japanese diet and several of them previously have been found to produce goiter in animals or man (2–4).

METHODS

A total of 105 Wistar albino male rats weighing 50 to 60 g each was divided into 7 groups of 15 rats each, and matched closely for body weight. The animals were housed in individual wire cages which were arranged so as to prevent cross contamination by foodstuffs or excreta. Each animal was fed a low iodine basal diet — the Remington ration (5).[4] Including any additional food items tested, all rats of the 7 groups were offered 15 g of experimental diet per day, an amount which was somewhat more than would normally be eaten (table 1). The diets were prepared in bulk and kept refrigerated to assure a constant daily intake of each dietary constituent. Group 1 rats were given a measured volume of double-distilled water containing 2 μg of iodide (as 2.4 μg sodium iodide) per animal per day, while group 2 animals were maintained with double-distilled water without added iodide in an attempt to induce a state of iodine deficiency. Rats of groups 3 through 7 received the basal Remington diet and double-distilled water plus one of the following: Chinese cabbage, whole turnip, Japanese pure buckwheat noodle (soba), powdered soybean, or seaweed (table 1). All diets were rendered isocaloric by the addition of chemically pure sucrose at the time of bulk preparation.

Each group of rats was maintained with the designated experimental diet for 5 months. Those that died before termi-

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[4] Obtained from Nutritional Biochemicals Corporation, Cleveland.

TABLE 1

Diets, iodine intake and final body weight of rats

Group no.[1]	Daily diet [2]	Iodine intake	Avg final wt
		μg/animal/day	*g*
1	Remington diet,[3] 15 g + "iodide," 2 μg [4]	6.4	149.5
2	Remington diet, 15 g, no additive	4.4	150.0
3	Remington diet, 7.5 g + Chinese cabbage, 7.5 g	3.0	212.5
4	Remington diet, 7.5 g + turnip, 7.5 g	2.2	214.7
5	Remington diet, 7.5 g + buckwheat, 7.5 g	3.0	327.2
6	Remington diet, 7.5 g + soybean, 7.5 g	3.8	266.0
7	Remington diet, 7.5 g + seaweed, 7.5 g [5]	167.2	256.2

[1] Fifteen rats/group.
[2] Diets of all groups were rendered isocaloric by the addition of chemically pure sucrose. Each rat was given 15 g of food daily, an amount somewhat greater than would normally be eaten.
[3] See reference (5).
[4] In the form of sodium iodide, 2.4 μg daily.
[5] Contains equal parts by weight of both "flat" and "long" seaweed.

nation of the experiment were autopsied promptly to determine the cause of death. Prior to the end of the feeding period, all animals were injected intraperitoneally with 2.5 μc of I^{131} and were killed at the end of 24 hours. At that time, final body weight was measured, blood was drawn from the inferior vena cava for determination of serum total I^{131} and serum protein-bound iodine-131 (PBI^{131}), and the thyroid glands were removed, weighed immediately on a torsion balance and fixed in 4% formaldehyde. Serum total I^{131} was determined by counting 2 ml of serum in a well-type scintillation counter. Serum proteins were then precipitated with 8 ml of 10% trichloroacetic acid. The precipitate was washed 3 times with water and then dissolved in 2 ml of 1 N sodium hydroxide for determination of PBI^{131} by means of radioassay in a well-type scintillation counter.

TABLE 2

Iodine content of test diets [1]

Diets	Iodine
	μg/100 g
Remington ration [2]	29.1
Chinese cabbage	11.5
Turnip	not detectable
Buckwheat (soba)	10.5
Soybean	21.4
Flat seaweed (nori) [3]	1833.6
Long seaweed (wakame) [3]	2568.0

[1] Analyzed at the National Institute for Nutrition Research, Tokyo, Japan.
[2] See reference (5).
[3] Present in equal amounts by weight in diet ration of group 7.

The 24-hour I^{131} uptake was determined by counting the formaldehyde-fixed thyroid glands shortly after removal, using a probe-type scintillation counter. The thyroid glands were then processed for histologic examination. All of the test diets were analyzed for iodine content at the National Institute for Nutrition Research, Tokyo, by a gravimetric method of Ishibashi et al. (6) (table 2).

RESULTS

Table 3 shows the mean values of thyroid gland weights at the time of autopsy as both absolute gland weight and weight per 100 g of body weight. The thyroid gland weight relative to body weight was normal in animals of group 7, slightly increased in those of group 1, and significantly increased in those of groups 2, 3, 4, 5 and 6 ($P < 0.01$). Thyroid glands of group 2 were significantly heavier than those of any other group of animals with

TABLE 3

Thyroid weights at autopsy of rats fed various additives

Group (additive fed)	Avg of absolute wt	Avg wt/100 g body wt
	mg	*mg*
1 (NaI, 2.4 μg/rat/day)	22.6	15.0
2 (None)	145.1	95.7
3 (Chinese cabbage)	122.0	56.3
4 (Turnip)	90.6	42.4
5 (Buckwheat)	148.8	51.6
6 (Soybean)	143.2	51.5
7 (Seaweed)	20.3	8.1

TABLE 4

Twenty-four hour uptake of I^{131} by thyroid glands, serum total I^{131} and serum PBI^{131} of rats fed various additives

Group (additive fed)	I^{131} uptake	Serum I^{131}	Serum PBI^{131} [1]
	%	% dose/l	% dose/l
1 (NaI, 2.4 μg/rat/day)	48.7	0.246	0.205
2 (None)	62.3	0.371	0.322
3 (Chinese cabbage)	62.3	0.287	0.253
4 (Turnip)	47.8	0.480	0.429
5 (Buckwheat)	52.5	0.270	0.242
6 (Soybean)	52.0	0.248	0.225
7 (Seaweed)	1.8	0.035	0.005

[1] PBI indicates protein-bound iodine.

enlarged thyroid glands ($P < 0.01$). Table 4 shows the results of 24-hour I^{131} uptake by the thyroid glands, serum total I^{131} and serum PBI^{131}. All of the uptake values were somewhat elevated except in group 7 animals where a marked depression occurred, presumably because of the extremely high daily iodine intake in these animals. The data for serum total I^{131} and serum PBI^{131} show that the latter accounts for between 80 and 90% of the total serum radioactivity with little variation between groups, except in animals of group 7 where again a marked depression was noted. Histologic sections of the thyroids of group 1 animals revealed low cuboidal epithelium and follicles filled with abundant colloid (fig. 1). Group 2 animals that differed from those of group 1 only in that they received 2 μg of iodide less per day, revealed markedly hyperplastic follicles with scanty colloid (fig. 2). In sections of the thyroid glands of group 6 (soybean diet) similar hyperplastic follicles and colloid depletion were observed (fig. 3), but to a somewhat lesser degree than observed in the group 2 animals. Histologic features similar to those shown for group 6 rats were also exhibited by thyroid glands of animals in groups 3, 4

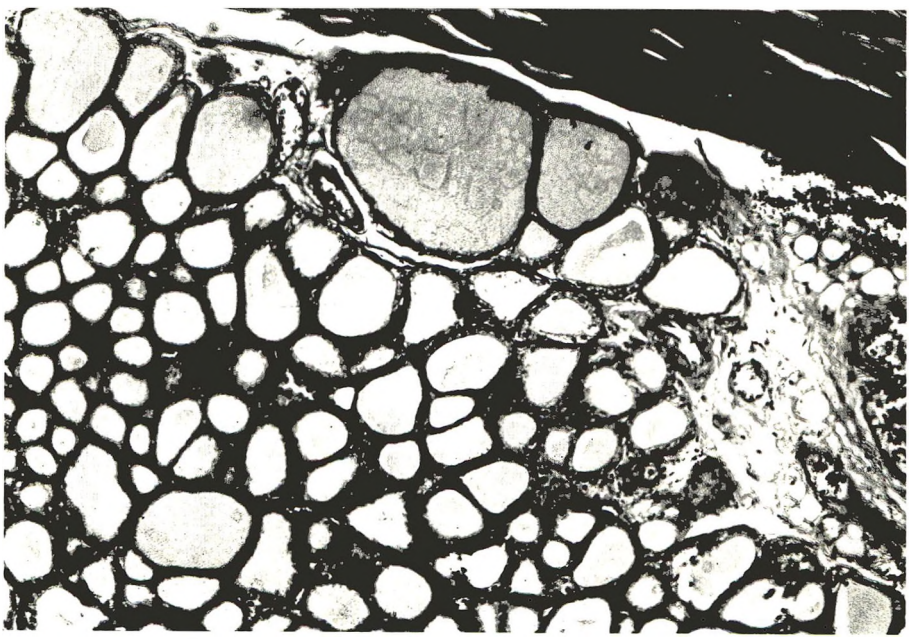

Fig. 1 Thyroid glands of group 1 rats, fed Remington ration and 2 μg iodide/day, revealed low cuboidal follicular epithelium and abundant colloid. H & E stain. × 33.

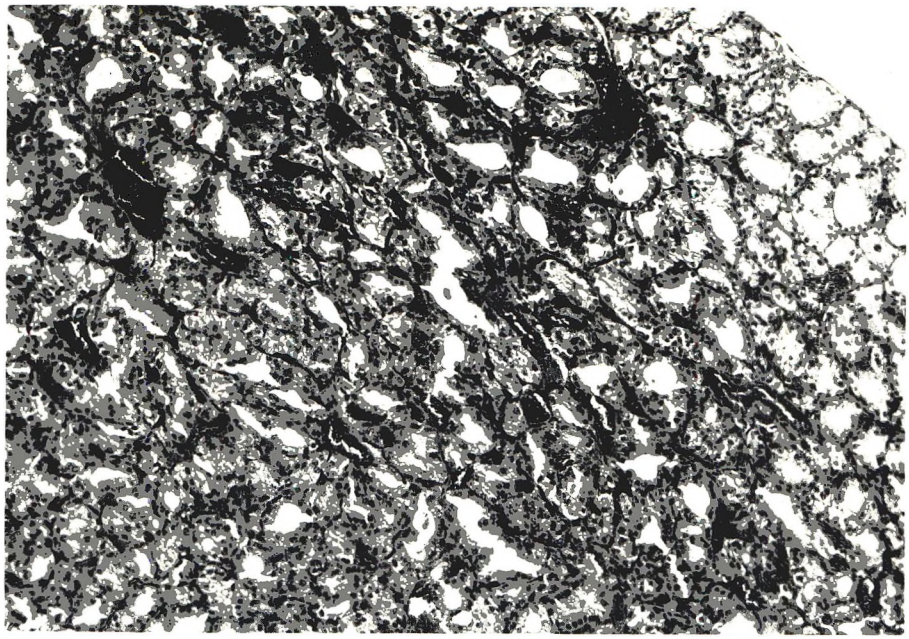

Fig. 2 Thyroid glands of group 2 rats, fed Remington ration alone, revealed markedly hyperplastic follicles with scanty colloid. H & E stain. × 33.

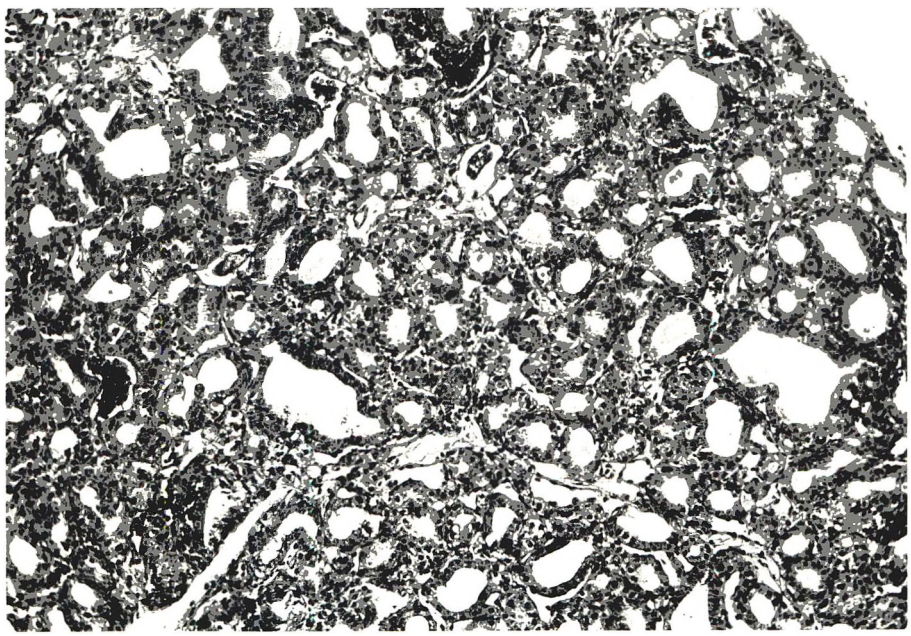

Fig. 3 Thyroid glands of group 6 rats, fed Remington ration and soybean, revealed hyperplastic follicles and colloid depletion similar to figure 2. H & E stain. × 33.

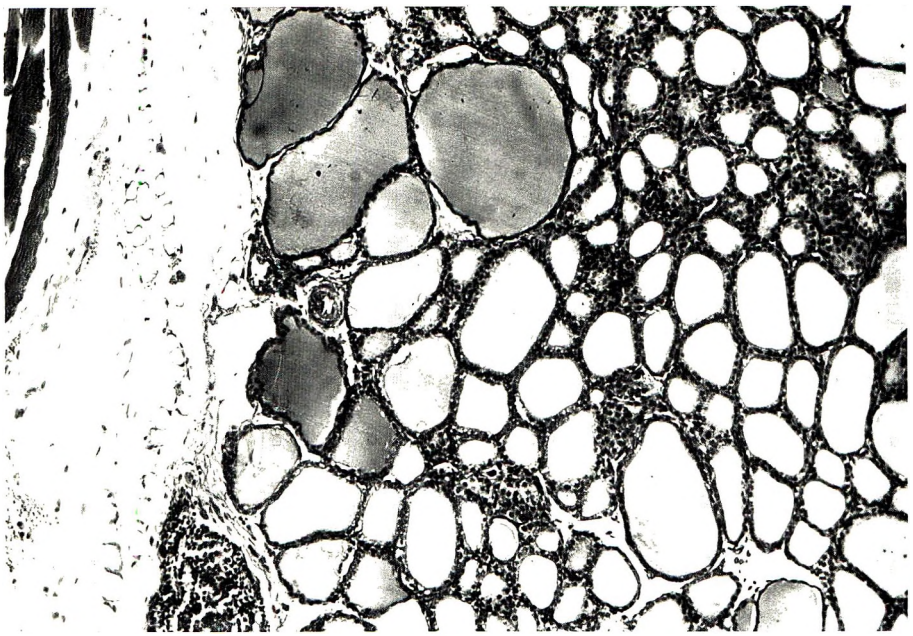

Fig. 4 Thyroid glands of group 7 rats fed Remington ration and seaweed were histologically indistinguishable from those of group 1 or of normal rat thyroid. H & E stain. × 33.

and 5 which are not described separately. The histology of the thyroids in the seaweed-fed animals of group 7 was virtually indistinguishable from that of group 1 or that of normal rat thyroid (fig. 4).

During the 5-month feeding period each rat was weighed at weekly intervals. The final body weights were low for animals in groups 1 and 2, suggesting that the Remington ration may not always support normal rat growth, even when iodine supplements are added (5) (table 1).

DISCUSSION

The experimental diets were initially devised so that the diet of group 1 animals would be a theoretical normal because of the constant daily supply of 2 μg of iodide (as 2.4 μg sodium iodide) to the reputedly low iodine Remington ration. In contrast, group 2 animals were fed a low iodine diet and it was felt that animals of groups 3 through 7 should also be maintained with an iodine-deficient diet so that the effect of the individual food additive in each group could be readily assessed by comparison with glands of group 2 animals. Moreover, previous experiments using rats have shown that the goitrogenicity of soy flour, of some cereals intended primarily for infant use, and of vegetables of the Brassica family such as cabbage, turnip, and rutabaga usually can be completely reversed by the addition of 2 or 3 μg of iodide to the daily ration of each animal (7–10). Therefore, if any of the foods surveyed in this study were to prove goitrogenic, such an action could be brought out clearly only if the animal's dietary iodine intake was restricted.

The thyroid glands of all animals in group 1, the supposed normal group, revealed relatively normal histology. However, the mean thyroid weight of this group was 15.0 mg/100 g body weight, slightly greater than the upper normal limit of 14 mg/100 g reported by Van Middlesworth (8). Group 2 animals received the same basal diet as group 1 except that the diet was initially designed to be iodine-deficient. Rats in group 2 consistently revealed the largest thyroid glands in this series, and the most severe hyperplasia was observed microscopically. Although the value for iodine content of the Remington ration analyzed at the Na-

tional Institute for Nutrition Research is higher than the value reported by Van Middlesworth, the calculation based on these analytical results reveals that 15 g of the Remington ration would provide 4.4 μg of iodide. It appears likely that the goiters in animals of groups 1 and 2 were the result of the Remington diet and it may be postulated that the Remington ration increased iodine requirement in these groups to at least 6.4 μg per rat per day as shown by the minimal goiter in group 1 animals and the severe goiter in group 2 animals if each rat consumed 15 g of the diet daily.

In groups 3 through 6 each animal received only half as much of the Remington diet as did animals in groups 1 and 2, and the daily iodine intake was considerably less (2.2 to 3.8 μg/day) than that of iodine-deficient animals of group 2. In view of the lower iodine intake it would be expected that if any of the test foods added to the diet of these groups were goitrogenic, more marked hyperplasia would have occurred along with larger goiters in the animals in these groups than those in group 2. Such an effect was shown by Halverson et al. (4) who reported that the addition of soybeans increased the degree of epithelial hyperplasia in Sprague Dawley rats previously maintained with a low iodine diet. The present study, however, showed just the opposite result as the glands of the animals fed soybean weighed significantly less and showed slightly less hyperplasia when compared with the thyroid glands of group 2 animals. Thus, using these parameters, the present experiment failed to prove the goitrogenicity of the foods tested.

The thyroid glands of group 7 animals fed seaweed are histologically similar to those of group 1. However, the mean weight of thyroid glands for this seaweed-fed group is 8.1 mg/100 g body weight, compared with 15.0 mg/100 g for the animals supplemented with 2 μg of iodide per day in group 1, a highly significant difference ($P < 0.01$). The value of 8.1 mg/100 g is well within normal range for rat thyroid weight and most likely can be attributed to the high iodine content of the added seaweed which apparently completely suppressed the goitrogenic effect of the Remington diet. The amount of iodine consumed by these animals is, however, far in excess of the normal daily requirement of about 2 μg (11).

Goitrous thyroids produced in this experiment revealed hyperplastic follicles with columnar epithelium and scanty colloid that are dissimilar to colloid goiter commonly occurring in man or to experimental colloid goiter in animals reported by Follis (12). Therefore, the development of goiters in the present experimental model may not be directly implicated in the pathogenesis of human goiters. However, the results of this experiment indicate that constant high iodine intake can prevent the development of goiter as shown in the rats fed seaweed. Follis (13) produced colloid goiter by giving large amounts of iodine to hamsters with hyperplastic goiters induced by an iodine-deficient diet or by goitrogens. It is conceivable that an analogous situation might ensue if humans suddenly ingest foodstuffs containing large amounts of iodine after a certain period of an iodine-deficient state.

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Effects of Dietary Antibiotics and Uric Acid on the Growth of Chicks[1,2]

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ABSTRACT One-day-old (Vantress × Arbor Acre) male chicks, fed a diet containing 2% uric acid, showed a significant weight depression after 4 weeks. This growth depression was not observed in chicks that received the same uric acid-containing diet but supplemented with 11 mg/kg of bacitracin and 44 mg/kg of procaine penicillin G. It is proposed that uric acid depresses growth by acting as an irritant and thus interfering with the absorption of nutrients from the intestinal tract; or uric acid inhibits the microbial biosynthesis of known vitamins or other nutrients essential to the host. Chemical analyses of the intestinal contents revealed an increased degradation of uric acid in the tract of the "uric-antibiotic"-fed chicks. No significant reduction or elevation was noted in the level of serum uric acid in any of the chicks. Antibiotic assays showed rapid penicillin inactivation in the tract, but persistence of the bacitracin. Although the beneficial response and increased uricolysis were observed in the "uric-antibiotic"-fed chicks, the level of antibiotic was lower in these birds. A growth response was obtained with diets supplemented with bacitracin only when an "old" environment appeared to have been established.

Wiseman et al. (1) noted that when chickens were fed folic acid-deficient diets containing a bacitracin-penicillin mixture, there was an increase in numbers of folic acid - synthesizing coliform bacteria, primarily *Aerobacter* spp., and a decrease in numbers of folic acid-requiring lactobacilli. Wiseman[5] concluded that the growth response of chicks fed vitamin - deficient diets which contained antibiotics was dependent upon the "vitamin-sparing" action of the antibiotics and the "vitamin-supplementing" action, resulting from an increase in numbers of a vitamin-synthesizing microflora, or possibly increased intestinal absorption as a result of thinner, more permeable intestinal walls caused by the antibiotics. It was further stated that a previously undescribed mode of action should be considered, since the increased numbers of folic acid-synthesizing *Aerobacter* spp. found in the intestinal contents were able to utilize uric acid. He proposed that chicks grow better when the conditions of diet and antibiotic supplement encourage the increase in numbers and activity of bacteria which remove a potentially toxic substance, uric acid, from the intestinal tract.

The studies reported in the present paper were undertaken to determine the effects of dietary antibiotics and uric acid on the growth of chicks.

EXPERIMENTAL

Experiment 1. One hundred and sixty one-day-old (Vantress × Arbor Acre) male chicks were received from a local hatchery and immediately distributed randomly into 4 equal groups. Each chick was wing-banded and weighed. The chicks were maintained on raised wire-mesh floors in an electrically heated battery in a continuously illuminated room which had not been used previously to house chicks. Diet and water were supplied ad libitum. Four groups of 40 chicks each were fed the following diets: a glucose-soybean oil meal diet (table 1), hereafter referred to as the basal diet; the basal diet plus 2% uric acid;[6] the uric acid-containing diet plus

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[5] Wiseman, R. F. 1956 Influence of dietary antibiotics and other conditions upon bacteria in the intestines of chickens. Ph.D. Thesis, University of Wisconsin (August).

[6] Purchased from Nutritional Biochemicals Corporation, Cleveland.

TABLE 1

Composition of the basal diet

	%
Glucose [1]	45.0
Fat (lard)	3.0
Soybean meal (50% protein)	48.0
Calcium carbonate	1.0
Dibasic calcium phosphate	2.0
Sodium chloride	0.5
Vitamin and mineral mixture in glucose [2]	0.5

[1] Cerelose, Corn Products Company, Argo, Illinois.
[2] Provided the following per kg of diet: KI, 2.2 mg; $MnSO_4$, 255 mg; ZnO, 85 mg; biotin, 0.13 mg; choline chloride, 2.3 g; folic acid, 0.83 mg; niacin, 40 mg; Ca pantothenate, 16 mg; pyridoxine·HCl, 5.2 mg; riboflavin, 4.3 mg; thiamine·HCl, 3 mg; vitamin A, 3970 USP units; vitamin B_{12}, 13 μg; vitamin D_3, 298 ICU; vitamin E (*d*-α-tocopheryl acetate), 25 mg; vitamin K (menadione sodium bisulfite), 0.32 mg; butylated hydroxytoluene, 34 mg; methionine (supplied as the calcium salt of the hydroxy analogue), 1.7 g.

11 mg/kg of bacitracin (58.7 units/mg)[7] and 44 mg/kg of procaine penicillin G (985 units/mg);[8] and the basal diet containing only the antibiotic supplement.

Twenty chicks were selected at random from each group of 40 and weighed individually at 1, 7, 14, 21 and 28 days of age. These chicks were returned to their respective diet and used only for weight gain studies. The remaining 20 chicks in each group were killed at various intervals to obtain samples for bacteriological and chemical analyses.

At 7, 14, 21 and 28 days, 5 chicks from each group were decapitated, and blood samples for uric acid analysis were collected in clean test tubes. Precautions were taken to keep crop content from the blood samples to prevent contamination by uric acid in the diet. The blood samples were allowed to clot at room temperature. After partial contraction, the clots were centrifuged and the serum was removed and refrigerated. Blood was obtained from the brachial vein of chicks over 28 days old.

Two segments of the alimentary tract were sampled, the ileum from the yolk stalk to the ilio-cecal junction (hereafter referred to as small intestine) and the ceca. Five chicks from each group were opened and the respective segments of the tract removed aseptically. The contents were expressed into a sterile petri dish and mixed. One gram of pooled contents was transferred to a rubber-stoppered tube containing 9 ml of sterile tap water and 2 to 3 glass beads. After thorough shaking, decimal dilutions were made in sterile tap water.

Bacitracin and penicillin assays were performed in duplicate on sterile filtrates[9] of 10^{-1} or 10^{-2} dilutions of intestinal contents, by the tube dilution procedure. A sensitive strain of *Staphylococcus aureus* served as the test organism. Dilutions of the filtrates were assayed in a liquid infusion medium,[10] with and without 1% penicillinase.[11] The difference between total antibiotic activity (bacitracin and penicillin) and antibiotic activity after treatment with penicillinase (bacitracin) was considered to be a measure of the amount of penicillin in the filtrate. Samples of the antibiotic-containing diets taken from the feed troughs were also assayed for antibiotic content.

The quantity of uric acid in serum and intestinal contents was determined by the procedure of Bare and Wiseman (2).

Experiment 2. This experiment was conducted to determine the effect of different levels of dietary uric acid on the growth of chicks. Four groups of 40 chicks each were fed the following diets; the basal diet used in experiment 1 (table 1); and the basal diet containing 0.5, 1.0 and 2.0% uric acid, respectively. Weight gain studies were conducted as previously described.

Experiment 3. This experiment was designed to detect any changes in the growth response of the chicks to dietary antibiotics since growth of the basal chicks had declined from the initial experiment. Four groups of 40 chicks each were fed the following diets: the basal diet (table 1); the basal diet plus 11 mg/kg of bacitracin; the basal diet plus 44 mg/kg of procaine penicillin G; and the basal diet supplemented with both antibiotics. Weight gain studies and antibiotic assays of intestinal contents were performed as prevously described. Penicillinase was not used when the intestinal contents from chicks which did not receive both antibiotics were assayed.

[7] Zinc bacitracin, donated by Commercial Solvents Corporation, Terre Haute, Indiana.
[8] Donated by Eli Lilly and Company, Indianapolis, Indiana.
[9] Seitz bacteriological filter, Republic Seitz Filter Corporation, Newark, New Jersey.
[10] Bacto-Brain Heart Infusion, Difco Laboratories Incorporated, Detroit.
[11] Bacto-Penase, Difco Laboratories Incorporated, Detroit.

In all the experiments, statistical analyses were determined on all the data for the experimental period by the analysis of variance and the multiple range test of Duncan (3), with the critical values of Harter (4).

RESULTS

Results of the weight gain studies from experiment 1 are shown in table 2. The chicks fed the basal diet plus 2% uric acid showed a significant weight depression ($P < 0.05$) when compared with the weights of the basal chicks. This weight depression was eliminated ($P < 0.01$) when the antibiotics were added to the uric acid-containing diet. No significant growth response to antibiotics was noted in the chicks fed the bacitracin-penicillin mixture when compared with the basal chicks.

The effect of different amounts of dietary uric acid on the growth of chicks (exp. 2) is shown in table 2. Chicks fed 0.5% uric acid had slightly lower weights when compared with the weights of the basal chicks, but the difference was not statistically significant. Chicks fed 1% uric acid also had lower weights which almost reached significance at the 5% level, whereas the chicks fed 2% uric acid showed a highly significant weight depression ($P < 0.01$).

Growth response studies (exp. 3) are shown in table 2. The penicillin-fed chicks had only slightly greater weights when compared with the weights of the basal chicks. The bacitracin-fed chicks and the chicks fed both antibiotics showed a highly significant weight increase ($P < 0.01$) when compared with the basal chicks. The bacitracin-fed chicks and the chicks fed both antibiotics showed a significant weight increase ($P < 0.05$) when compared with the penicillin-fed chicks.

Bacitracin and penicillin assays of intestinal contents (exp. 1) are shown in table 3. These studies revealed a rapid reduction of penicillin activity in contents from chicks fed diets containing the antibiotic mixture. Statistical analysis of the penicillin levels showed that the decreases that occurred from the first to the second week and the greater amounts in the small intestine compared with the ceca were both highly significant ($P < 0.01$). Little, if any, active penicillin was shown beginning with the third week.

Much higher levels of bacitracin persisted in the intestinal contents during the

TABLE 2

Effect of dietary antibiotics, uric acid and antibiotics-uric acid combination on the growth of chicks

Exp. no.	Dietary treatment	Avg body wt of 20 chicks, weeks			
		1	2	3	4
		g	g	g	g
1	Basal	105	222	414	553
	2% Uric acid	102	216	400	501 [2a]
	2% Uric acid + antibiotics [1]	108	234	432	560 [2b]
	Antibiotics [1]	115	232	438	559
2	Basal	104	219	380	510
	0.5% Uric acid	104	219	374	504
	1.0% Uric acid	102	206	335	483
	2.0% Uric acid	100	203	317	448 [2c]
3	Basal	104	211	388	506
	Penicillin [1]	103	214	395	512
	Bacitracin [1]	108	232	417	556 [2d,f]
	Bacitracin + penicillin [1]	109	230	422	563 [2e,f]

[1] Antibiotic in diet at level of 11 mg of bacitracin or 44 mg of procaine penicillin G, or both, per kg of feed.

[2] Statistical analysis includes all data for the 4-week period.
a. Uric acid vs. basal significant at $P < 0.05$.
b. Uric acid + antibiotics vs. uric acid significant at $P < 0.01$.
c. 2% uric acid vs. basal significant at $P < 0.01$.
d. Bacitracin vs. basal significant at $P < 0.01$.
e. Bacitracin + penicillin vs. basal significant at $P < 0.01$.
f. Significant at $P < 0.05$ when compared with penicillin.

TABLE 3

Levels of antibiotics in the intestinal contents of chicks fed a bacitracin-penicillin mixture, with and without 2% uric acid (exp. 1)

Dietary treatment	Segment of gut [1]	Sampling periods, weeks							
		1	2	3	4	1	2	3	4
		μg penicillin/g wet contents [3]				*μg bacitracin/g wet contents* [4]			
Uric acid + antibiotics [2]	S	9.2	4.2	0	0	34.5	28.5	30.0	24.2
	C	3.2	0	0	0	37.0	31.0	31.7	25.4
Antibiotics	S	10.8	6.6	1.7	0	42.0	36.3	36.2	32.0
	C	4.3	0.8	0	0	53.7	38.5	34.6	32.9

[1] S indicates small intestine; C, ceca.
[2] Antibiotic in diet at level of 11 mg of bacitracin and 44 mg of procaine penicillin G per kg of feed.
[3] Differences between first and second week and between gut segments significant at $P < 0.01$.
[4] Differences significant between treatments ($P < 0.01$) and between first and subsequent weeks ($P < 0.05$).

entire test period but a decrease ($P < 0.05$) of the antibiotic occurred beginning with the second week. Cecal contents from both treatments almost always gave higher levels of bacitracin than samples from the small intestine, but this difference was not statistically significant.

Contents from chicks fed both uric acid and the antibiotic mixture always showed lower levels ($P < 0.01$) of both antibiotics than did the contents from chicks fed only the antibiotics.

Results of antibiotic assays from experiment 3 were similar to those obtained in experiment 1 and are not shown. These also demonstrated rapid penicillin inactivation but persistence of the bacitracin.

Assays of samples of the antibiotic-containing diets taken from the feed troughs showed that the amounts of bacitracin and penicillin remained at their original levels.

Results of serum uric acid assays (exp. 1) are shown in table 4(a). They indicate that the level of uric acid did not appear to be related either to the period of sampling or to the treatment of chicks from which the serum was obtained. No statistically significant differences were noted in the amount of serum uric acid in chicks in any of the treatments or weeks during the test period. The overall range of serum uric acid was 4.7 to 7.2 mg/100 ml.

At the end of 4 weeks, 5 birds from the group fed 2% uric acid (exp. 1) were changed to a diet containing 5% uric acid. Table 4(b) shows that the level of serum uric acid did not rise. Again no significant differences occurred from week to week. But, individual chicks showed highly significant variations in the level of serum uric acid.

TABLE 4

Effect of dietary antibiotics, uric acid and antibiotics-uric acid combination on the amount of uric acid in the serum of chicks (exp. 1)

(a) Average level in pooled serum from 5 chicks determined weekly in quadruplicate through the fourth week of age

Dietary treatment	*mg/100 ml*
Basal	6.2
2% Uric acid	6.2
2% Uric acid + antibiotics [1]	5.6
Antibiotics [1]	5.9

(b) Average level determined weekly in duplicate from the fifth through the ninth week in individual chicks fed 5% uric acid

		Multiple range test		
Chick no.	Mean ($\bar{x}$)	$\bar{x} - 3.9$	$\bar{x} - 4.8$	$\bar{x} - 5.3$
	mg/100 ml			
1	6.3	2.4*** [2]	1.5**	1.0*
2	5.5	1.6**		
3	5.3	1.4**		
4	4.8	0.9*		
5	3.9			

[1] Antibiotics in diet at level of 11 mg of bacitracin and 44 mg of procaine penicillin G per kg of feed.
[2] Asterisks indicate statistical significance:
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

The quantity of uric acid in the intestinal contents (exp. 1) is shown in table 5. Very little, if any, uric acid could be detected in the contents from chicks that were not fed uric acid. The highest uric acid levels were obtained from the contents of chicks fed 2% uric acid. The amount of uric acid in the contents from chicks fed the combination "uric--anti-

TABLE 5

Levels of uric acid in the intestinal contents of chicks fed antibiotics and 2% uric acid (exp. 1)

Dietary treatment	Segment of gut [1]	Sampling periods, weeks			
		1	2	3	4
		mg uric acid/g wet contents			
Basal	S	1.4	0	0.7	0
	C	0	0	2.2	0.8
Uric acid	S	37.6	46.3	42.4	39.6 [2,3]
	C	22.4	34.5	23.7	26.2
Uric acid + antibiotics [4]	S	27.4	21.3	16.5	13.7 [2]
	C	14.6	10.2	8.4	7.8
Antibiotics [4]	S	0	0.8	1.2	0
	C	1.7	0	2.3	0

[1] S indicates small intestine; C, ceca.
[2] Small intestine vs. ceca significant at $P < 0.01$.
[3] Differences due to treatment (uric acid vs. uric acid + antibiotics) highly significant ($P < 0.001$).
[4] Antibiotics in diet at level of 11 mg of bacitracin and 44 mg of procaine penicillin G per kg of feed.

biotic" diet was lower ($P < 0.001$) than in corresponding samples from the chicks fed the "uric" diet. In the chicks fed the "uric" and "uric-antibiotic" diets, the levels of uric acid in the cecal contents were lower ($P < 0.01$) than in the small intestinal contents.

DISCUSSION

The results of this study show that uric acid, when ingested by the chick, may be growth-depressing. The mechanism of the growth-depressing effect of dietary uric acid is not known. Since uric acid is an insoluble waste product and it attained high levels in the intestinal tract when ingested by the chick, this substance may have acted as an irritant and thus interfered with the passage of nutrients through the gut wall. The addition of antibiotics to the uric acid-containing diet apparently brought about a decrease in the amount of uric acid and thus reduced its "irritating" effect. This decrease was probably brought about by the uricolytic activity of the *Aerobacter* spp. Lev et al. (5) stated that antibiotics may stimulate the growth of the host animal by eliminating organisms that produce substances which irritate, thicken, and hence decrease the permeability of the gut, with consequent impairment of the absorption of nutrients. In this study, the irritating substance was fed to the chicks, and the antibiotics enhanced the growth of organisms which removed the irritating substance.

Recently, Lau and Wiseman (6) reported that 10 to 750 μg of uric acid/ml of synthetic medium inhibited the in vitro production of riboflavin by cultures of *Escherichia coli, Paracolobactrum* spp. and *Aerobacter aerogenes* isolated from the intestinal contents of rats and from human feces. They reported that uric acid did not affect the growth of the bacteria. This report suggests a second explanation for the growth-depressing effect of uric acid. The high level of uric acid in the tract may have inhibited the synthesis of vitamins (or other unidentified nutritional factors for the chick (7)) by the microflora without affecting their growth. Although the basal diet used in these studies was designed to exceed the requirements of known vitamins for optimal growth of chicks (8), this explanation would appear to be valid if the altered intestinal flora were not synthesizing sufficient quantities of some unidentified factor(s).

It has been reported that dietary antibiotics often depress the vitamin-utilizing flora, especially the lactobacilli, and stimulate the vitamin-synthesizing flora and, thus, make more of the vitamin available to the host (1, 9). Bacteriological analyses (10)[12] of the contents of the small intestines from the chicks fed the "uric-antibiotic" combination revealed an increase in numbers of uricolytic *Aerobacter*

[12] Bare, L. N., and R. F. Wiseman 1963 Synergistic effect of antibiotics and uric acid upon the intestinal bacteria and weight gains of chicks. Bacteriol. Proc., p. 12 (abstract).

spp. which also have been reported to be good vitamin synthesizers.[13] The counts of latcobacilli were also lowest in this group of chicks. It appears that when the antibiotics were added to the uric acid-containing diet, the increase in numbers of *Aerobacter* spp. not only aided in the removal of uric acid, which may have interfered with vitamin production by other coliforms, but also produced an additional source of vitamins for the chick. In addition, the concurrent reduction in numbers of lactobacilli could have spared vitamins for the host.

Failure to obtain a significant growth response in the chicks that received only the antibiotic supplement in experiment 1 may be attributed to the housing of chicks in quarters that had not been used previously for chickens. That the growth response to antibiotics will not be expressed when chicks are kept in a "new" environment is well established (11–13). The poorer growth occurring subsequently in the basal groups and the response to bacitracin in experiment 3 suggest that an "old" environment may have been established.

Failure to obtain a growth response in the penicillin-fed chicks in experiment 3 may be attributed to the rapid inactivation of the antibiotic. Following the demonstration of penicillinase in *Escherichia coli* by Abraham and Chain (14), substances that inactivate penicillin have been reported in other coliform bacteria (15, 16). Wiseman et al. (17) reported that, since paracolon bacteria are the predominate coliform group in the bovine rumen and the lactose-fermenting bacteria of this group (*Escherichia* and *Aerobacter* spp.) often predominate in the intestines of chickens, the penicillin inactivation which occurs in the rumen may not occur in the intestinal tract of chickens. This suggestion was not supported in this study, since the penicillin activity disappeared rapidly and was not detectable after 4 weeks of feeding the antibiotic.

Since the contents from chicks fed the "uric-antibiotic" combination gave higher numbers of *Aerobacter* spp. and less penicillin activity than those from chicks fed the "antibiotic," it is therefore suggested that the *Aerobacter* spp. were primarily responsible for the penicillin degradation. Sabath and Finland (18) reported that of the gram-negative organisms able to inactivate penicillin G, methicillin, biphenylpenicillin, oxacillin and ampicillin (semisynthetic penicillins), 12 strains of *Klebsiella-Aerobacter* were the most effective inactivators and 11 strains of *Escherichia coli* were the next most active. This inactivation of penicillin may explain the temporary failure of Waibel et al. (19) to obtain a growth response in penicillin-fed chicks, since they reported that a decrease in the growth-promoting effect of antibiotics occurred in an environment continuously occupied by chicks. Subsequent growth studies in that laboratory have shown that growth responses have been recovered (20).

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Effect of Protein Level of Diet on Nitrogen Excretion in Fowls

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ABSTRACT Nitrogen metabolism in the fowl was investigated using White Leghorn cockerels with artificial anuses. When fed protein-free diets the excretion of uric acid and ammonia increased with an increase in nitrogen intake. The excretion of urea and total creatinine was relatively constant. Total nitrogen excretion in the urine of birds fed a protein-free diet was higher than that estimated from the regression equation calculated with the data of varying levels of protein. This high nitrogen excretion is due to the ammonia, urea and creatinine excretion. Since a protein-free diet lowers the pH of urine, the excess of excreted ammonia with this diet is considered to be a regulator of the disturbed acid-base balance. There is a highly significant correlation not only between total urinary nitrogen and the sum of uric acid and ammonia nitrogen, but also between total urinary nitrogen and uric acid nitrogen alone. This suggests that the total urinary nitrogen can be estimated by determining the uric acid nitrogen of the fowl's droppings. Birds receiving diets containing more than 3% of casein gained body weight, whereas birds receiving a protein-free diet lost body weight. When birds, weighing 1.5 to 2.0 kg, were supplied with 2.8 g of casein and 51.5 g of starch-oil mixture (calculated metabolizable energy: 280 kcal), they maintained their body weight, but birds receiving 33 g or less of starch-oil mixture lost body weight.

Because the fowl excretes both urine and feces together from the cloaca, only a limited number of experiments have been conducted on the urine component of the fowl. Katayama (1) studied the urine component of fowls fed various kinds of diets and found a high correlation between total urinary nitrogen and the sum of urinary uric acid and ammonia nitrogen. O'Dell et al. (2) reported that chicks fed purified diets excreted ammonia in larger quantities than those fed practical diets. From these results they suggested that purified diets would not allow maintenance of normal acid-base balance, as do practical diets. In their experiments, however, the effect of protein and energy levels was obscure. The present study deals with the effect of protein and energy levels of diets on the urinary nitrogen excretion in fowls.

MATERIALS AND METHODS

Single Comb White Leghorn cockerels, about 5 months old, and weighing from 1.5 to 2.0 kg, were used. To collect feces and urine separately, an artificial anus was surgically produced in the birds by the method of Ariyoshi and Morimoto (3). Thus, feces and urine could be collected quantitatively in polyethylene bags through funnels attached to both the artificial anus and the natural anus, respectively. The birds were fed a practical stock diet for about 2 weeks after the operation and behaved normally. When the birds had recovered their body weight, they were divided into 9 experimental groups of 3 birds each, and placed in individual metabolism cages. Birds of 5 of the groups received 80 g of the experimental diets once a day (9:30 AM). Semipurified diets, modified from those of Ariyoshi (4) were used as the experimental diets, and their composition is shown in table 1. Casein, fortified with methionine, was used as the sole source of protein. The amounts of vitamins, minerals and calories contained in the diet appeared to be adequate for the maintenance of body weight of the experimental birds. Birds of the 4 other groups received a constant amount of casein (2.8 g/day) but varying amounts of starch-oil mixture (10:1) to regulate energy intake (table 2). After prefeeding the experimental diet for 5 days, it was continued for an additional 3 days during which urine and feces were collected. Dur-

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TABLE 1

Composition of diets with varying protein levels

Casein content	0%	3%	6%	9%	12%
	%	%	%	%	%
Cornstarch	82.8	79.8	76.8	73.8	70.8
Soybean oil	8	8	8	8	8
Salt mixture [1]	5	5	5	5	5
NaCl	0.5	0.5	0.5	0.5	0.5
Al silicate	1	1	1	1	1
Agar-agar	1	1	1	1	1
Cellulose [2]	1.5	1.5	1.5	1.5	1.5
Vitamin premix [3]	0.2	0.2	0.2	0.2	0.2
Casein [4]	0	3	6	9	12

[1] Supplied the following minerals, as a percentage of the mineral mixture: NaCl, 4.68; $MgSO_4$, 7.19; $Na_2HPO_4 \cdot H_2O$, 9.38; K_2HPO_4, 25.80; $CaH_4(PO_4)_2 \cdot H_2O$, 14.60; Ca lactate, 35.15; Fe citrate, 3.11; $MnSO_4$, 0.5.
[2] Wood pulp cellulose powder provided by Sanyo Pulp Company, Ltd., Gozu, Japan.
[3] Contained: (per g of premix) vitamin A, 2,500 IU; thiamine mononitrate, 1 mg; nicotinamide, 10 mg; folic acid, 0.15 mg; vitamin B_{12}, 1 μg; tocopherol, 1 mg; vitamin D_3, 250 ICU; riboflavin, 1.5 mg; pyridoxine·HCl, 1.5 mg; Ca pantothenate, 2.5 mg; ascorbic acid, 37.5 mg; and vitamin K, 0.1 mg in lactose.
[4] The casein was fortified with 0.8% methionine.

TABLE 2

Daily consumption of diets with varying energy levels

	Energy supplied compared with control diet			
	100%	75%	50%	25%
	g	g	g	g
Starch-oil [1]	69.8	51.5	33.3	15.0
Casein [2]	2.8	2.8	2.8	2.8
Salt mixture [3]	4	4	4	4
NaCl	0.4	0.4	0.4	0.4
Al silicate	0.8	0.8	0.8	0.8
Agar-agar	0.8	0.8	0.8	0.8
Cellulose [4]	1.2	1.2	1.2	1.2
Vitamin premix [5]	0.16	0.16	0.16	0.16

[1] Cornstarch, 10 parts, soybean oil, 1 part.
[2] The casein was fortified with 0.8% methionine.
[3] See footnote 1, table 1.
[4] See footnote 2, table 1.
[5] See footnote 3, table 1.

ing the experimental period the birds were allowed water ad libitum, and consumed pelleted diets completely.

Urine and feces were quantitatively collected in polyethylene bags at 9:30 AM and 4:00 PM. A few drops of toluene were placed in the bags for urine collection to prevent putrefaction of the collected urine. Urine samples were frozen in the refrigerator at −20°C until ready for analysis. The feces samples were air-dried at 70°C.

Urine samples were thawed and homogenized with a Waring Blendor (1800 RPM) in the presence of a small quantity of isoamyl alcohol to prevent foaming.

Total nitrogen of both urine and feces was determined by the semimicro-Kjeldahl method. Uric acid nitrogen in the homogenized urine was determined by the iodimetric method of Bose (5) as modified by Baker (6). One milliliter of the homogenized urine was transferred to a 200-ml flask, and appropriate amounts of sodium borate buffer (pH 8.8 to 9.3) and lithium carbonate powder were added. The solution was shaken well and titrated with 0.1 N iodine solution. Conway's method (7) was used to determine urea and ammonia nitrogen. Creatinine and creatine nitrogen were determined photometrically according to the Jaffe reaction (8).

RESULTS AND DISCUSSION

It is generally recognized that the rate of nitrogen metabolism, as well as of energy metabolism, is not directly proportional to simple body weight, but to metabolically effective body weight (metabolic

body size) defined as W^b, in which W represents body weight and the power *b* indicates a constant peculiar to each species. Since Mitchell (9) proposed the power 0.705 for White Leghorn fowls, we used this figure to estimate the metabolic body size in the present experiment. Nitrogen excretion per $W_{kg}^{0.705}$ of metabolic body size is shown in table 3. Excretion of total urinary nitrogen increased as the dietary protein level increased. Urinary components showing an increase were uric acid and ammonia, with the exception of ammonia in the casein-free diet, whereas the excretion of urea and total creatinine was not affected by dietary protein levels.

Some characteristic differences in urine exist between mammals and fowls. According to Folin (10), there are 2 types of end-products of nitrogen metabolism in the human; urea is considered to be exogenous, and uric acid, creatine and creatinine endogenous. From the present experiment, however, the major component of urinary nitrogen compounds is uric acid, and both uric acid and ammonia increase as dietary protein level increases, whereas urea, creatine and creatinine are relatively constant in fowls.

As nitrogen excretion is linearly proportional to the nitrogen intake, endogenous urinary nitrogen may be estimated from the regression equation calculated with the data of increasing increments of protein-containing diets; endogenous nitrogen thus obtained is 175 mg/$W_{kg}^{0.705}$. However, urinary nitrogen observed with the birds fed the protein-free diet in this study (generally recognized as the endogenous nitrogen) is 234 ± 34 mg/$W_{kg}^{0.705}$, and is greater than the estimated one. This high excretion of nitrogen with the protein-free diet was the result of the high excretion of ammonia, urea and total creatinine (table 3).

The pH value of fowl's urine is on the acid side as opposed to that of mammals; Ariyoshi and Morimoto (3) reported that the pH value of fowl's urine is 6.4 ± 0.7 and O'Dell et al. (2) reported values of 5.4 to 5.5. The fact that the urine is acid indicates that the fowl may differ from the mammal in the metabolism of electrolytes. Cations such as Na^+ and NH_4^+ may play a significant role in the control of acid-base balance. According to the authors' unpublished data, urine pH with protein-free diets is more acid than that with protein-containing diets. From these facts, the excess of ammonia excreted is considered to be a regulator of the disturbed acid-base balance, and not a product of the normal nitrogen metabolism. This suggests that it is difficult to express the urinary nitrogen as endogenous nitrogen when a protein-free diet is fed. Ariyoshi (4) reported that the amount of urinary nitrogen with a protein-free diet was almost the same as that with a diet containing 1 g of dried whole egg protein, and he suggested that the endogenous nitrogen might be determined with a low egg-protein diet. However, the urinary nitrogen with a protein-free diet does not indicate the true endogenous nitrogen level but rather a high value. Consequently, it is very difficult to determine the true endogenous nitrogen directly. In the case of estimating the biological value of feed protein by Mitchell's method (11), true endogenous nitrogen must be determined. If a protein-free diet is used for the endog-

TABLE 3

Major urinary nitrogenous compounds (mg N per $W_{kg}^{0.705}$ in 24 hours) and body weight gains at different levels of protein intake

Casein in diet	Total N	Uric acid N	Ammonia N	Urea N	Total creatinine N	(Preformed creatinine)	Undetermined N	Avg body wt gain, 8 days
%								*g*
0	235 ± 34 [1]	138 ± 17	69 ± 3	8 ± 0	16 ± 1	(4 ± 1)	34 ± 4	−12
3	218 ± 21	164 ± 18	31 ± 2	4 ± 1	7 ± 1	(2 ± 0)	9 ± 2	38
6	278 ± 24	214 ± 18	44 ± 5	4 ± 0	7 ± 2	(1 ± 0)	9 ± 2	37
9	395 ± 19	304 ± 17	58 ± 3	6 ± 1	6 ± 0	(1 ± 0)	24 ± 3	58
12	423 ± 16	335 ± 18	63 ± 5	5 ± 1	4 ± 0	(1 ± 0)	19 ± 3	239

[1] Mean ± SE of mean.

enous nitrogen determination, it will give a higher value than the true one. On the other hand, Allison et al. (12–14) proposed the nitrogen balance index, by which the biological value of protein could be estimated without determining the endogenous nitrogen. It appears that Allison's method gives a more reliable biological value for fowls than Mitchell's method.

Because of the exogenous origin, excretion of uric acid increased linearly as nitrogen intake increased. The excretion of urea and total creatinine was almost constant when birds were fed the protein-containing diets. On the other hand, birds fed the protein-free diet excreted urea and total creatinine at higher levels. The high excretion of creatinine appears to be the result of excessive breakdown of body protein. This hypothesis is explained by the fact that the birds fed the protein-free diet lost body weight.

O'Dell et al. (2) reported that, in birds, the preformed creatinine was about 20% of the total creatinine, and the remainder is creatine. The present experiment shows comparable results. Although urea is excreted in the same way as the total creatine, the significance of urea metabolism in fowls is still not clear.

Katayama (1) proposed that urinary nitrogen excretion with diets of various levels of protein could be estimated from the sum of uric acid and ammonia nitrogen. In the present experiment, when birds were fed the diets containing between 3 and 12% casein, Katayama's proposal agrees well, because a correlation coefficient between total urinary nitrogen and the sum of the urinary uric acid and ammonia nitrogen is calculated as + 0.998. The regression equation is $Y = 0.98X + 29.6$, where Y is total urinary nitrogen and X is urinary uric acid plus ammonia nitrogen (fig. 1). Figure 2 also shows that total urinary nitrogen and urinary uric acid nitrogen are highly correlated, showing a correlation coefficient of $r = 0.998$, and a regression equation of $Y = 1.15X + 42.2$, where Y is total urinary nitrogen and X is urinary uric acid nitrogen. This fact suggests that with usual feeding conditions, the total urinary nitrogen might be estimated from the urinary uric acid nitrogen alone, instead of urinary uric acid and ammonia nitrogen. However, since this correlation between the total urinary nitrogen and the urinary uric acid nitrogen was obtained only from the experiment with a casein diet, it is not certain whether it can be applied in the case of other protein diets. Further study will be needed for a discussion of the pertinence of this correlation. Figure 3 indicates the nitrogen balance for each bird, and suggests that a daily intake of 2.8 g of casein (corresponding to 420 mg of ab-

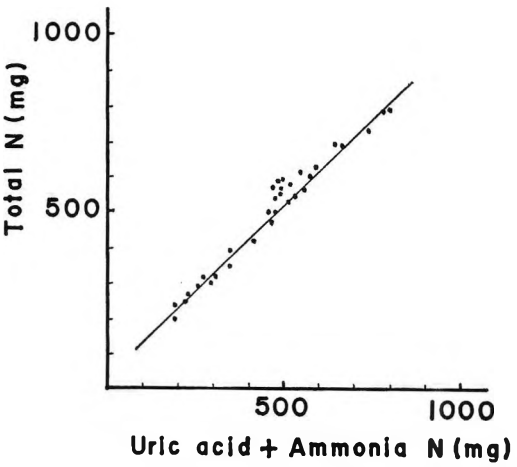

Fig. 1 Correlation between urinary total nitrogen and the sum of uric acid and ammonia nitrogen. Correlation coefficient, $r = + 0.988 \pm 0.011$; regression equation, $Y = 0.98X + 29.6$.

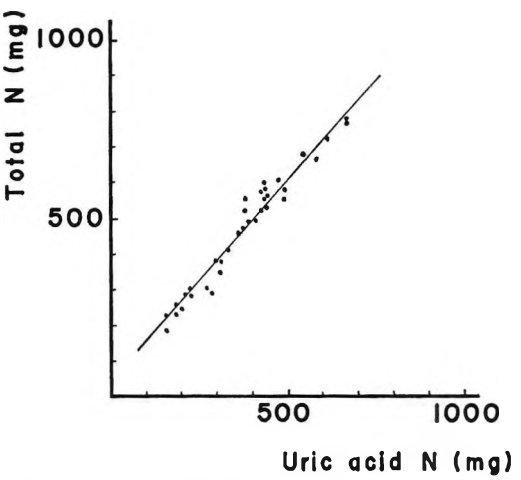

Fig. 2 Correlation between urinary total nitrogen and uric acid nitrogen except for protein-free diet. Correlation coefficient, $r = + 0.998 \pm 0.008$; regression equation, $Y = 1.15X + 42.2$.

TABLE 4

Major urinary nitrogenous compounds (mg N per $W_{kg}^{0.705}$ in 24 hours) and body weight gains with diets of restricted energy intake

Energy supplied compared with control diet	Total N	Uric acid N	Ammonia N	Urea N	Total creatinine N	Avg body wt gain, 8 days
%						g
100	271±29 [1]	178±7	58±6	6±2	9±3	6
75	265±27	185±19	45±5	3±0	9±3	8
50	429±66	318±45	51±5	8±0	15±2	−150
25	1628±95	1183±82	178±7	46±7	51±4	−550

[1] Mean ± SE of mean.

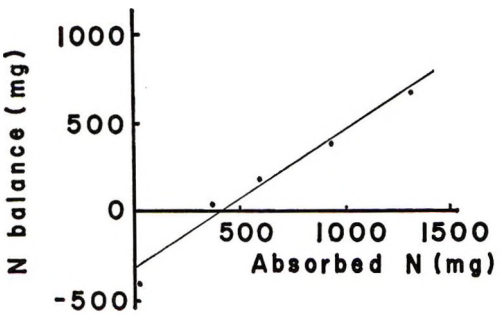

Fig. 3 Nitrogen balance of fowls fed casein diet.

sorbed nitrogen) is sufficient for maintaining nitrogen equilibrium.

Table 4 shows the results of the experiment comparing different amounts of dietary energy. Birds receiving 75% of the energy of the control diet maintained a constant body weight, but the birds receiving 50 and 25% of energy lost body weight at an average of 150 and 550 g/8 days respectively. As shown in table 4, total nitrogen excretion of birds receiving 100% energy and that of birds receiving 75% energy did not differ, but the restricted feeding of 50 and 25% of the energy significantly increased the levels of total urinary nitrogen. Especially the birds on the 25% energy level excreted much larger amounts of nitrogen which included all the components of urinary nitrogen. These data show that sufficient energy could be provided by the diet containing 75% of energy compared with the control diet.

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The Relative Anti-muscular Dystrophy Activity of the *d*- and *l*-Epimers of α-Tocopherol and of Other Tocopherols in the Chick[1]

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ABSTRACT The relative anti-muscular dystrophy activities (AMDA) of the *d*- and *l*-epimers of α-tocopheryl acetate and of β- and γ-tocopherols determined in chick bioassay were found to be 1.46, 0.36, 0.12 and 0.05 units/mg, respectively, as compared with a reference standard *dl*-α-tocopheryl acetate (unofficial ANRC) which was assigned an AMDA value of 1.0 unit/mg. Blood plasma tocopherol analyses showed that prevention of muscular dystrophy was correlated directly with total plasma tocopherol levels and that 900 to 1025 μg tocopherol/100 ml of blood were present when muscular dystrophy was prevented completely.

The biological activity of synthetic *dl*-α-tocopherol has been compared with that of *d*-α-tocopherol and with other natural tocopherols using a wide variety of laboratory animals. The most generally accepted bioassay of vitamin E is based upon prevention of fetal resorption in the female rat as established by Evans and Burr (1) and standardized by Mason (2). Using this method, Joffe and Harris (3) and Harris and Ludwig (4, 5) found the natural *d*-α-tocopherol to be 1.36 times more potent than the synthetic *dl*-α-tocopherol. Based upon this work, the tenth revision of the National Formulary has stated that *d*-α-tocopherol or its acetate ester is 1.36 times more potent than *dl*-α-tocopherol or its acetate ester, respectively. Studies on other vitamin E deficiency diseases such as erythrocyte hemolysis in rats by Friedman et al. (6), muscular dystrophy prevention in rabbits (7), tocopherol levels in human plasma (8), and plasma and liver tocopherol levels in chicks (9), have indicated that *d*-α-tocopherol or its acetate is more potent than *dl*-α-tocopherol or its ester by a factor ranging from 1.22 to 1.47.

Following the successful isolation of the *l*-epimer of natural *d*-α-tocopherol by Robeson and Nelan (10), the biological activity of this *l*-epimer has been compared in rats by the resorption-gestation bioassay with that of natural *d*-α-tocopherol by Ames et al. (11). The activities of the *d*- and l-epimers also were compared for prevention of encephalomalacia in chicks by Søndergaard and Dam (12). Ames has calculated, using the vitamin E activity as measured by the resorption-gestation bioassay of Mason and Harris (13), that the potency of the compound, 2*l*,4′D,8′D-α-tocopheryl acetate is approximately 21.1% that of the synthetic 2*d*,4′D,8′D-α-tocopheryl acetate or of *d*-α-tocopheryl acetate derived from natural sources. Søndergaard and Dam (12) reported that the *l*-epimer had approximately 25% the activity of the *d*-α-tocopheryl acetate in the prevention of encephalomalacia in chicks. Using an *l*-isomer prepared by Isler et al. (14), Brüggemann and associates (15) reported the *l*-isomer to have approximately 50% the activity of *dl*-α-tocopheryl acetate which in turn averaged a little more than half the activity of *d*-α-tocopheryl acetate for prevention of erythrocyte hemolysis in rats. These results, therefore, also indicate that the acetate ester of the *l*-isomer is about 25% as effective as *d*-α-tocopheryl acetate. The comparison of *d*- and *dl*-α-tocopheryl acetates, conducted in the above studies, indicated a relative potency of 1.67 for *d*-α-tocopheryl acetate as compared with the activity of the racemic mixture.

The present report presents results showing the relative biological activities of the

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d-, and *l*-epimers of α-tocopheryl acetate, singly and in racemic mixture, and of 2 forms, β- and γ-tocopherol, for the prevention of muscular dystrophy in vitamin E-deficient chicks.

EXPERIMENTAL

One-day-old White Plymouth Rock × Vantress chicks from vitamin E-depleted hens were used in the study. Each treatment consisted of replicated lots of 10 male and 10 female chicks housed in electrically heated battery brooders with wire-mesh floors. Feed and water were supplied ad libitum. The basal diet used was the casein-isolated soybean protein-torula yeast diet as described by Scott and Calvert (16) with modifications as follows: the diphenyl-*p*-phenylenediamine was omitted and the diet was supplemented with 1.0% L-arginine·HCl, 0.0125% ethoxyquin and 1.0 ppm selenium (as Na selenite). This diet contains sufficient synthetic antioxidant to prevent encephalomalacia and sufficient selenite to prevent exudative diathesis. The basal diet was fed alone and supplemented with *d*-, *l*-, and *dl*-α-tocopheryl acetate,[2] and with β- and γ-tocopherol[3] in graded levels as shown in table 1.

Chicks were killed at the end of the 4-week experimental period and scored for muscular dystrophy by a panel of 3 members working independently. The scoring scale ranged from zero for absence of dystrophy to 4 for maximal dystrophy. The average scores were determined by dividing the total scores for each group by the number of chicks in that group. Pectoral muscles were removed and frozen immediately in liquid nitrogen. Blood samples

[2] The *d*- and *l*-α-tocopheryl acetates were characterized as to purity and kindly supplied by Distillation Products Industries, Rochester, New York. The *dl*-α-tocopheryl acetate was a gelatin-coated product especially prepared for testing as a possible Animal Nutrition Research Council (ANRC) standard.

[3] The β- and γ-tocopherol were the natural forms contained in vacuum-sealed ampules obtained from Merck and Company, Rahway, New Jersey.

TABLE 1

Plasma tocopherol levels resulting from the feeding of various forms of tocopherol and the corresponding prevention of muscular dystrophy in chicks

Dietary tocopherol level	Muscular dystrophy, 4 weeks of age		Plasma tocopherol levels
	Incidence [1]	Score (0–4)	
mg/kg diet	%		*μg/100 ml* [2]
None	100	2.70	161 ± 21
d-α-Tocopheryl acetate			
1.25	100	2.22	264 ± 20
2.50	89	2.19	400 ± 40
5.00	42	0.37	589 ± 20
10.00	0	0.0	902 ± 45
dl-α-Tocopheryl acetate			
2.50	90	2.18	364 ± 18
5.00	80	1.55	486 ± 28
7.50	42	0.61	589 ± 58
10.00	22	0.47	802 ± 41
12.50	0	0.0	1034 ± 45
l-α-Tocopheryl acetate			
5.00	100	2.34	298 ± 29
10.00	94	1.91	377 ± 37
20.00	35	0.82	577 ± 27
40.00	0	0.0	1025 ± 83
β-Tocopherol			
10.00	95	2.55	430 ± 45
40.00	80	1.44	555 ± 42
γ-Tocopherol			
10.00	100	2.70	307 ± 17
40.00	89	2.13	477 ± 47

[1] Chicks showing muscular dystrophy as percentage of the total number alive at end of experiment. All lots contained 20 chicks at start.

[2] SD.

were taken from the chicks by heart puncture just prior to killing. The syringe and test tubes used for handling blood were rinsed with heparin and all precautions were taken to keep the blood cold and free from hemolysis. The heparinized blood was centrifuged immediately at 2500 rpm for 10 minutes and the plasma was carefully pipetted off and frozen immediately for subsequent tocopherol analysis. Total plasma tocopherols were determined by the method of Quaife and Harris (17).

RESULTS

All chicks consumed the diets about equally and live weights were approximately equal for all lots at 4 weeks of age.

Bioactivity curves for the *d*-, and *l*-epimers of α-tocopheryl acetate, for the racemic mixture, and for β- and γ-tocopherol, showing their relative activities in the prevention of muscular dystrophy in chicks, are presented in figure 1. The logs of the average muscular dystrophy scores are plotted against the logs of the levels of the various tocopherols added to the diet. The activity of each of the tocopherols followed a definite pattern which showed, at each dietary level, the distinct relative potency of each of the tocopherols tested. The sequence of biological effectiveness was as follows: *d*-α-tocopheryl acetate, *dl*-α-tocopheryl acetate, *l*-α-tocopheryl acetate, β-tocopherol and γ-tocopherol, respectively.

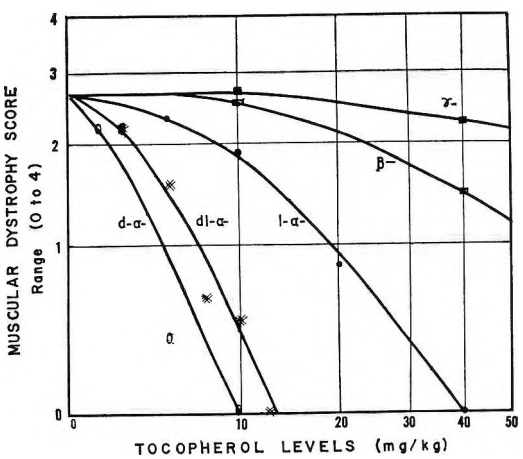

Fig. 1 Anti-muscular dystrophy activity curves of *d*-, *dl*- and *l*-α-tocopheryl acetate and β- and γ-tocopherol in chick bioassays.

The total plasma tocopherol levels of chicks receiving graded dietary amounts of each of the tocopherols, the incidence of muscular dystrophy, and the severity scores obtained, are presented in table 1. These results demonstrate a direct correlation between the effectiveness of these tocopherols and the total tocopherol levels in the plasma. With the increase in dietary amounts of the effective tocopherols, proportionate increases were observed in the total plasma tocopherol levels up to concentrations of 900 to 1025 μg total tocopherols per 100 ml of plasma. In each instance, when total tocopherols reached this level, muscular dystrophy was completely prevented. The differences in the activities of these tocopherols, therefore, can be explained on the basis of their relative blood tocopherol patterns. Thus *d*-α-tocopheryl acetate was more active, requiring only 10 mg/kg diet to reach complete protection and a plasma tocopherol level of 902 μg/100 ml of plasma. On the other hand, 40 mg *l*-α-tocopheryl acetate/kg of diet were required to prevent dystrophy and to increase plasma tocopherol to 1025 μg/100 ml. The β- and γ-tocopherol did not show full effectiveness even at the level of 40 mg/kg of diet. This was apparently because of their poor absorption, since this high dietary level of these tocopherols brought plasma tocopherol levels to only 555 and 477 μg/100 ml, respectively.

The calculated anti-muscular dystrophy activities (AMDA) of the various tocopherols, as related to the *dl*-α-tocopheryl acetate (ANRC gelatin-coated granules) used as reference standard, are presented in table 2. These values represent the ratio of the amount of dietary *dl*-α-tocopheryl acetate required to the relative amounts of the other forms of tocopherols required for prevention of muscular dystrophy in chicks. These ratios were calculated from the bioactivity curves represented in figure 1. Assigning a value of 1.0 AMDA units/mg for the activity of *dl*-α-tocopheryl acetate, the average relative values for *d*-α-tocopheryl acetate, *l*-α-tocopheryl acetate, β-tocopherol and γ-tocopherol were found to be 1.46, 0.36, 0.12 and 0.05 AMDA units/mg, respectively.

TABLE 2

Relative anti-muscular dystrophy activity (AMDA) of various forms of tocopherol in chicks

Muscular dystrophy score (Range 0–4)	AMDA units per milligram relative to *dl*-α-tocopheryl acetate			
	d-α-Tocopheryl acetate	*l*-α-Tocopheryl acetate	β-Tocopherol	γ-Tocopherol
0.0	1.350	0.338		
0.25	1.375	0.355		
0.50	1.424	0.352		
0.75	1.455	0.356	0.104	
1.00	1.473	0.368	0.117	
1.50	1.538	0.368	0.132	
2.00	1.600	0.376	0.139	0.046
2.50	1.500	0.369	0.109	0.054
Average	1.464	0.360	0.120	0.050

[1] The anti-muscular dystrophy activity (AMDA) of *dl*-α-tocopheryl acetate was assigned a value of 1.0 unit/mg. Ratios for other tocopherols were calculated from the bio-activity curves presented in figure 1.

DISCUSSION

The biological activities of various forms of tocopherol have been assayed previously for their relative effectiveness in the prevention of muscular dystrophy in rabbits (7), using creatinuria as the bioassay test, and in rats (18) using incidence of dystrophic lesions in the thigh musculature. However, no previous studies have been reported on the relative biological activities of the 2 epimers (*d*-α- and *l*-α-) or other forms of tocopherol for prevention of muscular dystrophy in chicks.

The results of the present experiments show that the inverted, unnatural epimer, *l*-α-tocopheryl acetate, possesses approximately 25% of the activity of natural *d*-α-tocopheryl acetate for prevention of muscular dystrophy. The results obtained with the racemic mixture indicate, however, a possible synergistic effect of the 2 epimers in the prevention of dystrophy in the chick, since the calculated relative potency of the *dl*-mixture should be 62.5% that of pure *d*-α-tocopheryl acetate, whereas the actual average potency in these experiments for the *dl*-α-tocopheryl acetate was 68.5% that of the pure *d*-epimer. These results, thus, are in general agreement with the observation of Ames et al. (11), Søndergaard and Dam (12), and Brüggemann et al. (15) concerning the relative potencies of the *d*-α- and *l*-α-epimers and yet they are also in accord with the earlier results of Harris and Ludwig (4, 5), Friedman et al. (6), Pudelkiewicz (9), and others indicating that *d*-α-tocopheryl acetate is superior to *dl*-α-tocopheryl acetate by a factor of 1.46, which is midway between the previously determined factors which ranged from 1.22 to 1.67.

The observed variation in the AMDA of these tocopherols may be related to differences in 1) the degree of absorption across the mucosa of the intestinal tract; 2) rate of excretion; 3) rate of destruction before or after absorption; 4) the affinity of the various tocopherols for carriers for their active transport to the proper loci or for the specific tissues requiring tocopherol, or both; or to true differences in chemical activity as influenced by structural configuration. The results presented in table 1 demonstrate that the prevention of muscular dystrophy in the chicks was directly correlated with the plasma tocopherol concentrations with all forms of tocopherol administered. Since the plasma tocopherol level must reflect the net difference between rate of absorption and rate of excretion of these tocopherols, it appears that the biological value or the AMDA of the various tocopherols is largely dependent upon one or both of the first 2 possibilities listed above. It is possible that differences in affinities for active sites may be responsible for the observed differences in retention of the various forms and also for differences in their functioning at cellular and subcellular active loci in the tissues of other parts of the body. Recently Weber et al. (19) indicated a synergistic relationship when *d*- and *l*-α-tocopherol were administered together, which they attributed to an increased absorption of the racemic

mixture as compared with the absorption of the separately administered epimers.

The AMDA values for β- and γ-tocopherol as compared with standard *dl*-α-tocopheryl acetate were 0.120 and 0.050, respectively. According to these results, β- and γ-tocopherol are only 12 and 5% as active, respectively, as *dl*-α-tocopheryl acetate for prevention of muscular dystrophy in chicks. These values are somewhat lower than those obtained by liver storage bioassay in growing chicks by Griffiths (20) where the relative values of α-, β- and γ-tocopherol were 100, 41 and 19, respectively. These differences may indicate that the liver levels of tocopherols are not indicative of their relative biological effectiveness. Although the basal diet contained ethoxyquin, some loss of β- and γ-tocopherol could have occurred since the acetate forms of the tocopherols may be somewhat more stable than the free alcohol forms.

Early work by Dam et al. (21) showed β-tocopherol to be more effective for rats than for chicks. This indicates that there may be inter-species differences in retention of the tocopherols, but the results of the present studies and those of Søndergaard and Dam (12) with chicks, compared with the results of Ames et al. (11) and of Brüggemann et al. (15) with rats indicate that the relative potency of the *d*- and *l*-epimers and of *dl*-α-tocopheryl acetate are similar in chicks and rats.

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Mineral Utilization in the Rat

II. RESTORATION OF NORMAL TISSUE LEVELS OF MAGNESIUM AND CALCIUM FOLLOWING MAGNESIUM DEFICIENCY

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ABSTRACT One hundred and ten weanling male albino rats were used in a 7-week feeding trial to investigate the rate of restoration of blood serum and bone ash Mg and to study during a repletion period the fate of Ca deposited in the kidneys consequent to a Mg deficiency. After a 4-week depletion, blood serum Mg increased to normal values within 7 days of repletion, bone ash Mg values increased significantly only during the second week of repletion, and kidney calcium values, elevated as a result of the deficiency, were not significantly decreased during the 3-week recovery period. The data also show the Mg requirement to be higher for rats fed the isolated soy protein, lactose diet employed in this test than for rats fed a casein glucose diet in a previous study.

Calcification of kidney and depletion of Mg levels in bone and blood serum of rats fed a Mg-deficient diet have been reported by many investigators since the early demonstrations by Greenberg et al. (1) and Orent et al. (2). However, there is little information about the reversal of these changes in Mg-deficient rats subsequently fed adequate diets. Duckworth and Godden (3) reported that restoration of normal concentration of Mg in bone ash of depleted rats proceeded more slowly during a 10-day repletion study than depletion of bone Mg of rats fed a Mg-deficient diet. McAleese et al. (4) reported that Mg^{28} was more slowly incorporated in the bone shank of depleted lambs than in that of normal lambs. It has been suggested that the slow repletion is a result of Ca ions occupying sites on bone crystal lattice that have been vacated by Mg and hence these sites are no longer available (4, 5). Sanderson (6) observed that the calcification produced in rat kidneys by the feeding of a diet high in Ca and P was not reversible in a 3.5-week recovery period. The calcifying diet he used contained about 2% Ca, 2.5% P and 450 ppm Mg; hence it is not certain whether the calcification was a result of the high Ca and P concentration per se or whether it arose from a relative deficiency of Mg. It has also been reported (7) that the kidney calcification produced by feeding a trace-mineralized milk diet to rats for 12 months is not reversed by 4 months' feeding of a stock diet.

The experiment reported here was designed to investigate the rate of restoration of blood serum and bone Mg to normal levels following a depletion period and to study the fate of Ca deposited in the kidneys of Mg-deficient rats given a variety of recovery treatments.

METHODS

One hundred and ten weanling male albino rats of the Sprague-Dawley strain were individually caged and fed the experimental diets and distilled water ad libitum. Feed intake was measured and animals were weighed at weekly intervals. The basal diet used was selected on the basis of our own unpublished observations that Mg-deficient diets containing isolated soy protein and lactose produced greater kidney calcification than did diets containing a similar mineral content but with casein and glucose replacing soy protein and lactose.

The ingredients of the basal diet are listed in table 1. This diet was supplemented with $MgCO_3$ or $NaH_2PO_4 \cdot H_2O$, or both, at the expense of glucose to provide 4 diets with mineral levels as shown in table 2. Diet A was adequate in Ca and P but was designed to contain insufficient Mg for maintenance of normal concentra-

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tion of Mg in bone ash or blood serum or of normal kidney Ca concentration. Diet B was formulated to provide an amount of Mg adequate to meet the requirement of the rat. Diets C and D were designed to test the effect of increased Mg intake on the increased kidney calcification resulting from raising the P level of the otherwise adequate diet.

Thirty-five rats were fed diets A and C, and 20 rats were fed diets B and D for a 4-week period. At the end of this time 5 animals from each group were killed, and one-half of the remaining rats fed diets A and C were subsequently fed diets B and D, respectively. All other animals continued to be fed their original diets. Thereafter, at weekly intervals, 5 animals from each treatment combination were killed. Blood samples, kidneys and femurs were obtained from all rats and were subsequently analyzed for Ca and Mg by the method of Malmstadt and Hadjiioannou (9) following removal of P and interfering cations by the ion exchange procedure of Carlson and Johnson (10).

TABLE 1

Ingredients of basal diet

	%
Isolated soy protein [1]	15.0
DL-Methionine	0.4
Corn oil	10.0
Cellulose [2]	3.0
Vitamin mixture [3]	5.0
Vitamins A and D concentrate [4]	0.5
Salt mixture [5]	2.32
Lactose	25.0
Glucose	38.78

[1] Archer-Daniels-Midland Company, Minneapolis.
[2] Solka-Floc, Brown Company, Chicago.
[3] Forbes and Yohe (8), but without antibiotic.
[4] Forbes and Yohe (8).
[5] Contained: (in per cent) NaCl, 15.95; K_2CO_3, 21.58; $CaHPO_4$, 58.65; $FeSO_4 \cdot 7H_2O$, 1.72; $MnSO_4 \cdot H_2O$, 0.52; $CuSO_4 \cdot 5H_2O$, 0.25; KI, 0.015; $ZnCO_3$, 0.085, $MgCO_3$, 1.23.

TABLE 2

Mineral analyses of diets

Diet	Ca	P	Mg
	%	%	*ppm*
A	0.41	0.48	120
B	0.40	0.48	430
C	0.41	0.92	420
D	0.40	0.92	720

During the first 4 weeks the average daily gain of rats in group A was 3.36 ± 0.13 (SE) g and was significantly ($P < 0.01$) less than the gains of the other groups: 4.57 ± 0.16, 4.05 ± 0.09, and 4.32 ± 0.17 for B, C and D, respectively. Rats receiving diet C also gained less than those receiving diets B or D. Eight of the 35 rats in group A exhibited the erythema typical of Mg deficiency, starting at about 2 weeks. During the repletion period, rats shifted from diet A to diet B showed an increase in weight gain not shown by animals that continued to be fed diet A.

The concentration of blood serum Ca averaged 10.5 mg/100 ml and was not affected by treatment or time on experiment. The blood serum Mg data are shown in table 3. At the end of the depletion period of 4 weeks the blood serum Mg values for group A animals were definitely in the subnormal range. Analysis of variance conducted on the data obtained during weeks 5, 6 and 7 show that dietary P did not affect the level of serum Mg but that dietary Mg did so. The data show that shifting animals from a lower dietary level of Mg to a higher one resulted in an increase in serum Mg within 7 days to a

TABLE 3

Mg in blood serum

Group	Week				Mean, 5,6,7 [1]
	4	5	6	7	
	mg/100 ml	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*
A	1.23	1.35	1.21	0.99	1.18^{a}
A–B		2.22	2.10	2.08	2.13^{cd}
B	2.45	1.93	1.94	1.98	1.95^{bc}
C	1.84	2.17	1.61	1.95	1.91^{b}
C–D		2.59	2.26	2.22	2.36^{e}
D	2.23	2.40	2.03	2.24	2.22^{de}

[1] Duncan's multiple range test (15). Means not bearing the same superscript are different at the 1% level.

TABLE 4

Dry weight of kidneys (% of body weight) and Ca (%) in kidney dry matter

Group	Week								Avg of 5,6,7 [1]	
	4		5		6		7			
	Wt	Ca	Wt	Ca	Wt	Ca	Wt	Ca	Wt	Ca
A	0.485	4.49	0.370	3.03	0.310	1.57	0.352	2.97	0.344	2.52[c]
A–B			0.345	2.98	0.251	1.68	0.248	1.51	0.281	2.06[bc]
B	0.234	0.17	0.221	0.30	0.214	0.69	0.213	0.88	0.216	0.62[a]
C	0.279	1.03	0.272	1.28	0.335	1.85	0.278	2.00	0.295	1.71[b]
C–D			0.329	1.91	0.250	1.24	0.246	1.48	0.275	1.55[b]
D	0.278	0.79	0.295	2.58	0.253	1.38	0.225	0.53	0.258	1.50[b]

[1] Duncan's multiple range test (15). Means with the same superscript are not statistically different at the 1% level.

level that was not increased during 14 subsequent days.

In table 4 are shown data concerning deposition of Ca in kidneys. That a treatment effect exists was verified by analysis of variance which showed an effect of treatment on the percentage of Ca in the dried kidney ($P < 0.01$). The calcification consequent to a Mg deficiency (group A) was clearly prevented by Mg supplementation (group B). Inclusion of extra P in the diet increased calcification in the kidney, an abnormality not prevented by the addition of Mg to the higher P ration (groups C and D). The case for removal of Ca from affected kidneys is not so clear because of the large variation between periods within treatments, but it is clear that at the end of a 3-week recovery period a large part of the excess Ca remains in the kidney. Calculations show a high degree of correlation between the percentage of Ca in the dry kidney and kidney dry weight expressed as a percentage of body weight, the correlation coefficient being 0.92.

The data relative to Mg concentration in the femur ash are shown in table 5. The concentration of Mg in femur ash was definitely lowest in animals fed the basal diet (group A). During the recovery period the concentration of Mg in the ash did not increase until the second week. The addition of P to the normal Mg diet (group C) did not affect femur ash Mg values.

The data presented in figure 1 were assembled from this experiment and from a previous one [1] in which weanling rats were killed at weekly intervals during the development of Mg deficiency while receiving a casein-glucose diet containing 80 ppm Mg, 0.4% Ca and 0.4% P. The abrupt and parallel decrease of blood serum and bone Mg values as the deficiency progressed are in contrast with the abrupt increase in serum Mg and the delayed increase in bone Mg values of animals receiving 400 ppm Mg in this experiment.

TABLE 5

Mg in femur ash

Group	Week				Mean of 5,6,7 [1]
	4	5	6	7	
	%	%	%	%	%
A	0.33	0.16	0.23	0.37	0.25[a]
A–B		0.21	0.56	0.54	0.44[b]
B	0.60	0.57	0.47	0.66	0.57[bc]
C	0.58	0.48	0.55	0.48	0.51[bc]
CD		0.63	0.65	0.50	0.59[bc]
D	0.74	0.62	0.63	0.75	0.67[c]

[1] Duncan's multiple range test (15). Means with the same superscript are not statistically different at the 1% level.

DISCUSSION

The development of frank symptoms of Mg deficiency in animals fed the basal diet containing 120 ppm Mg is at variance with our earlier observation (11) which indicated that maximal weight gain and freedom from erythema could be attained with diets containing 110 ppm Mg with Ca and P at levels similar to those employed here. The present diets, however, contained isolated soy protein and lactose, whereas those used previously contained casein but not lactose. We have previously reported (12) that inclusion of lactose in an isolated soy protein diet decreased re-

[1] Unpublished data, D. M. McAleese and R. M. Forbes, 1961.

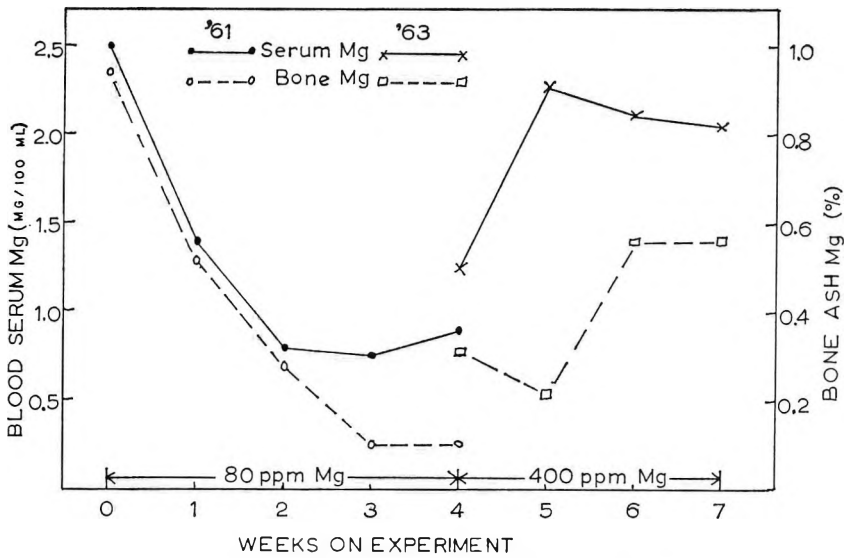

Fig. 1 Change in blood serum and bone ash Mg values during development of Mg deficiency and during recovery therefrom.

tention of Mg by increasing urinary excretion. The apparently higher requirement for Mg observed for rats fed lactose-containing diets is in harmony with our earlier observations.

As previously indicated, the rats exhibited a large amount of individuality in their response to dietary treatment with respect to calcification of the kidneys. The high degree of correlation between kidney size and Ca content indicates this variation to be inherent in the animals and not a result of analytical difficulties. The Mg content of the kidneys was relatively constant, varying threefold, whereas the Ca content varied 25-fold between treatments. The highly calcified kidneys could be detected at the time of autopsy, since they were obviously enlarged, pale, often had pitted surfaces, and the Ca deposits could be detected by scraping a knife across a cut surface.

The data obtained in this experiment clearly show that Mg-deficient rats fed a Mg-adequate diet for 3 weeks still retain a major portion of the Ca deposited in their kidneys during the development of the deficiency. It is possible that the rate of decalcification might be enhanced by further manipulation of the diet to produce a renal tubular acidosis, namely, by use of NH_4Cl. On the other hand, it has been reported (11) that Mg-deficient rats do not respond normally to an ammonium chloride load. An impaired ability to conserve P passing through the kidney (13) and dramatic changes in the structure of the kidney tubule and in the enzyme levels in kidney tissue have been reported.[2] Hess et al. (14) have demonstrated that calcification is initiated in kidney tubular cells undergoing necrosis as a consequence of Mg deficiency. Recovery of normal kidney function may proceed independently of removal of Ca deposits, but this has not been studied.

The delay in restoration of bone Mg (fig. 1) is in agreement with previous observations indicating a slow replacement of Mg in deficient bone during the first 10 days of repletion (3) or soon after administration of Mg^{28} to deficient animals (4). The present data not only confirm these observations but also indicate that after a lag period the restoration of bone Mg can proceed at nearly as rapid a pace as does depletion. Although it may be assumed that the increased concentration of Mg in the bone represents a combined

[2] Linder, A., T. Kutkam, W. O. Smith and D. Baxter 1962 Changes in enzymes and RNA in kidneys of magnesium deficient rats. Federation Proc., 21: 310 (abstract).

effect of repletion of old bone and formation of new bone of normal Mg content, it would be difficult to differentiate these factors quantitatively in this type of experimentation.

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Energy Utilization by Sheep as Influenced by the Physical Form, Composition and Level of Intake of Diet[1,2]

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ABSTRACT The efficiency of utilization of the energy of 3 diets (chopped hay; pelleted, ground hay; pelleted mixture of 55% of ground hay and 45% of corn meal) was studied in metabolism and slaughter-chemical analysis experiments with 63 sheep. The hay used in the 3 diets was prepared from the same source. Each diet was fed at 3 levels during a 196-day period. Sheep of 2 ages were used; at the beginning of the feeding period, 18 were 8 months old and 27 were 20 months old. Nine animals of each age group were killed and analyzed to obtain reference body composition and energy measurements at the beginning of the feeding period. The gain in body energy represented the following percentages of the gross energy and metabolizable energy, respectively, ingested above the maintenance level: chopped hay, 15.1 and 31.0; pelleted, ground hay, 20.8 and 43.3; and pelleted corn-ground hay, 31.3 and 56.6. Although the degree of energy absorption was about the same for the 2 forms of hay, the heat increment for body gain and wool growth was 21.7% greater for the chopped hay than for the pelleted, finely ground hay. Sheep fed ad libitum ingested 28.4% more dry matter and 63.4% more of net energy for body gain as pelleted, ground hay than as chopped hay. The greater energy retention by sheep ingesting the pelleted, ground hay than by those fed chopped hay was 78.5% attributable to the greater intake of dry matter and 21.5% attributable to the greater nutritive effect per unit of feed ingested. No difference in the proportion of energy absorbed or in the net utilization of metabolizable energy was observed between the lambs and older wethers. The lambs were 65% more efficient converters of feed weight to body weight, but the energy concentration of the body gain was 56% greater in the older sheep.

During the last 8 years much attention has been given to the influence of the physical form of the diet upon the responses of ruminants. This interest resulted from the observation that the rate of body weight gain by growing-fattening ruminants fed ad libitum pelleted, mixed diets or pelleted, finely ground hay diets is invariably greater than that of similar animals ingesting unpelleted mixed diets of the same composition or the same hays in chopped or long form, respectively (1–14). These investigations also demonstrated that the ad libitum intake of pelleted diets is greater than that of the same diet in unpelleted form.

Despite the results of many feeding trials, it has not been resolved whether the increased rate of body weight gain is attributable solely to the greater intake or partly to an increased nutritive value per unit weight of pelleted diets. In experiments in which diets in the pelleted and unpelleted form were fed at the same levels of intake to sheep, Esplin et al. (5) and Meyer et al. (9) observed no difference in the rate of weight gain or in the gain per unit of feed ingested. On the other hand, Wright et al. (15) reported that the rate of gain and efficiency of feed use for body gain are higher in sheep fed hay chopped through a 19.1-mm screen, reground through a 6.4-mm screen and pelleted than in sheep fed the same amount of unpelleted hay chopped through a 19.1-mm screen.

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[2] The data presented here are a part of those presented in the Ph.D. Thesis by O. L. Paladines to the Graduate School, Cornell University, 1963.

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The purpose of the present experiment was to examine by means of the balance-trial and slaughter-chemical analysis methods the utilization of energy by sheep of 2 ages ingesting pelleted and unpelleted diets.

EXPERIMENTAL PROCEDURE

Animals. Sixty-three wethers were used in this experiment. These consisted of 36 western wethers of Rambouillet-Columbia origin about 20 months old (referred to as "older" animals) at the beginning of the experimental feeding period and 27 Suffolk-Shropshire-Hampshire cross-breds 8 months old (referred to as "lambs"). During the 4-month period prior to the experiment, a timothy-hay ration was fed at levels intended to improve the uniformity of body composition. During the last 2 months preceding the experimental feeding period, the ration was fed at about the maintenance level and consisted of long hay from the same source as that fed during the experimental period.

Just prior to the beginning of the experimental feeding period, 9 animals selected at random from each age group were slaughtered and analyzed. Each of the remaining 27, 20-month and 18, 8-month-old sheep was allotted to one of the dietary treatments.

Design and dietary treatments. The experiment was of the factorial design involving 2 ages of sheep × 3 kinds of diet × 3 levels of feed intake. Thus, 3 older wethers and 2 lambs constituted each of the diet groups (9 intake levels).

The 3 diets were composed of: chopped hay; pelleted, finely ground (1.6-mm sieve) hay from the same harvest; and a pelleted mixture of 55% of the same finely ground hay and 45% of corn meal. The proximate chemical composition and gross energy (GE) values of the 3 diets are shown in table 1.

TABLE 1

Chemical composition and gross energy values of diets

	Chopped hay	Pelleted hay	Pelleted corn-hay
Energy, kcal/g	4.531	4.559	4.555
Protein, %	12.4	13.7	12.3
Ether extract, %	2.1	2.9	3.6
Carbohydrates, %	79.2	76.9	79.7
Ash, %	6.3	6.5	4.4

Each diet was fed at 3 levels designated as follows: low (slightly above maintenance); medium (intermediate between low and high); and high (ad libitum). The chopped and pelleted hay diets were fed in equal amounts of dry matter per unit of metabolic size (body weight$^{0.734}_{kg}$) at each of the low and medium levels of intake. Table 2 summarizes the mean daily intakes of dry matter, GE and protein per unit of metabolic size. Water, salt with trace elements, and dicalcium phosphate were available to the animals at all times.

General mechanics of experimentation. The feeding treatments were imposed for 196 days at the end of which the 45 sheep were slaughtered and the chemical com-

TABLE 2

Mean daily intakes of dry matter, gross energy and protein during feeding period

Level of intake	Intake of matter and energy [1]	Diet		
		Chopped hay	Pelleted hay	Pelleted corn-hay
Low	Dry matter, g	48.1	46.9	41.3
	Gross energy, kcal	218	214	188
	Protein, g	6.0	6.4	5.1
Medium	Dry matter, g	57.4	56.4	53.1
	Gross energy, kcal	260	258	242
	Protein, g	7.1	7.7	6.6
High	Dry matter, g	68.0	87.3	76.9
	Gross energy, kcal	308	398	350
	Protein, g	8.6	12.0	9.6

[1] Mean daily intakes/kilogram of body weight$^{0.734}$.

position and energy value of their bodies were determined. At this time the older wethers were about 27 months old and the lambs were 15 months old.

For convenient mechanics, the animals were allotted to a given diet in groups of three on the same day that one sheep from the group in the same condition was slaughtered for a measurement of "initial" body composition. The beginning dates were arranged so that one group of 3 animals was begun on each of 3 consecutive days with 2 days intervening between the allotment of the next 3 groups on 3 consecutive days. Thus, 23 days were required for the allotment of the animals. The animals were slaughtered after the 196-day feeding period in the same order that they were begun. Just prior to allotment to the experiment and just before slaughtering, all animals were closely shorn. The wool produced during the feeding period was measured and its content of protein, ether extract and ash and its energy value were determined.

Each animal was housed in a metabolism unit with a floor area of one square meter, permitting considerable freedom of movement, the separate collection of feces and urine, and quantitative, individual feeding. The dry-matter content of the feeds offered and refused was determined daily throughout the 196-day period. Aliquots of both were analyzed for moisture, nitrogen, ether extract, ash and heat of combustion.

The performance of both of the lambs of the high-intake, pelleted corn-hay group was unsatisfactory. One lamb developed masculine characteristics such as horns, a heavy neck and aggressiveness. Upon slaughter an active testicle was found in the abdominal cavity. It was suspected that the energy expense for maintenance was greater in this lamb than in its mates. The other lamb in this group had entered the experiment 67 days later than its replicate mates as a replacement for an animal that died. Thus, this animal completed the 196-day feeding period 67 days later than its mates. This required exposure to the high temperatures of July, August, and early September to which the animal's mates had not been exposed. Although the losses of energy in the feces, urine and methane were comparable to those of the older sheep, the utilization of absorbed energy was lower. As a consequence, the data obtained for the 2 lambs were excluded from considerations involving the estimation of the energy requirements of maintenance and the utilization of energy ingested above the maintenance level of intake.

Metabolism trials. Metabolism trials were conducted with each animal at 2 intervals during the 196-day period. The collection of feces and urine for the first trial was begun at the 40th day of the feeding period. In the second trial, excreta collections were made during the last 10 days of the feeding period. During the 7-day period preceding the collection period and during the collection periods, the daily allowance of feed was constant for each animal. Aliquots of diets and feces were analyzed for moisture, nitrogen and heat of combustion as described previously (16).

Slaughter and body composition analyses. The methods of slaughtering, sampling of body parts, preparation of samples and the analysis of body samples are described in previous reports (17, 18). Wool was analyzed for protein, ether extract, ash and heat of combustion separately from the remainder of the body (19).

Estimation of gain in body energy. The gain in body energy during the feeding period was computed as the difference between the energy values of the body at the beginning and end of the experiment. The final energy value of the body of each animal was determined by direct analysis at the end of the feeding period. Initial energy values were estimated from the direct analysis of reference animals slaughtered at the beginning of the experimental feeding period.

The conventional method of determining the initial energy value of the body is to average the analyses made of the reference animals, or to employ the energy value of one (or, at most, a few) animal as the initial energy value of one replicate of animals of similar history, size, and visually estimated condition.

In the present experiment the mean energy value of the ingesta-free body of the 9 reference lambs was 2,093 $\pm$ 298

TABLE 3

Relationship between energy value and weight of ingesta-free body of the reference sheep

Age group	Regression equations [1]	SE of estimate	Coefficient of variation [2]
months		*kcal*	*%*
8	Y = 4,032 X − 33,935	4,557	12.3
20	Y = 4,929 X − 70,395	14,178	14.2

[1] Y = energy value (kcal) of ingesta-free body, and X = weight (kg) of ingesta-free body.
[2] Coefficient of variation between predicted and measured energy values. Mean energy values: 8-month-old lambs, 37.14 megcal; 20-month-old sheep, 100.04 megcal.

kcal/kg and of the 9 older, reference sheep was 2,872 ± 421 kcal/kg. The mean energy values of the ingesta-free bodies of the sheep of the 2 reference groups were: lambs, 37.14 megcal, and the 20-month-old sheep, 100.04 megcal. The standard deviations of the means were 7.61 and 19.30 megcal, respectively.

Because of the magnitude of the variation, the energy value of the body was regressed on the weight of the ingesta-free body. The statistics, concerned with this relationship are summarized in table 3. This method was used to derive the initial body energy values because it reduced the coefficient of variation from 20.5 to 12.3% and from 19.3 to 14.2% for the 8- and 20-month-old sheep, respectively. Nevertheless, the error is still appreciable. As a consequence of such errors, the feeding treatments imposed in most slaughter-analysis experiments must produce large gains of energy.

RESULTS AND DISCUSSION

The results of the 2 metabolism trials are summarized in table 4.

Fecal energy loss. The energy loss in the feces of sheep ingesting the pelleted corn meal-hay diet was significantly lower ($P < 0.01$) than that of the sheep receiving the chopped or pelleted hay diets. No

TABLE 4

Intakes of gross energy and losses of fecal and urinary energy during the two metabolism trials

Diet		Level of intake		
		Low	Medium	High
	Trial 1			
Chopped hay	Intake [1]	237	285	311
	Feces, % [2]	42.3	40.3	42.0
	Urine, % [2]	4.6	4.2	3.8
Pelleted hay	Intake	234	278	319
	Feces, %	40.6	40.4	41.0
	Urine, %	4.4	4.1	4.2
Pelleted corn-hay	Intake	200	276	381
	Feces, %	27.3	28.7	35.3
	Urine, %	3.5	3.2	2.7
	Trial 2			
Chopped hay	Intake	197	224	294
	Feces, %	38.9	40.6	40.7
	Urine, %	4.6	4.3	3.5
Pelleted hay	Intake	197	247	380
	Feces, %	40.3	41.3	43.7
	Urine, %	4.4	4.3	3.3
Pelleted corn-hay	Intake	157	235	304
	Feces, %	26.9	28.8	32.9
	Urine, %	4.0	3.4	3.1

[1] Intake expressed as gross energy (kcal/body weight $^{0.734}_{kg}$/day).
[2] Fecal and urinary energy losses expressed as percentages of the gross energy intake.

difference in the fecal energy loss was observed between the chopped-hay and pelleted-hay treatments. In both trials the fecal energy loss as a percentage of the GE intake increased as the level of intake of the 2 pelleted diets increased. This relationship was mathematically significant for the pelleted corn meal-hay diet in both trials ($P < 0.01$) and for the pelleted-hay diet only in the second trial ($P < 0.05$). The range of intakes of pelleted hay was considerably greater during the second trial than during the first trial and could account for the differences observed. On the other hand, the size of the fecal energy loss by sheep ingesting chopped hay was not related to the level of intake. In the first trial the mean losses of fecal energy by the older sheep and lambs were 38.5 and 36.2%, respectively, of the gross energy intake. In the second trial the respective mean losses were 37.0 and 37.3%. Although the difference in the first trial was significant ($P < 0.01$), no difference attributable to age was evident in the pooled data for both trials.

Urinary energy loss. The urinary energy loss expressed as a percentage of the GE intake was not different for the animals ingesting the chopped and pelleted hay diets. However, the energy output in the urine of sheep ingesting the pelleted corn meal-hay was lower ($P < 0.01$) than that of the animals fed the 2 hay diets. As the level of intake of all 3 diets increased, the proportion of energy lost in the urine decreased. This effect was significant in trial 1 ($P < 0.05$) and 2 ($P < 0.01$).

Metabolizable energy values. To compute metabolizable energy (ME) values, the losses of methane were estimated by means of the equation proposed by Swift and French (20) which is based on digestible dry-matter intake as the predictor. Both linear and second-degree polynomial equations were fitted to the relationship between the intakes of GE and ME for the 2 pelleted diets. These are summarized in table 5. Since the proportion of the GE ingested as chopped hay that was lost in the feces was the same at all levels of intake, only the linear equation was computed for the data obtained with the chopped hay diet.

Although the standard errors of estimate are not much different for linear and curvilinear equations, some refinement in the prediction of ME at the extreme intakes of GE of the 2 pelleted diets is effected by the curvilinear equations.

Relationships between body energy gain and the gross and metabolizable energy intakes. The regression of body (including wool) energy gain on the GE and ME intakes of the 3 diets resulted in the data summarized in table 6. Polynomial equations fitted to the data did not reduce significantly the error variance.

The energy value of the body gain represented the following percentages of the ME of the 3 diets ingested: chopped hay, 31; pelleted, finely ground hay, 43; and pelleted corn-ground hay, 57.

Metabolizable energy requirement for maintenance. The equations in table 6 were used to compute the quantities of ME required just to maintain energy equi-

TABLE 5

Relationship between metabolizable energy and gross energy intake

Diet	Regression equations [1]	SE of estimate
		kcal/body wt $^{0.73}_{kg}$ */day*
	Linear equations	
Chopped hay	$Y = 0.488X + 1.73$	4.94
Pelleted, ground hay	$Y = 0.414X + 20.28$	6.21
Pelleted, corn-hay	$Y = 0.503X + 22.76$	7.61
	Polynomial equations	
Pelleted, ground hay	$Y = 0.661X - 0.00039X^2 - 16.5$	5.52
Pelleted, corn-hay	$Y = 0.642X - 0.00025X^2 + 5.0$	7.60

[1] Y = metabolizable energy (*kcal/body wt* $^{0.73}_{kg}$ */day*), and X = gross energy (*kcal/body wt* $^{0.73}_{kg}$ */day*).

TABLE 6

Relationship between the gain in body energy and the gross and metabolizable energy intake

Diet	Regression equations [1]	SE of estimate
		kcal/body wt $^{0.73}_{kg}$ */day*
	Gross energy	
Chopped hay	Y = 0.1504X − 27.7	2.1
Pelleted, ground hay	Y = 0.1796X − 28.9	4.5
Pelleted corn-hay	Y = 0.2916X − 34.9	5.6
	Metabolizable energy	
Chopped hay	Y = 0.3084X − 28.2	2.1
Pelleted, ground hay	Y = 0.4339X − 37.6	4.4
Pelleted corn-hay	Y = 0.5693X − 46.9	5.7

[1] Y = body energy gain (*kcal/body wt* $^{0.73}_{kg}$ */day*), and X = gross or metabolizable energy intake (*kcal/body wt* $^{0.73}_{kg}$ */day*).

librium (namely, the maintenance requirement in terms of ME). For the 3 diets, these amounts (kcal/body weight$^{0.734}_{kg}$/day) were: chopped hay, 91.5; pelleted, ground hay, 86.7; and pelleted corn-ground hay, 82.4. Using only the positive energy balance data obtained in respiratory exchange and C-N balance experiments conducted by Blaxter and Graham (21), the present authors computed the maintenance requirements for ME of sheep ingesting a hay in long form and a pelleted, finely ground hay. These values were 95.8 and 88.9 kcal / body weight$^{0.734}_{kg}$ / day, respectively. Marston (22) reported an ME requirement of 86 kcal/body weight$^{0.734}_{kg}$/day for sheep ingesting a pelleted diet of crushed wheat, cane molasses, and finely ground alfalfa hay. Thus, the data obtained in the present study are of similar magnitude to those determined by respiration calorimetry and the C-N balance method.

Net utilization of GE and ME for body gain. The extent to which the energy ingested above the maintenance level was utilized for the combined functions of body gain and wool growth was computed by dividing the amount of energy retained by the amount of energy ingested above the level required for energy equilibrium. These coefficients for GE and ME are summarized by diet, age-of-sheep and level-of-intake groups in table 7. As would be expected in view of the small energy gain, the variation in the utilization of energy by sheep on the lowest level of energy intake was large. However, at the medium and high levels of intake the variation was small.

The GE and ME of the chopped-hay diet were utilized significantly less efficiently ($P < 0.01$) at all levels of intake than the GE and ME of either the pelleted hay or the pelleted corn-ground hay diet. Also,

TABLE 7

Net utilization of gross and metabolizable energy for body gain and wool growth

Level of intake	Age group	Chopped hay		Pelleted hay		Pelleted corn-hay	
		GE [1]	ME [2]	GE [1]	ME [2]	GE [1]	ME [2]
		%	%	%	%	%	%
Low	lambs	20.4	41.9	24.7	48.5	22.3	39.3
	older	10.9	22.3	18.8	36.7	41.2	73.1
Medium	lambs	14.6	30.0	21.2	43.3	30.3	55.3
	older	14.9	30.6	22.4	45.1	32.5	58.8
High	lambs	14.0	28.7	17.3	40.7	(23.5) [3]	(44.5) [3]
	older	15.8	32.4	20.3	45.3	30.0	56.7
Mean	Both	15.1	31.0	20.8	43.3	31.3	56.6

[1] GE indicates gross energy.
[2] ME indicates metabolizable energy.
[3] Values not included in means.

the GE and ME of the pelleted corn-ground hay diet were utilized significantly more efficiently ($P < 0.01$) than the GE and ME of the pelleted, ground hay diet.

The utilization of the GE and ME ingested above the maintenance level was not different ($P = 0.05$) for sheep of the 2 age groups or for the 3 levels of intake. Disregarding the data for the high level of the pelleted corn-hay diet, for which the values obtained with lambs were unsatisfactory, the net utilization of the GE as an average for the remaining 8-level diet groups was 21 and 22% for the lambs and older sheep, respectively. The mean net utilization of ME by the lambs and older sheep was 41 and 43%, respectively. The complete energy balances determined by Schürch (23) in sheep ranging from 6 to 15 months and from 3 to 4 years of age also indicate that age has very little, if any, effect on the net utilization of ME by sheep within the range of ages studied.

Physical nature of diet and heat increment. The heat increment values shown in table 8 are expressed as percentages of the ME. These represent the difference between 100% of ME and the percentage of the ME retained. Because of the small energy gains on the low level of intake, the data obtained for this level are more variable and not as reliable as those obtained at the medium and high levels of intake. Within diet, the heat increments were similar at the medium and high intakes. On the average, the heat increment value of the chopped-hay diet for body gain and wool growth was 22% greater than that of the pelleted, ground hay diet. The heat increment value of the pelleted hay diet was 31% higher than that of the pelleted corn-ground hay diet.

These observations for the 2 forms of hay diet confirm those made by Blaxter and Graham (24) in respiration calorimetric experiments. Although they found that sheep ingesting pelleted, finely ground hay retained a greater proportion of ME than did sheep ingesting the same hay in chopped form, the proportion of dietary GE lost in the feces was greater for the pelleted hay than for the chopped hay. Because of the heat increment compensating for the fecal energy loss, the net utilization of the GE was the same for the 2 forms of hay. With respect to fecal energy losses, the results of the present study are different from those of Blaxter and Graham (24). However, the effect of physical form of the diet on digestibility has not been uni- from one study to another. Although the mesh size of sieves used to grind rations is usually specified, this is not a good index of the spectrum of particle sizes.

Many experiments have demonstrated that physical form of the diet affects the nature of the end-products of ruminal digestion. For example, the molar proportions of acetic acid are lower and those of propionic acid are higher in the ruminal ingesta of sheep fed pelleted, ground hay than in the ingesta of sheep fed chopped or long hay (15). If the volatile fatty acids are absorbed in approximately the same proportions in which they are produced in the rumen, it is expected that the matter absorbed from pelleted, ground hay would be more efficiently utilized for body gain than that absorbed from the same hay ingested in chopped or long form.

TABLE 8

Heat increment for body gain and wool growth

Level of intake	Age group	Chopped hay	Pelleted hay	Pelleted corn-hay
		% metabolizable energy		
Low	lambs	58.1	51.5	60.7
	older	77.7	63.3	26.9
Medium	lambs	70.0	56.7	44.7
	older	69.4	54.9	41.2
High	lambs	71.3	59.3	(55.5) [1]
	older	67.6	54.7	43.3
Mean	both	69.0	56.7	43.4

[1] Value not included in mean.

This conclusion is predicated on the results of studies by Armstrong et al. (25) demonstrating that the heat increment of propionic acid is considerably lower than that of acetic acid when these fatty acids are metabolized for body gain.

On the other hand, in conventional calorimetric measurements the heat produced in metabolism is not distinguishable from that produced by the muscular work of the gut. As a consequence, the compensating greater loss of energy as heat by sheep ingesting long hay as observed by Blaxter and Graham (24) might be the result of long hay requiring greater muscular work in digestion than pelleted, finely ground hay. If this is the case, the additional heat produced should be charged to digestive losses rather than to the heat increment.

Net energy value of the diets. Net energy (NE) values for the combined functions of body gain and wool growth were computed from the data obtained at the medium and high levels of intake, using the intake at energy equilibrium as the baseline. The mean values (megcal/100 kg dry matter) and standard deviations of the means are: chopped hay, 68.3 ± 3.6; pelleted, ground hay, 93.7 ± 5.1; and pelleted corn meal-hay diet, 134.5 ± 6.6. Thus, the mean NE value for body gain and concomitant wool growth was 37.1% greater for the hay ingested in pelleted, finely ground form than for the same hay fed in chopped form.

Based on the data obtained at the 2 higher levels of intake of the pelleted, ground hay and pelleted, corn meal-ground hay diets, the NE value of corn meal was estimated to be 196.9 megcal/100 kg of dry matter.

It is known that the NE value of some diets diminishes as the level of intake increases. By means of the equations in tables 5 (polynomials) and 6 (concerning ME), the NE values of the diets used in the present studies were computed for several intake levels using energy equilibrium as the baseline. At intakes of dry matter equivalent to 2 and 3 times the maintenance intake the respective NE values were: chopped hay, 68.3 and 68.3; pelleted, ground hay, 90.8 and 86.0; and pelleted corn meal-hay, 142.2 and 133.8. The lower NE values of the 2 pelleted diets at the 3 times maintenance level of intake is traceable to the reduction in digestibility effected by level of intake.

Effect of physical form on intake and nutritive value. Per unit of metabolic size the dry-matter intakes (g) per day under ad libitum feeding were: chopped hay, 68.0; pelleted, ground hay, 87.3; and pelleted corn-ground hay, 76.9. Although the dry matter intake was 28.4% greater with the pelleted hay than with the chopped hay, the NE intake was 63.4% greater for the pelleted hay. The difference between the energy gains for the 2 hay diets was partitioned as follows: 78.5% due to the greater intake and 21.5% due to the greater nutritive value per unit of weight of the pelleted, ground hay diet.

Body weight gain and amount of feed required per unit of body weight gain. The responses of animals to the ingestion of diets are commonly expressed in terms of body weight gain per unit of time and the quantity of feed required per unit gain in weight. Both criteria have short-comings. For example, the apparent body weight gain in ruminants can be greatly affected by the amount of ingesta in the gastrointestinal tract and it can vary greatly in chemical composition. Efficiency of feed utilization expressed in terms of feed required per unit of gain is markedly affected by the amount of feed ingested above the level required for maintenance.

It has been demonstrated in many experiments that the ad libitum feeding of a diet in pelleted form results in a greater rate of body gain and a lower feed requirement per unit of gain than that of a diet in unpelleted form. The results of 2 earlier studies (5,9), in which no difference was observed in the rate of body gain or in the gain per unit of feed ingested when equal quantities of pelleted and unpelleted diets of the same composition were eaten, suggested that the increased rate of gain with pelleted diets is attributable solely to the higher intake.

As a part of the present studies, it was observed that the proportion of the total body weight constituted by the weight of the gastrointestinal contents is considerably greater in sheep ingesting chopped hay than in those fed the 2 pelleted, finely

ground diets. For example, an animal whose total body weight is 45.4 kg (100 pounds) would have the following ingesta-free body weights (in kilograms) as a result of ingesting the following diets: 37.6, chopped hay; 39.5, pelleted, ground hay; and 40.4, pelleted mixture of corn meal and finely ground hay. The standard error of estimate attached to these means is of the order of 1 kg. Thus, it is possible that a difference in empty-body gain of 0.045 kg/day effected by the feeding treatments in an experiment of 60 days' duration could be entirely masked by the difference in weight of the gastrointestinal contents.

If it is assumed that the weights of the gastrointestinal contents of the sheep used by Meyer et al. (9) were the same at the beginning of the 56-day feeding period, the gain in ingesta-free body of the sheep fed the pelleted hay could have been as much as 20 to 25% greater than that of the sheep fed chopped hay. Similarly, for the 79 pairs of sheep for which Esplin et al. (5) summarized the data from 9 laboratories, the difference in the empty-body gain of lambs fed pelleted, mixed diets could have been as much as 30% greater than that of the lambs fed the same amount of unpelleted feed.

On the other hand, in the present experiment the rate of body weight gain was greater and the amount of feed required per unit of gain was lower for sheep ingesting the pelleted, ground hay diet than for those fed the same amount of chopped hay at each of the low and medium levels of intake (table 9). (The responses to these 2 diets at the high intake level cannot be compared because the amounts ingested are different.) Table 9 also shows that the quantity of feed required per unit of body weight gain decreased progressively for all diets as the feed intake increases. This is the result of the diminishing proportion of the ration required for maintenance.

For all diets and levels of intake, the older sheep required 34.2 kg of feed/kg of body gain and the lambs needed 16.7 kg on the average. However, as shown in table 10, the energy value of the gain in lambs was markedly lower than that of the body

TABLE 9

Body weight gain and efficiency of feed utilization for body weight gain

Level of intake	Age group	Chopped hay		Pelleted hay		Pelleted corn-hay	
		Gain [1]	Efficiency [2]	Gain [1]	Efficiency [2]	Gain [1]	Efficiency [2]
Low	lambs	11	46.2	25	20.7	18	25.3
	older	6	124.1	22	46.1	26	41.0
Medium	lambs	44	15.3	56	12.2	112	6.7
	older	29	32.4	64	15.1	103	10.1
High	lambs	90	9.8	192	7.7	204	6.5
	older	67	18.5	130	12.4	190	7.7
Mean	both		41.0		19.1		16.2

[1] Gain is expressed in grams of body weight/day.
[2] Efficiency expressed as feed intake (kg)/body weight gain (kg).

TABLE 10

Energy concentration of gain of whole body [1]

Level of intake	Age group	Chopped hay	Pelleted hay	Pelleted corn-hay
		kcal/kg of body gain		
Low	lambs	7,804	4,456	7,626
	older	7,642	5,396	13,037
Medium	lambs	3,143	3,915	4,539
	older	5,992	4,639	6,639
High	lambs	2,598	3,634	4,678
	older	5,204	6,073	6,535

[1] Weight gain of total body includes ingesta.

gain of the older sheep. The mean energy value of 1 kg of body weight gain (including ingesta) was 4710 kcal in the lambs and 6795 kcal in the older sheep. Thus, although the lambs were more than 2 times as efficient as the older sheep in converting feed to body weight, the energetic efficiency of the sheep of the 2 age groups was the same (as explained earlier in this report).

Since the amount of feed required per unit of gain reaches a maximum near the maintenance level of intake, the data obtained at the low level of intake are useless for the purpose of comparing the rates of feed conversion and the energy values of the body gain. Considering only the medium and high levels of intake, the lambs were 65% more efficient converters of feed to body weight than were the older sheep. On the other hand, the mean energy concentration of the body mass gained by the older sheep was 56% higher than that of the lambs at these 2 levels of intake.

The observations discussed above emphasize the significance of gastrointestinal fill and the composition of body gain in experiments with ruminants in which body weight is the criterion of response. In addition, they demonstrate the inadequacy of body weight gain and feed required per unit of body weight gain as indices of energy storage.

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Effect of Magnesium and Sulfur upon Cellulose Digestion of Purified Rations by Cattle and Sheep[1,2]

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ABSTRACT Fistulated steers and intact lambs were used in an experiment designed to study the effect of magnesium and sulfur upon cellulose digestion and of magnesium upon voluntary feed intake. Purified diets with and without magnesium and sulfur were fed to steers, and rations with and without magnesium were fed to lambs. In vivo cellulose digestion was determined with steers and in vitro digestion measured using inoculum from steers and lambs. Digestion of cellulose was significantly decreased in steers fed the purified rations without added magnesium and sulfur. In vitro digestion was also significantly reduced when rumen fluid from the steers and lambs fed the deficient rations was used as the inoculum for in vitro fermentation. Rumen fluid from lambs fed the complete ration was used for inoculation of a series of in vitro media without magnesium. The serial dilution of magnesium from the original inoculum resulted in a progressive decrease in cellulose digestion. Voluntary intakes of the magnesium-deficient ration were significantly reduced by the third day of consumption. Oral administration of magnesium oxide by capsule to lambs resulted in restoration of appetite, but intravenous injection of magnesium sulfate had a lesser effect.

The rate and extent of cellulose digestion are important in ruminant nutrition and many dietary factors are known to affect the microbial degradation of cellulose. The role of the various mineral elements and their interrelationship in this process are generally unknown. Magnesium is required by the body, but its function in the rumen has not been identified. Many of the studies of magnesium have been concerned with requirements and, in ruminants, much attention has been given to studies of magnesium tetany (1–3). Chamberlain and Burroughs (4) studied the effect of magnesium upon cellulose digestion in vitro and reported that absence of the element from the nutrient media did not result in a significant reduction in cellulose digestion. McAleese et al. (5) observed that the voluntary intake of a semipurified ration by sheep was reduced when the ration was deficient in magnesium.

A requirement for sulfur by rumen microorganisms has been demonstrated in several experiments (6–8) which have shown that rumen microorganisms utilize elemental sulfur in the synthesis of microbial protein. Cellulose digestion, urea utilization, riboflavin and vitamin B_{12} synthesis were stimulated in vitro when sulfur either as the sulfate or methionine was added to the media (9).

The present study was undertaken to determine the effect of magnesium and sulfur upon in vivo and in vitro cellulose digestion and the effect of dietary magnesium upon voluntary feed intake by ruminants fed a purified ration.

METHODS

Four fistulated steers and 8 wether lambs served as experimental animals. The complete ration fed to both species was prepared from purified ingredients in the proportions shown in table 1. The steers were fed one of four rations; complete, Mg-deficient, S-deficient, and Mg-

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[3] Present address: Monsanto Chemical Company, St. Louis.

TABLE 1

Composition of purified ration

	%
Glucose monohydrate [1]	28.8
Cornstarch	28.0
Cellulose [2]	30.0
Cottonseed oil [3]	4.0
Crystalline urea [4]	4.0
Mineral mixture [5]	5.1
Vitamin A palmitate (10,000 IU/g) [6]	0.044
Vitamin D_2 (9,000 IU/g) [6]	0.048
D-α-Tocopherol	0.001

[1] Corn Products Company, Argo, Illinois.
[2] Solka Floc, SW-40 grade, Dicalite Company, New Orleans, Louisiana.
[3] Stabilized with ethoxyquin, donated by Monsanto Chemical Company, St. Louis.
[4] Donated by E. I. duPont de Nemours & Company, Wilmington, Delaware.
[5] One hundred grams of mineral mixture contained: (in grams) $CaHPO_4 \cdot 2H_2O$, 62.24; KCl, 18.22; Na_2SO_4, 8.62; NaCl, 4.31; $FeC_6H_5O_7 \cdot 2H_2O$, 3.23; MgO, 3.16; $MnCO_3$, 0.076; $CuCl_2 \cdot 6H_2O$, 0.057; $ZnCO_3$, 0.039; KI, 0.038; $CoCl_2 \cdot 6H_2O$, 0.001.
[6] Donated by Commercial Solvents Corporation, New York.

and S-deficient, in a 4 × 4 Latin square design. Each experimental period was followed by a recovery period of 7 days in which animals received the complete ration. The deficient rations were prepared by omitting MgO and Na_2SO_4 from the mineral mixture. The daily intake for steers was calculated to furnish 2000 kcal in excess of maintenance requirement and a constant intake was insured by adding unconsumed feed to the rumen via the rumen fistula. Steers were fed the experimental rations for 21 days and during the last 7 days of the period, total fecal collections were made for in vivo cellulose digestion measurements. At the same time, rumen fluid was taken via the rumen fistula for use as inoculae for in vitro studies.

Intact lambs were offered 800 g daily of either the complete or magnesium-deficient ration, but this amount was not completely consumed by lambs provided with the magnesium-deficient treatment. After a 14-day period of adjustment to the complete purified ration, the 8 lambs were randomly allotted to treatments for 8 days. The lambs were then reallotted to treatment after a 60-day recovery period with the complete ration. Rumen fluid was taken by stomach tube on each of the first 3 days of each treatment period for in vitro cellulose digestion studies.

In vitro cellulose digestion was determined in triplicate using cellulose [4] as the substrate and a nutrient medium described by Bentley et al. (10). The inoculae were prepared by straining rumen fluid through cheesecloth. After 24 hours' fermentation, the residual cellulose was determined using the method of Crampton and Maynard (11) for all cellulose determinations.

A portion of the inoculum prepared from lambs consuming the complete ration was used in a transfer fermentation procedure designed to serially dilute the carryover of magnesium from the original inoculum but allowing cellular proliferation between transfers. Five successive daily transfers of 25% portions of each daily fermentation were made into fresh media containing no magnesium. Control transfer fermentations were conducted with magnesium added to the medium. Either cellulose or the corresponding complete ration served to supply the cellulose substrate. The digestion of cellulose during each successive fermentation period was taken as a measure of the effect of magnesium on the activity of cellulolytic rumen microorganisms.

The depression of appetite observed in steers and lambs fed the magnesium-deficient ration was studied in more detail in lambs. Two animals exhibiting severe loss of appetite after receiving the magnesium-deficient ration for 3 days were given daily oral doses of 1.5 g of MgO. Two additional lambs fed the magnesium-deficient ration were given 5 daily intravenous injections of 2.5 ml of a 25% solution of $MgSO_4$ from the day that magnesium-deficient rations were started. Changes in voluntary feed intake and comparisons with intakes of animals consuming the complete and unsupplemented deficient rations throughout the experiment were measures of the effect of magnesium upon appetite.

Statistical analyses of the data were made by a matrix vector product analysis of variance [5] and by factorial analysis of split block designs described by Snedecor (12).

[4] Solka Floc, BW-40 grade, Dicalite Company, New Orleans, Louisiana.
[5] Uupublished data, A. E. Brandt, University of Florida.

RESULTS AND DISCUSSION

Values representing the digestion of cellulose in vivo by steers and in vitro using inoculae from steers are recorded in table 2. Treatment effects were highly significant ($P < 0.01$). Digestibility in vivo by steers was reduced by 35% in the absence of magnesium ($P < 0.05$), 70% in the absence of sulfur ($P < 0.001$) and 72% when both elements were omitted from the ration. In vitro digestion of cellulose with inoculum from steers was also significantly reduced in the absence of each element. The reductions observed were 88, 95 and 83% in the absence of magnesium, sulfur and both magnesium and sulfur, respectively.

The data obtained for in vitro cellulose digestion using rumen inoculae from experimental lambs are recorded in table 3. On the third day after lambs were offered the magnesium-deficient ration, digestion was significantly reduced ($P < 0.05$) and appetite had also decreased. The decrease in digestion by the third day further suggested a specific dietary requirement for magnesium for optimal cellulose digestion.

Cellulose digestion was progressively decreased in the in vitro transfer inoculation technique when magnesium was omitted from the media (table 4). This decrease resulted when either cellulose or the experimental rations were used to provide the cellulose substrate. With the rations,

TABLE 2

Effect of magnesium and sulfur upon in vivo cellulose digestion by steers and in vitro digestion using rumen inoculum from steers

Period	Ration treatment			
	Complete	Without Mg	Without S	Without Mg and S
	%	%	%	%
	Cellulose digested in vivo			
1	71.4	53.0	40.4	39.7
2	72.8	50.8	12.2	0.0
3	74.9	49.0	22.8	37.6
4	55.0	25.4	7.6	0.0
Mean [1]	68.5 [1a]	44.5 [2ab]	20.8 [3b]	19.4 [3b]
	Cellulose digested in vitro			
1	30.1	5.9	5.2	8.0
2	52.6	8.7	0.6	3.1
3	25.3	0.7	0.0	0.2
4	25.9	0.4	0.3	11.6
Mean [1]	33.5 [a]	3.9 [b]	1.6 [b]	5.7 [b]

[1] Means with different superscript numbers significantly different ($P < 0.05$); different superscript letters significantly different ($P < 0.01$).

TABLE 3

Cellulose digested in vitro by rumen microorganisms from lambs fed purified rations with and without magnesium

Ration treatment	Replication	Cellulose digested		
		Days		
		1	2	3
		%	%	%
Complete	1	82.0 [1]	78.0	83.0
	2	97.5	61.0	44.0
	Mean	89.8	69.5	63.5 [2]
Without Mg	1	82.0	76.0	31.0
	2	58.4	56.6	00.0
	Mean	70.2	66.3	15.5 [2]

[1] Average of inoculae from 4 lambs.
[2] Difference between means significant ($P < 0.05$) on third day.

which contained soluble carbohydrate, inhibition of cellulose degradation was much more severe in the absence of magnesium. The presence of soluble carbohydrate in the ration substrate may have created a competition between amylolytic and cellulolytic microorganisms for the magnesium present and resulted in the inhibition. With the cellulose substrate, digestion was not significantly restricted until the fourth transfer indicating adequate carryover of magnesium to the fourth transfer. Such a carryover effect may help explain the apparent discrepancy between these results and those of Chamberlain and Burroughs (4).

Gross microscopic examination of the morphological types of the microorganisms in the rumen indicated that magnesium deficiency induced changes in the rumen flora. The complete ration supported a population including both gram-

TABLE 4

Cellulose digested in vitro per day using transfer technique

Media	Substrate [1]	Days				
		1	2	3	4	5
		g	*g*	*g*	*g*	*g*
Without Mg	cellulose	0.410 [2]	0.289	0.284	0.174	0.097
With Mg	cellulose	0.422	0.371	0.324	0.302	0.329
Without Mg	ration, without Mg	0.172	0.039	0.010	0.000	
With Mg	ration, with Mg	0.306	0.268	0.243	0.207	

[1] One gram cellulose (Solka Floc, BW-40 grade, Decalite Company, New Orleans) per fermentation tube and 0.6 g cellulose (Solka Floc, SW-40 grade) per tube in ration substrate.
[2] Average of duplicate flasks in 2 replicates.

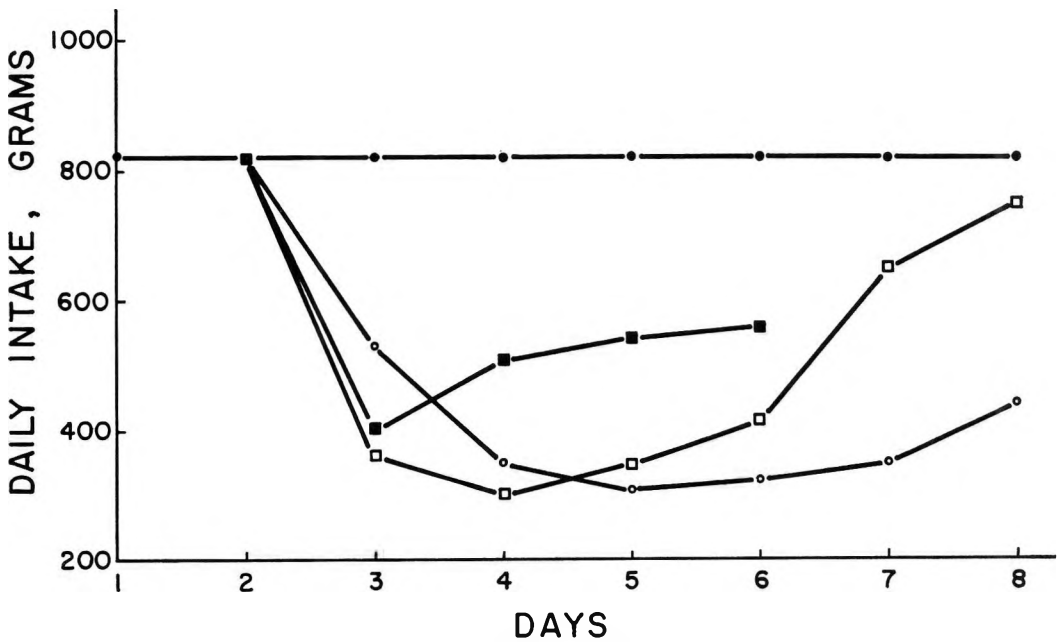

Fig. 1 Voluntary feed intake of lambs consuming complete and magnesium-deficient rations and response to oral and intravenous administration of magnesium. Key: ●——● indicates complete ration; ○——○, Mg-deficient ration; □——□, oral Mg daily from third day; ■——■, intravenous Mg daily from first day.

positive and gram-negative microorganisms. The magnesium-deficient ration supported a population composed primarily of gram-negative organisms.

Feed intake measurements were made throughout the study and it was consistently observed that the voluntary consumption of the magnesium-deficient ration decreased on or about the third day after offering the deficient ration. The decrease was apparent in both species and in lambs was generally concurrent with the observed decrease in in vitro cellulose digestibility by inoculae from lambs. Intakes by the third day were equivalent to 66% of that for lambs consuming the complete ration. The daily oral administration of magnesium oxide to deficient lambs, beginning on the third day after change to the deficient ration, resulted in improvement in voluntary feed intake (fig. 1). Daily intravenous injections of magnesium sulfate solution did not prevent the characteristic decrease in consumption but did cause a slight increase by the fourth day. The amount of magnesium administered intravenously was less than the amount given orally or that present in the complete ration; however, it caused a slight increase in feed intake.

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Physiological Aspects of Aging

V. CALCIUM AND MAGNESIUM METABOLISM IN SENESCENT MICE[1]

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ABSTRACT Mice representing 3 adult ages (7, 21 and 26 months) were subjected to a low-calcium, low-magnesium diet for a period of 10 days to determine their relative capacities to adapt to a reduced intake of these elements. Whereas the youngest group attained calcium equilibrium during this period, the older animals were in significant negative balance which was attributable to an increased loss of endogenous calcium from the skeleton. The net digestibility coefficients for dietary calcium, determined by the isotope dilution method, were similar for the 3 groups. An analogous age effect was observed with respect to magnesium balance. The results indicate that the capacity for calcium absorption is unimpaired in aged mice, but that following a reduction in calcium intake old animals are more susceptible to a negative calcium balance as a result of an accelerated rate of skeletal catabolism.

The subject of calcium absorption and retention by aged animals is of interest not only from a general gerontological standpoint but also because of its possible relevance to the etiology of senile osteoporosis. The results of several independent studies, recently reviewed (1), have shown that calcium balance in animals and man may be achieved over a considerable range of intakes. These experiments indicate that a major factor in adaptation to a change in the level of calcium intake is an adjustment in the efficiency of calcium absorption from the intestine, which evidently is receptive to some stimulus from the physiological mechanism responsible for maintaining calcium homeostasis. The existence of an "endogenous factor" regulating calcium absorption was proposed by Nicolaysen (2) to explain his observation that the efficiency of calcium uptake, both in vivo and by isolated gut loops, was increased following a period of low intake. Kimberg and co-workers (3) have also shown that the active transport of calcium in vitro by everted gut sacs is enhanced by previous calcium deprivation.

The decline in physical activity which ordinarily accompanies senescence presents the aging individual with the choice of either reducing his caloric intake or incurring increasing obesity. If the composition of the diet remains unchanged, a reduction in food consumption entails a corresponding decrease in the supply not only of those nutrients for which the requirement is related to energy intake, but also those, such as calcium, for which the requirement is related to body weight. Thus the aging subject who moderates his caloric intake in accordance with his declining energy requirement is liable to a negative calcium balance unless he is capable of adaptating to the concomitant reduction in the intake of this element.

Several studies on aged rats (4–7) indicate that senescent individuals do not differ qualitatively from young adults with respect to their ability too re-establish calcium equilibrium at a lowered level of intake, although the adaptive process may be of longer duration (6). Experiments on human adults (8–10) generally have supported the observations on rats, but it may be noteworthy that some individuals were observed to be in prolonged negative balance following a sharp reduction in calcium consumption (8). Although only about one-quarter of the calcium consumed by healthy adults in Western countries may be absorbed, calcium supplementation has been reported to lead to an increased retention in osteoporotic subjects (11, 12), and it has been proposed that calcium deficiency is a major factor in this disease.

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By contrast with normal subjects, in severe osteomalacia in remission the efficiency of calcium absorption may reach 80 to 85%, even at an intake of 1.5 g/day (13). If calcium insufficiency is a primary cause of osteoporosis, a similar avidity for dietary calcium might be anticipated. An alternative possibility is that in osteoporosis there may be a decreased capacity for adaptation, with the result that the absorption mechanism is no longer responsive to the needs of the skeleton for additional calcium to compensate for the losses caused by an accelerated catabolism.

The present experiments were designed to compare mice of 3 adult ages with respect to the efficiency of calcium absorption and the ability to adapt to a sharp reduction in calcium intake. Similar observations were made with respect to magnesium.

EXPERIMENTAL

Animals. One hundred female mice of the C57BL/6 Bar Harbor strain were obtained as weanlings and maintained continuously with a commercial laboratory chow. Groups of 10 animals in apparent good health were chosen at random for absorption measurements at 7, 21 or 26 months of age, selection of the oldest group being made from the surviving 15% of the original population. All 3 groups averaged 21 to 22 g in body weight at the beginning of the test.

Diet. The commercial stock diet, which contained 1.30% calcium, 0.90% phosphorus and 0.20% magnesium, was augmented by a supplement of 1500 IU of vitamin A and 5 mg of vitamin E given monthly by mouth. Ad libitum consumption of this diet was observed to average 3.5 g/day, which provided 45 mg of calcium, 31 mg of phosphorus and 7 mg of magnesium.

For a 3-day preliminary period and a collection period of 10 days the animals were maintained with a constant daily intake of 3 g of a standard test diet. It was predetermined that this quantity of food would be consumed consistently and would maintain the animals without a significant change in weight during the collection period. The composition of the experimental diet was as follows: (%) starch, 35.6; glucose,[2] 35.6; soybean protein,[3] 15.0; corn oil, 5.0; vitamin mixture (in equal parts starch and glucose), 4.0, cellulose,[4] 2.5; trace minerals, 0.72; $CaCO_3$, 0.50; H_3PO_4, 0.56, choline chloride, 0.5; DL-methionine 0.05. The vitamin supplement provided 1.5 IU of vitamin A and 0.6 IU of vitamin D/g, plus the following amounts of other vitamins: (μg per g) thiamine, 4; riboflavin, 4; Ca pantothenate, 4; nicotinic acid, 10; pyridoxine, 1; menadione, 0.4; folic acid, 0.2; biotin, 0.1; and vitamin B_{12}, 0.1. The diet furnished, in 2 successive experiments, approximately 2 or 6 mg of calcium/day at a Ca:P ratio of 1:1.33, and 0.5 or 1.5 mg of magnesium. To prevent spillage, the diet was mixed with 3 ml of double-distilled water; free access was also available to calcium-free drinking water.

Procedure. During the collection period the test animals were housed in a metabolism cage which was observed to prevent coprophagy and effect a clean separation of urine and feces. The details of this cage have been presented elsewhere (14). At the end of the 3-day preliminary period approximately 10 μc of $Ca^{45}Cl_2$ (3×10^4 μc/mg) were administered intraperitoneally and the animals were maintained in the metabolism cages for a period of 10 days. The feces were collected daily and analyzed for calcium, magnesium and Ca^{45}; the urine was collected on no. 42 Whatman filter paper at two 5-day intervals and analyzed similarly. Analysis of the filter paper for calcium and magnesium yielded values of 3.6 and 6.6 μg, respectively, and these amounts, although generally negligible, were subtracted from the values obtained for the urine samples.

The daily fecal samples and 5-day composite urine samples were wet-ashed using HNO_3 and H_2O_2, and calcium was determined by titration with ethylenediaminetetraacetic acid after removal of phosphorus and trace minerals according to the procedure of Malmstadt and Hadjiionnou (15, 16). Magnesium was determined by a similar procedure using Eriochrome Black T as indicator. Calcium-45 was

[2] Cerelose, Corn Products Company, Argo, Illinois.
[3] ADM C-1 Assay Protein, Archer-Daniels-Midland, Minneapolis.
[4] Solka Floc, Brown Company, Berlin, New Hampshire.

estimated in a gas-flow counter, appropriate corrections being made for counting efficiency and decay. Isotope excretion curves were plotted for each age group and the net absorption of dietary calcium was calculated by the isotope dilution method (7, 17).

The net absorption of dietary calcium by young adult and aged mice was further investigated using 2 groups of animals aged 9 and 27 months. Eight mice representing each age group were given an intraperitoneal injection of Ca^{45}, placed in metabolism cages and fed 3 g of the test diet daily. Beginning 48 hours after injection, 5-day composite samples of urine and feces were taken for calcium, magnesium and Ca^{45} analysis and calculation of absorption coefficients.

RESULTS

Calcium-45 excretion. The fecal Ca^{45} excretion pattern for 3 mice representative of their respective age groups (7, 21 and 26 months) is shown in figure 1. As the total calcium excreted in the feces was relatively uniform from day to day, the data also give an indication of the total Ca^{45} excreted by this route during the 10-day experimental period. The most significant aspect of the data is that they can be represented by 2 straight lines which intersect at a point on the x axis corresponding to 4 to 6 days post-injection. There was no indication that the slope of the 2 lines, or their point of intersection, was different for the 3 age groups. The biphasic character of the data is similar to that observed for rats (7, 18), and reflects the presence in the bones of at least 2 metabolic pools of calcium with differing turnover rates.

Cumulative fecal Ca^{45} excretion curves (as averages for the 3 age groups) are shown in figure 2. The total fecal excretion, which amounted to approximately one-half of the administered dose, was similar for the 7- and 21-month-old animals but was greater for those 26 months of age, the figure for the oldest group being significantly greater ($P = 0.02$) than for the young adults. A similar difference ($P = 0.04$) was observed with respect to the urinary Ca^{45} excretion, the means and standard deviations for the oldest and youngest groups being $6.43 \pm 2.27\%$ and $2.77 \pm 0.83\%$ of the dose, respectively. These data show that the aged mice were

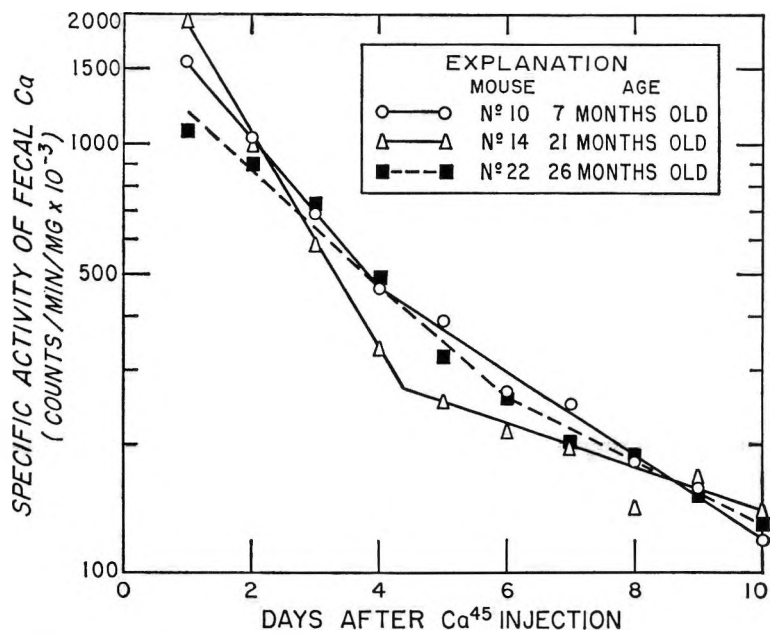

Fig. 1 Changes in the specific activity of fecal calcium excreted by mice of different adult ages following an intraperitoneal dose of Ca^{45}.

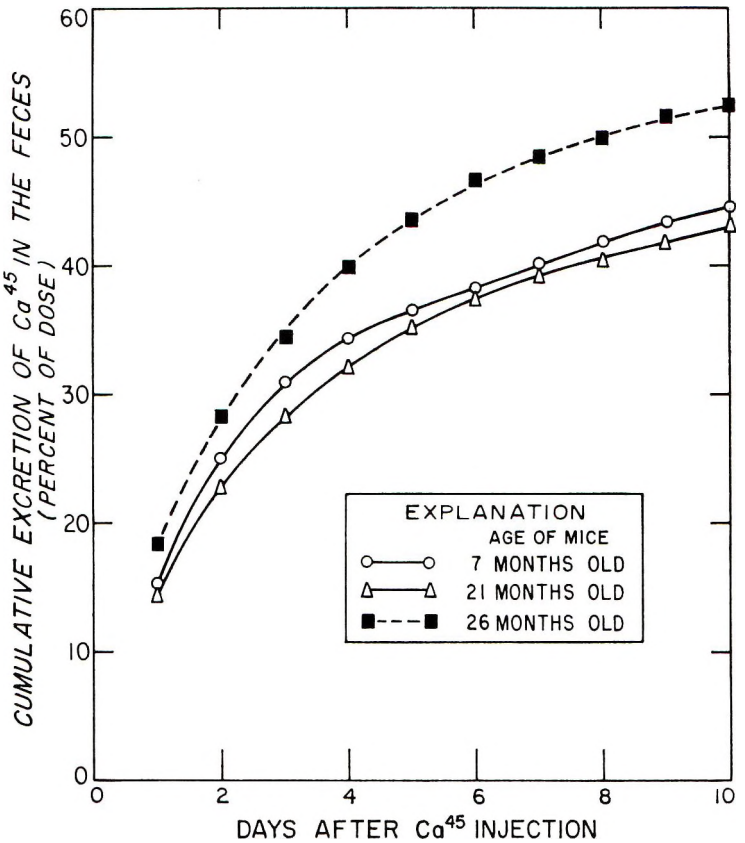

Fig. 2 Cumulative excretion of Ca^{45} in the feces of mice of different adult ages following an intraperitoneal dose.

subject to an accelerated rate of calcium exchange or catabolism with a resulting decrease in the length of time which calcium absorbed from the circulation was retained in the skeleton. This observation is analogous to that made previously with respect to aged rats (7). On the other hand, no indication was obtained that the retention of Ca^{45} by the 21-month-old mice was different from that of the 7-month-old animals. However, the oldest group exhibited characteristic signs of advanced age, including greyness and slow movement, whereas these symptoms were not generally noticeable in the mice 21 months of age.

The partition of Ca^{45} and total calcium between feces and urine is summarized in table 1. Two noteworthy age effects are indicated by the data, namely, an increase with age in the proportion of calcium excreted in the urine and an increase in the variability among animals. These changes were particularly evident in the oldest group and are therefore primarily an accompaniment of senescence. The increased proportion of calcium excreted in the urine by the oldest group coincided with an increase in the total calcium excretion, suggesting that the partition of calcium between the 2 excretory pathways is influenced by the rate at which this element leaves the circulatory pool. This conclusion was supported by the observation that when a group of five 21-month-old mice was permitted to consume the calcium-rich stock diet up to the time of injection (that is, with no preliminary period), 18.0% of the dose was excreted in the urine during the ensuing 10 days.

Calcium balance. The effect on calcium balance of subjecting mice of differ-

ent adult ages to a decrease of 85 to 95% in calcium intake is indicated in table 2. Despite the severity of this reduction, the 7-month-old animals remained, on the average, in overall calcium equilibrium. The older animals, however, were uniformly in negative balance, both groups excreting significantly more calcium than the younger group. The results show that aging is accompanied by a decreased ability to adapt to a low calcium diet, at least during the initial period of restricted intake. This observation is in accord with that of Henry and co-workers (6) that aged rats require a longer period of adaptation than younger adults.

Whereas no increase was observed in the rate of Ca^{45} excretion by the 21-month-old mice (fig. 2), a significant increase over that of the 7-month-old animals was noted with respect to the excretion of total calcium (table 2). The only explanation which can be offered for this apparent discrepancy is that the injected Ca^{45} did not come into equilibrium with the total pool of mobilizable calcium.

Calcium absorption. The apparent and net absorption coefficients for dietary calcium are shown in table 3. The results indicate that aging was accompanied by a decrease in the apparent digestibility of calcium but not by any significant change in net absorption. The increased excretion of fecal calcium by the 2 oldest groups was found to be largely of endogenous origin, and when the total excretion was corrected for this fraction, the unabsorbed portion was found to be similar for all 3 groups. Under the conditions of low calcium consumption which prevailed in this experiment, the endogenous fecal calcum represented about two-thirds of the total calcium excreted by this route.

Similar results with respect to the efficiency of calcium absorption were obtained in the second comparison between 2 groups

TABLE 1

Partition of excretory calcium and Ca^{45} between feces and urine of mice of different ages maintained with a low calcium regimen

Age, months	7	21	26
% Ca excreted in feces	94.8	93.4	92.0
% Ca excreted in urine	5.2	6.6	8.0*
Standard deviation	1.1	1.2	2.9
% Ca^{45} excreted in feces	93.8	93.2	90.7
% Ca^{45} excreted in urine	6.2	6.8	9.3*
Standard deviation	1.8	3.8	4.1

* *P* values for significance of mean differences between 7- and 26-month-old mice were 0.02 and 0.07, respectively, for Ca and Ca^{45}.

TABLE 2

Calcium balance in mice of different adult ages following a marked decrease in calcium intake

Age, months	7	21	26
No. animals	10	10	10
Ca consumed, mg/day			
In pre-test stock diet (approximately)	45	45	45
In test diet	2.19	1.89	1.89
Ca excreted, mg/day			
In feces	2.09	2.20	2.34
In urine	0.11	0.16	0.21
Total	2.20 ± 0.16 [1]	2.36 ± 0.15*	2.55 ± 0.25**
Ca balance, mg/day	-0.01 ± 0.13	-0.47 ± 0.15**	-0.66 ± 0.24**
No. animals in negative balance	4	10	10

[1] Mean ± SD.
* Significantly different from the mean for the 7-month-old group ($P = 0.02$).
** Significantly different from the mean for the 7-month-old group ($P = < 0.01$).

of mice aged 9 and 27 months. At an intake of 5.67 mg/day, the average net digestibility coefficients and their standard deviations were 65.4 ± 6.3% and 66.6 ± 5.7%, respectively. The combined results of these experiments indicate that there is no difference between young adult and aged mice relative to their ability to absorb calcium from the intestine under conditions of reduced intake, but that aged animals are more susceptible to calcium imbalance under such conditions because of their increased rate of skeletal catabolism. This conclusion is similar to that reached previously with respect to aged rats (7).

Magnesium balance. Table 4 illustrates the effect on magnesium balance of subjecting adult mice of different ages to a low magnesium diet. The results are similar to those obtained with respect to calcium, except that adaptation to the low intake was generally less satisfactory. All animals were in negative balance during the experiment, but the 2 older groups

TABLE 3

Calcium absorption by mice of different adult ages subjected to a marked reduction in calcium intake

Age, months	7	21	26
No. animals	10	9	10
Ca consumed, mg/day	2.19	1.89	1.89
Ca excreted in feces, mg/day	2.09	2.20	2.34
Apparent digestibility of dietary Ca, %	4.4 ± 11.6 [1]	−16.5 ± 11.4**	−23.7 ± 10.3**
Specific activity of urinary Ca, count/min/mg × 10^{-4}	25.48	24.81	95.44
Specific activity of fecal Ca, count/min/mg × 10^{-4}	16.89	16.77	66.65
Endogenous fecal Ca,[2] mg/day	1.39 ± 0.29	1.46 ± 0.34	1.64 ± 0.27
Unabsorbed fecal Ca, mg/day	0.70	0.74	0.70
Absorbed dietary Ca, mg/day	1.49	1.15	1.19
Net digestibility of dietary Ca, %	68.7 ± 11.2	62.2 ± 14.8	62.3 ± 8.0

[1] Mean ± SD.
[2] Estimated by dividing the total counts excreted in the feces by the specific activity of urinary Ca.
** Significantly different from the mean for the 7-month-old group ($P = < 0.01$).

TABLE 4

Magnesium balance in mice of different adult ages following a marked reduction in magnesium intake

Age, months	7	21	26
No. animals	10	9	10
Mg consumed, mg/day			
In pre-test stock diet (approximately)	7	7	7
In test diet	0.38	0.49	0.49
Mg excreted, mg/day			
In feces	0.25 ± 0.02 [1]	0.41 ± 0.04	0.39 ± 0.04
In urine	0.26 ± 0.03	0.32 ± 0.03	0.34 ± 0.04
Total	0.52 ± 0.04	0.72 ± 0.07	0.72 ± 0.06
Mg balance, mg/day	−0.14 ± 0.04	−0.23 ± 0.07**	−0.23 ± 0.06**
Apparent digestibility of dietary Mg, %	32.4 ± 6.4	18.0 ± 5.9**	23.2 ± 7.0*

[1] Mean ± SD.
* Significantly different from the mean for the 7-month-old group ($P = 0.02$).
** Significantly different from the mean for the 7-month-old group ($P = < 0.01$).

were in significantly greater imbalance than the young adults. Since the experiment did not permit the use of both a magnesium and calcium isotope, it is not possible to determine from the data whether the decrease in the apparent digestibility of magnesium which accompanied aging was due to a net reduction in intestinal uptake or, as in the case of calcium, to an increased endogenous excretion into the feces. In general, however, the data indicate that the effect of aging on the metabolism of magnesium is similar to that on calcium.

DISCUSSION

The present observations relative to the changes in calcium metabolism associated with aging in mice are in general accord with the results of an earlier study on aged rats (7). Apparently, from these and previous experiments on aged animals and man (1), an accelerated catabolism of bone mineral is a common, if not general, accompaniment of senescence. In some individuals this process may achieve pathological proportions; in a majority it probably has no practical consequences owing to the magnitude of the dispensable calcium reserves in the skeleton. Moore and co-workers (19) have estimated that there is a safety margin of 100% in the ash content of the femur of a normal rat above the minimal level at which any unfavorable effect on growth or general health is observed. A chronic negative balance may persist for protracted periods, therefore, without undue consequences, provided it is of low magnitude. It would be of interest to know whether the older mice in the present study would attain eventual equilibrium at the lowered level of calcium intake, as was the case in experiments on rats (6).

From the results of the present study and those of previous experiments on rats (7), it appears that the tendency of aged animals toward a negative calcium balance is not due to a decreased capacity for absorption from the intestine, but to an incomplete replacement of calcium eroded from the skeleton. Whether this phenomenon is due primarily to the decreased production of anabolic hormones in older subjects, as suggested by Albright (20), to an inadequate supply of calcium (11, 12) or both is unclear. A further consideration is that the increased skeletal losses may be related to declining physical activity in the aged; immobilization of human subjects has been shown to result in a "disuse atrophy" which is marked by an increased excretion of calcium in both feces and urine (21).

Studies are in progress to determine the effect of feeding mice various levels of dietary calcium throughout adult life on the ash content, volume, density and breaking strength of the bones, as well as on the concentration of calcium in the soft tissues.

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Effects of Dietary Cholesterol on Skin Lesions of Rats with Subacute Magnesium Deficiencies[1,2]

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ABSTRACT Skin lesions were observed in rats with both acute and subacute magnesium deficiencies when they were fed diets containing blood fibrin, glucose and saturated coconut oil as their main constituents. Cholesterol consistently increased the incidence of these skin lesions when added to diets with marginal magnesium levels. Further investigations showed that magnesium is not depleted from the skin immediately before the development of visible skin lesions. On the contrary a moderate elevation of skin magnesium levels on fresh weight basis was observed in skin that was taken from rats fed low magnesium diets just before or as skin lesions develop. The percentage dry weight of skin was lowered by lowering the dietary level of magnesium. The addition of cholesterol to diets induced the same changes in skin dry matter and magnesium content that were caused by the lowering dietary level of magnesium. It was also observed that cholesterol could promote the development of skin lesions without accumulating in the serum and without altering thyroid size or plasma protein-bound iodine levels. A magnesium balance experiment indicated that dietary cholesterol reduced the apparent intestinal absorption of magnesium. Thus it is possible that cholesterol may promote the development of skin lesions by reducing the amount of magnesium available to the tissues.

An increased dietary level of magnesium has been found to reduce the deposition of lipid in the aorta and in the heart valves of rats fed diets containing added cholesterol (1, 2). Vitale and co-workers[4] observed that magnesium deficiency in dogs caused lipid deposition in the heart, and fibrous thickenings of the medial ground substances of the heart. Reduced serum magnesium levels have also been reported in animals fed diets containing cholesterol (2, 3). These observations suggest that part of the atherogenic effect of dietary cholesterol may be related to a reduction in the ability of the animals to utilize dietary magnesium effectively. The observation that cholesterol and cholic acid promoted hyperemia, hyperexcitability and kidney lesions which are typical magnesium deficiency symptoms when added to a diet having a magnesium level of 240 ppm (1, 2) also suggests that cholesterol reduces the rat's ability to use magnesium.

Bersohn and Oelofse (4) have observed a negative correlation between serum cholesterol and serum magnesium levels. However, Brown et al. (5) found no correlation between serum magnesium and serum cholesterol when magnesium-lipid relationships were studied in 186 middle-aged men. With rats the dietary level of magnesium has not been noted to have any effect on serum cholesterol levels (1, 2).

It has been observed that the β-lipoprotein level increases with increasing serum cholesterol and with magnesium deficiency (6).

Magnesium also influences the ability of other agents to produce coronary lesions. Shimamoto and co-workers (7) observed that the oral administration of magnesium chloride will counteract coronary lesions produced by injecting bacterial polysaccharides into rats, whereas Mishra (8, 9) reported that a magnesium deficiency rendered rats more susceptible to heart lesions brought about by stress or steroid-phosphate treatments.

The initial purpose of the present research was to determine the effect of dietary magnesium on the amount and composition of tissue lipids. However, it was found during the course of the first ex-

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periment that cholesterol and sodium glycocholate could induce skin lesions when rats were fed a diet with a marginal level of magnesium. Subsequent experiments were directed toward determining how cholesterol promoted these skin lesions. Since cholesterol also induces coronary and arterial lesions, it was felt that these investigations on skin lesions had some bearing on the problem of atherosclerosis. Both types of lesions represent abnormalities in connective tissues.

METHODS

Weanling rats of the Wistar strain were placed on the experiments when they were 20 to 22 days of age and weighed 40 to 50 g. Rats from each litter were distributed as equally as possible among the experimental groups. They were confined in individual galvanized wire cages and given food and deionized water ad libidum. The animals were weighed and food consumption was recorded at weekly intervals. The composition of the basal diets, which were found to contain 38 ppm magnesium, is shown in table 1.

Cholesterol and sodium glycocholate were added at the expense of glucose. The cholesterol was dissolved in the melted saturated coconut oil which was then mixed with the rest of the diet.

TABLE 1

Composition of basal diets

	Diet A	Diet B
	%	%
Beef blood fibrin [1]	20	20
Saturated coconut oil	10	10
Powdered cellulose [2]	2	4
Cottonseed oil [3]	1	1
Salts [4]	3.9	3.9
Vitamins [5]	0.15	0.15
Sodium glycocholate	—	0.3
Glucose [6]	62.95	60.65

[1] Armour Adhesive Division, Chicago.
[2] Cellu Flour, Chicago Dietetic Supply House, Chicago.
[3] Wesson Oil, Wesson Oil and Snowdrift Sales Co., New Orleans, Louisiana.
[4] Mixture contained/kg of feed: (in grams) $MnSO_4$, 0.061; NaCl, 2.2; Na_2HPO_4, 4.5; K_2HPO_4, 11.1; $CaHPO_4$, 4.96; Ca lactate, 7.88; $FeSO_4 \cdot 7H_2O$, 1.36; $CaCO_3$, 7.24; and (in milligrams) $CuSO_4$, 10.6; $ZnCO_3$, 10.6.
[5] Mixture contained: (in milligrams) riboflavin, 0.5; thiamine, 37; niacin, 2.5; inositol, 15.00; pyridoxine, 0.5; choline, 100; biotin, 0.01; folic acid, 0.01; Ca pantothenate, 2.5; *p*-aminobenzoic acid, 15; 2-methyl-1,4-naphthoquinone, 0.25; vitamin B_{12}, 2.00; vitamin E, 10; and vitamin A, 30 IU; calciferol, 0.4 units.
[6] Cerelose, Corn Products Company, New York.

Lipids were extracted from hearts and livers by use of the procedure of Folch and co-workers (10). The procedure of Herb and Reimenschnieder (11) was used for polyunsaturated fatty acid analysis, and the Sperry-Webb procedure (12) for the analysis of total cholesterol.

The method described by Barker (13) was used for serum protein-bound iodine analysis.

The magnesium content of blood serum and tissue samples was determined by the method of Young and Gill (14). A modification of the Young and Gill method described by Gardiner et al. (15) was used to determine the magnesium content of basal diets.

The skin samples which were taken from the back of the neck were cleaned of hair, muscle and subcutaneous fat. They were then dried to constant weight in a vacuum oven at 80°C. All tissue, feed and feces samples were ashed in a muffle furnace at 600°.

EXPERIMENTAL

In the first experiment 54 male and 36 female rats were divided equally into 6 experimental groups. The rats in 3 of the groups were fed diet A supplemented with magnesium and contained 88, 138 or 538 ppm magnesium and no added cholesterol or sodium glycocholate. The rats in the other groups were fed diets that contained the same levels of magnesium with 1% cholesterol and 0.3% sodium glycocholate. Magnesium sulfate was added to the diets to obtain the desired levels of magnesium. After 8 weeks the animals were killed by a blow on the head. Hearts and livers were excised, frozen immediately and stored at − 20°C until analyzed. The lipid was extracted from hearts and livers for polyunsaturated fatty acid analyses and total liver cholesterol analysis. Polyunsaturated fatty acid analyses were run on heart and liver samples from animals fed diets without added cholesterol and the level of cholesterol in the liver was determined only on those animals fed diets containing cholesterol and sodium glycocholate.

Skin lesions were observed on rats fed diets containing 88 ppm of magnesium regardless of whether cholesterol was present. These skin lesions were also present

in rats fed diets containing 138 ppm of magnesium when cholesterol and sodium glycocholate were also present; however, no lesions were observed in rats fed diets containing this level of magnesium without cholesterol and sodium glycocholate. The skin lesions were first observed after the second week of the experiment and were usually preceded by hyperemia. They were generally more severe and sustained at the lower level of dietary magnesium.

The skin lesions first appeared as tiny oval scabs. These enlarged and sometimes fused to form open sores that occasionally bled. The skin lesions occurred most commonly on the face and neck although other parts of the body including the ears and paws were also subject to these lesions. Hair loss did not precede or accompany skin lesion development.

Lowering the level of magnesium in the diet reduced the lipid content of the liver (tables 2, 3). Within a given group rats that were free of skin lesions at the end of the experiment tended to have livers with higher lipid content than rats that had skin lesions.

The level of dietary magnesium did not significantly influence the amounts of dienoic and tetraenoic acids in the liver.

The hepatic storage of cholesterol tended to decrease as the dietary magnesium level was reduced but the differences were not statistically significant. There appeared to be greater variability in the hepatic storage of cholesterol at the 88- and 138-ppm magnesium levels than at the 538-ppm level of dietary magnesium.

A second experiment demonstrated that skin lesions also occurred with acute magnesium deficiency. When 7 male rats and 8 females were fed basal diet A without any added magnesium, skin lesions appeared in all the surviving animals. Four male rats and one female died during the experiment. No skin lesions developed in 15 paired littermate animals fed diet A with a magnesium level of 538 ppm.

TABLE 2

Effects of the magnesium level in diets without added cholesterol on the lipids in heart and liver tissues [1]

Group	Magnesium content of diet	Tissue	Lipid content	Tissue dienoic acid	Tissue tetraenoic acid	Avg value tetraenoic/ dienoic acid
	ppm		%	*mg/g*	*mg/g*	
1 [2]	88	Heart	2.26	1.72	1.97	1.20
		Liver	3.40	2.48	3.14	1.33
3	138	Heart	2.33	1.76	1.96	1.12
		Liver	3.74	2.76	3.28	1.21
5	538	Heart	2.32	1.68	2.07	1.33
		Liver	3.87	2.60	3.32	1.31

[1] All values were calculated on the basis of the fresh weight of tissue.
[2] Group having skin lesions.

TABLE 3

Effect of dietary magnesium on liver lipid and cholesterol storage when feeding 1% cholesterol and 0.3% sodium glycocholate

Group	Magnesium content of diet	Lipid content of liver [1]	Cholesterol content of liver lipids	Cholesterol content of liver [1]
	ppm	%	%	%
2 [2]	88	6.88 [3]	14.4	1.03
4 [2]	138	6.97	13.0	1.00
6	538	7.56	17.7	1.47

[1] Based on fresh weight.
[2] Experimental groups having skin lesions.
[3] Significantly lower than group 6 ($P < 0.05$) according to t test.

The acute magnesium deficiency brought about a marked reduction in growth and a moderate but significant reduction in feed efficiency.

In another experiment different levels of magnesium [$(MgCO_3)_4\ Mg(OH)_2 \cdot 4H_2O$] were added to basal diet A to make diets containing 38, 88, 138 and 538 ppm of magnesium. Each diet was fed to 15 weanling rats and the rats were thoroughly inspected each day for skin lesions. Skin lesions first appeared on the sixth day of the experiment when rats were fed the diet containing 38 ppm magnesium and on the eighth day when they were fed the diet with 88 ppm of magnesium.

On the fifth day 5 littermate pairs of animals from the groups at the 38- and 538-ppm magnesium levels were killed to obtain samples of blood plasma, skin and muscle tissue for magnesium analysis. The remaining 10 rats in the group at the 38-ppm level of magnesium were killed on the seventh day. Samples of blood plasma, skin and muscle were taken from 10 animals from each group at the 88-, 138- and 538-ppm magnesium levels on the eighth day of the experiment. The blood samples were taken by cardiac puncture and heparin was used as the anticoagulant. The skin samples were taken from the back of the neck and the muscle was taken from the hind leg.

The development of skin lesions was not immediately preceded by a reduction in the magnesium content of the skin (table 4). In fact the magnesium content of skin was higher when expressed on a fresh weight basis in the groups that developed skin lesions than in the groups without lesions. A significant reduction in the dry weight content of the skin was brought about by lowering the dietary level of magnesium. When the skin magnesium content was expressed as milligrams per 100 g of tissue water, no significant differences were observed. No differences were observed in the magnesium content of muscle.

The results of this experiment were confirmed by a second trial in which 14 rats were divided equally into 2 groups. Rats in one group were fed basal diet A and the others were fed basal diet A with enough magnesium added to raise the magnesium level to 538 ppm.

The observation of a higher magnesium level in skin from magnesium-deficient rats with skin lesions than in skin from rats fed diets with adequate magnesium has been confirmed in this laboratory using a different diet and using an atomic absorption spectrophotometer to facilitate rapid and accurate analyses for magnesium. The details of this experiment will be published later.

To determine the influence of dietary cholesterol on the composition of skin during the onset of skin lesions in rats with a subacute magnesium deficiency an experiment was conducted in which 40 rats were divided into 2 groups and were

TABLE 4

Skin magnesium and skin dry weight changes prior to the development of skin lesions

Group	Magnesium in diet	Days fed diet [1]	Dry wt skin	Skin magnesium wet wt	Plasma magnesium
	ppm		%	*ppm*	*ppm*
			Trial 1		
1	38	5(5)	37.4 ± 0.9 [2]	125 ± 11	9.6 ± 2.3
2	538	5(5)	40.6 ± 1.6	111 ± 4	19.4 ± 0.8
3	38	7(10)	31.2 ± 0.4	185 ± 16	4.9 ± 1.4
4	88	8(10)	30.7 ± 1.5	153 ± 7	6.8 ± 1.4
5	138	8(10)	39.6 ± 1.1	126 ± 4	10.4 ± 0.8
6	538	8(10)	41.8 ± 1.9	123 ± 8	21.5 ± 1.6
			Trial 2		
7	38	5(7)	29.2 ± 1.5	146 ± 10	3.8 ± 1.0
8	538	5(7)	37.6 ± 1.6	121 ± 5	17.8 ± 1.1

[1] Figures in parentheses denote numbers of animals in an experimental group.
[2] Mean and SE.

TABLE 5

Effect of dietary cholesterol on plasma cholesterol, the incidence of skin lesions, skin magnesium and dry skin weight at a dietary magnesium level of 138 ppm

Group	1% Cholesterol	Plasma cholesterol	Plasma magnesium	Skin magnesium	Skin dry wt	Rats with lesions [1]
		mg/100 ml	*ppm*	*ppm*	%	
1	–	50.9±4.2 [2]	5.0±0.5	124±17	35.5±1.6	7(10)
2	+	50.9±3.1	4.3±0.6	151±7	31.2±1.0	10(10)

[1] Figures in parentheses denote total number of animals in experimental group.
[2] Mean and SE of 10 observations.

TABLE 6

Effect of cholesterol on the apparent intestinal absorption of magnesium at a dietary magnesium level of 138 ppm

Group	1% Cholesterol	Apparent intestinal absorption of magnesium	Net magnesium intake	Avg wt gain	Food consumption
		%	*mg*	*g*	*g*
1	–	68.6±2.3 [1]	5.83	26	61.1
2	+	57.3±2.8	4.99	26	62.9

[1] Mean and SE of 5 observations. Group 2 was significantly lower than group 1 ($P < 0.05$) according to *t* test.

given diet B containing 138 ppm of magnesium. One per cent cholesterol was added to the diet for one group. Skin lesions were first noted on the eleventh day of this experiment and at this time 10 rats from each group were killed to obtain samples of skin and blood plasma.

The incidence of skin lesions was again higher in the group with added dietary cholesterol although skin lesions were also observed in the group without added dietary cholesterol. The skin analyses showed that adding cholesterol to the diet caused a decrease in the dry matter content and an increase in the magnesium content of the skin (table 5). These changes were the same as those noted when the dietary level of magnesium was decreased.

To determine the influence of dietary cholesterol on the apparent intestinal absorption of magnesium, 10 female rats were divided equally into 2 experimental groups. Both groups were fed type B diets containing 138 ppm magnesium. One of these diets contained 1% cholesterol, while the other served as a control.

Both groups of rats were fed these diets for 11 days. The rats were placed in individual metabolism cages on the third day of the experiment. Urine and feces samples were collected separately over a 7-day period and records were kept of the weight and food consumption of the animals. On the eleventh day blood was drawn by heart puncture for plasma protein-bound iodine analysis and the thyroid glands were removed and weighed.

Feeding cholesterol significantly ($P < 0.05$) reduced the apparent intestinal absorption of magnesium without reducing food consumption or weight gains (table 6).

Plasma protein-bound iodine and thyroid weight data were obtained to test the possibility that the secretion of thyroxine might be influenced by the dietary cholesterol level. The plasma protein-bound iodine levels of animals with and without dietary cholesterol were 3.3 and 3.4 μg/100 ml respectively. The average thyroid weights of animals with and without dietary cholesterol were 4.4 and 4.2 mg/100 g body weight, respectively. These data indicate that the addition of cholesterol to the diet did not cause any great change in thyroid activity.

DISCUSSION

The results of these experiments indicate that cholesterol may intensify the development of skin lesions in rats with subacute magnesium deficiencies by reducing the intestinal absorption of magnesium.

The observation that the introduction of cholesterol into the diet induced the same changes in skin magnesium content and skin dry weight as that caused by lowering the dietary level of magnesium is consistent with this interpretation. Furthermore cholesterol does not accumulate in the plasma of cholesterol-fed rats prior to skin lesion development.

However, cholesterol may have induced skin lesion development in other ways. Perhaps it is noteworthy that the dry weight of feces is increased significantly by feeding cholesterol. This increase could be accounted for only partially by cholesterol if it were completely unabsorbed. Cholesterol may have, therefore, altered the intestinal absorption of other substances.

Cholesterol may also have depleted glycine and L-cystine by being converted to the bile salts, glycocholic and taurocholic acid (16), and in so doing produced skin lesions.

The fact that magnesium carbonate prevented sores just as effectively as magnesium sulfate indicates that the skin lesions are induced by a magnesium rather than a sulfate deficiency. It was also noted that no skin lesions occurred in rats fed diets containing cholesterol and sodium glycocholate when the dietary magnesium level was adjusted to 538 ppm with magnesium carbonate. Therefore, it is not likely that cholesterol induced skin lesions by promoting a sulfate deficiency.

The specific biochemical abnormality responsible for the skin lesions associated with magnesium deficiency is not known. It is possible that the lack of magnesium decreases the resistance of the skin to infection. Some enzymic reaction vital to the synthesis or repair of skin tissue may be altered by the deficiency.

The observation that the magnesium level in skin was increased rather than decreased by the deficiency is difficult to explain. The magnesium level appeared to be associated with the tissue water. The magnesium level in skin is only about one-half of that observed in most other soft tissues such as muscle, heart and kidney. The development of the skin lesions may cause an increase in the substances which "bind" magnesium in the skin.

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Effects of Isoniazid, Chlortetracycline, and Copper upon Growth and Gastrointestinal Urease Activity of Chicks[1]

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ABSTRACT Results are presented from 4 experiments using 72 lots of 15 chicks and 4 lots of 10 chicks fed a sucrose-casein basal diet supplemented with chlortetracycline (100 ppm), isoniazid (50 or 100 ppm), and copper (3.5 ppm) alone and in all combinations over a 4-week experimental period. Chlortetracycline alone or in combination with isoniazid and copper resulted in increased weight gains, improved efficiency of feed utilization, and lowered gastrointestinal ammonia concentration and ureolytic activity. Incorporation of isoniazid, an anti-tuberculosis agent, resulted in greater weight gains, but the efficiency of feed utilization was not increased. Feeding of isoniazid resulted in lowered gastrointestinal ammonia and ureolytic activity. Copper showed similar but less marked effects. Additive or nearly additive increases in growth were observed when either copper or isoniazid was fed in combination with chlortetracycline, but when copper and isoniazid were combined the growth differences were less than additive. In general, increase in weight gain due to additives appeared dependent on overall growth rate. The data demonstrate that, under the experimental conditions used, isoniazid, chlortetracycline, and copper when added to a sucrose-casein basal diet resulted in increased growth of chicks concurrently with lowered gastrointestinal ammonia concentration and ureolytic activity.

Earlier studies have demonstrated that urea breakdown in the gastrointestinal tract may be altered by dietary anti-microbial agents (1) and by active immunization with crystalline jackbean urease (2). More recent work has demonstrated that barbituric acid and chlortetracycline, when fed to chicks, will enhance growth concurrently with a reduction in the urease activity of the gastrointestinal tract (3). This increase in growth appears to be dependent on the carbohydrate source (4).

Isoniazid, a very effective drug for the treatment of tuberculosis, is readily absorbed from the gastrointestinal tract (5). It is freely diffusible in all body fluids and is distributed to all organs (6). Using both microbial and mammalian enzymes, it has been shown that monoamine and diamine oxidase, guanidine deamidinase (7), transaminase (8), and a number of other enzymes are inhibited by low levels of isoniazid. Wing and Arnold observed that chlortetracycline and isoniazid were synergistic in promoting growth of young calves (9). Ševković et al. recorded considerable improvement of mean daily weight gains and of feed consumption in 2- to 3-month-old pigs fed diets supplemented with isoniazid (10, 11).

Although copper is known to be a dietary essential for chicks, the minimal requirements of this element for growth appear not to have been determined. However, the National Research Council recommends 2 ppm for growing chicks (12). Under ordinary dietary conditions, 90% or more of ingested copper appears in the feces (13). In some experiments the feeding of copper has given growth responses similar to those obtained with antibiotics (14), suggesting that the mode of action may be similar. In others, the effects have been additive (15), and in still others an antibiotic has been effective when copper has not (16). Recent evidence supports a view that the addition of copper to the diet has no observable effect on the bacterial content of the alimentary tract (17).

The effects of isoniazid, chlortetracycline, and copper on chick growth, when added to a purified diet fed for 4 weeks, are reported in the present paper. Data

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on gastrointestinal ammonia concentration and urease activity are also presented.

EXPERIMENTAL

One-day-old Vantress-Arboracre cockerels having an initial weight of 35 to 45 g were used in all experiments. A 28-day growth period was used as described previously (3). Four experiments, including 2 preliminary studies, were conducted with a total of 72 lots of 15 birds/lot and 4 lots of 10 birds/lot. The sucrose-casein basal diet (4) was supplemented with 100 ppm isoniazid or 100 ppm chlortetracycline, or both, in experiment 1 and 14 ppm $CuSO_4 \cdot 5H_20$ (3.5 ppm copper) or 100 ppm chlortetracycline, or both, in experiment 2.

These preliminary experiments were followed by 2 successive experiments, each with 32 lots of 15 chicks (exps. 3 and 4). Additives to the sucrose-casein basal diet in experiment 3 were as follows: (in ppm) diet 1, none; diet 2, isoniazid, 50; diet 3, chlortetracycline, 100; diet 4, copper, 3.5; diet 5, isoniazid, 50, plus copper, 3.5; diet 6, chlortetracycline, 100, plus copper, 3.5; diet 7, isoniazid, 50, plus chloreteracycline, 100; and diet 8, isoniazid, 50, plus chlortetracycline, 100, plus copper, 3.5. Additives in experiment 4 were the same as in experiment 3, with the exception that whenever isoniazid was included, the concentration was 100 ppm.

In experiments 3 and 4, the chicks were fasted 4 hours prior to killing at the end of the 28-day experimental period. Ten randomly selected chicks from each lot were killed and the small and large intestines plus contents were rapidly removed. Intestinal ammonia concentration and urease activity were estimated as described previously (4). The data from each experiment were subjected to analysis of variance using orthogonal comparisons as described by Snedecor (18).

RESULTS

The results of preliminary experiment 1 (table 1) involving 6 lots of chicks indicated increased weight gain when a sucrose-casein basal diet was supplemented by 100 ppm isoniazid or 100 ppm chlortetracycline, or both. The combination of the 2 additives produced essentially additive effects. Similar growth responses were obtained in experiment 2 (table 1) involving another 6 lots of chicks fed the same basal diet supplemented with 3.5 ppm copper or 100 ppm chlortetracycline, or both. No toxic symptoms were observed in any of the chicks over the 4-week period.

Data for growth and efficiency of feed utilization for experiments 3 and 4 are presented in table 2. In general, efficiency of feed utilization paralleled growth rate. In both experiments, increased weight gains were observed in each 2-week period

TABLE 1

Summary of preliminary investigations with cockerels fed a sucrose-casein basal diet supplemented with chlortetracycline, isoniazid, and copper [1]

Diet [2]	Weight gain		
	0–2 Weeks	2–4 Weeks	0–4 Weeks
	g	*g*	*g*
Experiment 1			
Basal (2) [3,4]	85 ± 22 [5]	203 ± 10	289 ± 49
Chlortetracycline (2) [4]	112 ± 19	198 ± 11	311 ± 48
Isoniazid (1) [6]	101 ± 26	211 ± 47	311 ± 71
Chlortetracycline + isoniazid (1) [6]	116 ± 26	218 ± 32	334 ± 56
Experiment 2			
Basal (2) [4]	104 ± 25	138 ± 49	242 ± 67
Chlortetracycline (2) [4]	127 ± 20	201 ± 41	328 ± 56
Copper (1) [6]	119 ± 23	175 ± 33	295 ± 47
Chlortetracycline + copper (1) [6]	139 ± 22	235 ± 43	374 ± 62

[1] Figures in parentheses indicate the number of lots.
[2] Concentrations of additives: chlortetracycline (Lederle Laboratories, Pearl River, N. Y.), 100 ppm; isoniazid (Hoffmann La Roche, Nutley, N. J.), 100 ppm; copper 3.5 ppm.
[3] Proportion of ingredients according to Alvares et al. (4).
[4] Fifteen chicks/lot.
[5] Mean of individual weights ± SD.
[6] Ten chicks/lot.

TABLE 2

Weight gain and efficiency of feed utilization of cockerels fed a sucrose-casein basal diet supplemented with isoniazid, chlortetracycline, or copper, or combinations of these [1]

Diet [2]	Weight gain							
	0–2 Weeks		2–4 Weeks		0–4 Weeks		0–4 Weeks	
	Exp. 3	Exp. 4	Exp. 3	Exp. 4	Exp. 3	Exp. 4	Exp. 3	Exp. 4
	g	*g*	*g*	*g*	*g*	*g*	*g/g feed*	*g/g feed*
Basal	97 ± 6 [3]	69 ± 3	189 ± 5	159 ± 15	286 ± 10	228 ± 18	0.62 ± 0.01	0.55 ± 0.02
Isoniazid	103 ± 6	71 ± 4	195 ± 3	185 ± 4	298 ± 9	257 ± 8 [5]	0.61 ± 0.01	0.58 ± 0.01
Chlortetracycline	104 ± 7	85 ± 3 [4]	199 ± 6 [6]	183 ± 3 [4]	303 ± 11 [6]	269 ± 3 [4]	0.61 ± 0.01	0.60 ± 0.01 [4]
Copper	106 ± 7	78 ± 2	197 ± 8	179 ± 10	304 ± 15	257 ± 11	0.62 ± 0.02	0.58 ± 0.01
Copper + isoniazid	100 ± 6	80 ± 2	191 ± 8	191 ± 9	291 ± 13	272 ± 9 [5]	0.61 ± 0.02	0.57 ± 0.02
Copper + chlortetracycline	109 ± 4	91 ± 5 [4]	206 ± 4 [6]	204 ± 6 [4]	315 ± 3 [6]	295 ± 10 [4]	0.63 ± 0.01	0.62 ± 0.01 [4]
Isoniazid + chlortetracycline	107 ± 3	99 ± 5 [4]	210 ± 6 [6]	201 ± 10 [4]	317 ± 8 [6]	301 ± 14 [4,5]	0.63 ± 0.01	0.60 ± 0.01 [4]
Copper + chlortetracycline + isoniazid	114 ± 7	89 ± 3 [4]	200 ± 6 [6]	197 ± 10 [4]	315 ± 12 [6]	286 ± 8 [4,5]	0.63 ± 0.01	0.61 ± 0.01 [4]

[1] Four lots/treatment, with 15 birds/lot.
[2] Concentrations of additives: Chlortetracycline, 100 ppm; copper, 3.5 ppm; isoniazid, 50 ppm in experiment 3 and 100 ppm in experiment 4.
[3] Mean ± SE.
[4] Main effect of chlortetracycline: 4 treatments with chlortetracycline vs. 4 treatments without chlortetracycline significant at $P < 0.01$.
[5] Main effect of isoniazid: 4 treatments with isoniazid vs. 4 treatments without isoniazid significant at $P < 0.05$.
[6] Main effect of chlortetracycline: 4 treatments with chlortetracycline vs. 4 treatments without chlortetracycline significant at $P < 0.05$.

for all groups receiving additives. In experiment 3, for groups fed chlortetracycline these differences in gains were statistically significant during the 2- to 4-week and zero- to 4-week periods ($P < 0.05$). The average growth for all groups in this experiment was 25% greater than in experiment 4. In the latter experiment, chlortetracycline increased weight gain by 22% ($P < 0.01$), isoniazid by 8%, and copper by 4% during the first 2 weeks. Weight gains during the 2- to 4-week period followed the trends observed in the first 2 weeks. For the overall experimental period of 4 weeks, chlortetracycline increased weight gain by 14% ($P < 0.01$), isoniazid by 6% ($P < 0.05$), and copper by 5%).

In experiment 4, statistical analysis revealed a significant negative interaction between copper and chlortetracycline ($P < 0.05$) during the zero- to 2-week period and a similar but less significant interaction between copper and isoniazid ($P < 0.10$). During the 2- to 4-week period the negative interaction between copper and the other 2 additives was also apparent. If the lots receiving copper were excluded, statistical analysis of the remaining lots revealed a significant increase in weight gain at the 5% level for lots supplemented with isoniazid. For the overall experimental period of 4 weeks the negative interactions were not significant. The combined effects of isoniazid and chlortetracycline appeared to be additive; however, the combined effects of copper, isoniazid, and chlortetracycline were not additive.

Gastrointestinal ammonia concentrations for experiments 3 and 4, expressed as micrograms of ammonia per gram of wet weight, are shown in table 3. The ammonia concentrations in the large intestine were consistently higher than in the small intestine. In experiment 3, the main effects of chlortetracycline, isoniazid, and copper upon the ammonia concentration of the small intestine were depressions of 24 ($P < 0.01$), 19 ($P < 0.01$), and 21% ($P < 0.01$), respectively. The 3 additives showed lower ammonia concentrations in the large intestine, but the effects were of a lesser magnitude. In experiment 4, copper and chlortetracycline significantly

TABLE 3

Ammonia concentration in gastrointestinal contents of 4-week-old cockerels fed a sucrose-casein basal diet supplemented with isoniazid, chlortetracycline, or copper, or combinations of these [1]

Diet	Ammonia			
	Experiment 3		Experiment 4	
	Small intestine	Large intestine	Small intestine	Large intestine
	μg/g wet wt	*μg/g wet wt*	*μg/g wet wt*	*μg/g wet wt*
Basal	155 ± 8 [2]	294 ± 49	143 ± 13	376 ± 48
Isoniazid	119 ± 7 [3]	216 ± 15	109 ± 9	291 ± 40
Chlortetracycline	131 ± 13 [4]	223 ± 62	100 ± 15 [5]	337 ± 40
Copper	141 ± 6 [6]	195 ± 31	99 ± 18 [6]	274 ± 26 [7]
Copper + isoniazid	102 ± 18 [3,6]	273 ± 18	110 ± 10 [6]	361 ± 24 [7]
Copper + chlortetracycline	75 ± 5 [4,6]	208 ± 14	72 ± 14 [5,6]	258 ± 28 [7]
Isoniazid + chlortetracycline	104 ± 19 [3,4]	285 ± 15	118 ± 11 [5]	371 ± 41
Copper + isoniazid + chlortetracycline	84 ± 4 [3,4,6]	179 ± 41	88 ± 8 [5,6]	261 ± 22 [7]

[1] Four lots/treatment, with 10 birds/lot.
[2] Mean ± SE.
[3] Main effect: isoniazid vs. no isoniazid significant at $P < 0.01$.
[4] Main effect: chlortetracycline vs. no chlortetracycline significant at $P < 0.01$.
[5] Main effect: chlortetracycline vs. no chlortetracycline significant at $P < 0.05$.
[6] Main effect: copper vs. no copper significant at $P < 0.01$.
[7] Main effect: copper vs. no copper significant at $P < 0.05$.

TABLE 4

Hydrolysis of urea by gastrointestinal contents of 4-week-old cockerels fed a sucrose-casein basal diet supplemented with isoniazid, chlortetracycline, or copper, or combinations of these [1]

Diet	Urea hydrolyzed			
	Experiment 3		Experiment 4	
	Small intestine	Large intestine	Small intestine	Large intestine
	μg/g wet wt	*μg/g wet wt*	*μg/g wet wt*	*μg/g wet wt*
Basal	68 ± 12 [2]	552 ± 156	115 ± 25	709 ± 57
Isoniazid	40 ± 7	429 ± 54 [3]	81 ± 18 [3]	524 ± 97 [4]
Chlortetracycline	50 ± 14	578 ± 50	111 ± 21	632 ± 76
Copper	58 ± 8	485 ± 91	74 ± 18	498 ± 68 [5]
Copper + isoniazid	57 ± 14	481 ± 48 [3]	70 ± 7 [3]	390 ± 47 [4,5]
Copper + chlortetracycline	59 ± 13	455 ± 14	100 ± 17	370 ± 106 [5]
Isoniazid + chlortetracycline	48 ± 11	327 ± 35 [3]	85 ± 7 [3]	433 ± 41 [4]
Copper + isoniazid + chlortetracycline	52 ± 4	438 ± 65 [3]	67 ± 23 [3]	391 ± 51 [4,5]

[1] Four lots/treatment with 10 birds/lot.
[2] Mean ± SE.
[3] Main effect: isoniazid vs. no isoniazid significant at $P < 0.06$.
[4] Main effect: isoniazid vs. no isoniazid significant at $P < 0.05$.
[5] Main effect: copper vs. no copper significant at $P < 0.01$.

depressed ammonia concentrations in the small intestine, but isoniazid had no effect. In the large intestine, copper significantly depressed ammonia concentration ($P < 0.05$), whereas chlortetracycline and isoniazid had little or no effect.

Gastrointestinal ureolytic activity, expressed as micrograms of urea hydrolyzed per gram of wet weight, is shown in table 4. In experiment 3, chlortetracycline and isoniazid lowered the ureolytic activity in the small intestine by 15 and 6%, respectively, but copper had no effect. In the large intestine, similar trends were observed. In experiment 4, isoniazid and copper suppressed ureolytic activity in the small intestine by 24 ($P < 0.06$) and 21%, respectively, whereas in the large intestine isoniazid, copper, and chlortetracycline suppressed ureolytic activity by 21 ($P < 0.05$), 28 ($P < 0.01$), and 14%, respectively. The gastrointestinal ureolytic activity was consistently higher in experiment 4 than in experiment 3.

DISCUSSION

The results of these experiments demonstrate that chlortetracycline, when fed to chicks either alone or in combination with isoniazid and copper in a sucrose-casein basal diet, results in increased weight gains over a 4-week experimental period. Concurrently with increased weight gain, there was a lowering of gastrointestinal ammonia concentration and ureolytic activity. The reduction in ammonia concentration with antibiotics has been shown by others (19), and the relationship between increased weight gains and depressed ureolytic activity confirms results previously obtained in this laboratory (3, 4). Isoniazid, an anti-tuberculosis agent, when fed at a level of 100 ppm, produced greater weight gains than when fed at 50 ppm. In experiment 3 the overall growth of the chicks was greater, which might account for the lack of effect of isoniazid. Gastrointestinal ammonia concentrations were lower in the groups fed isoniazid alone than in the basal groups. However, this reduction was less consistently observed when isoniazid was combined with other additives. Depressed ureolytic activity was always observed when isoniazid was added alone or in combination with one of the other agents. These data suggest that isoniazid may directly inhibit gastrointestinal urease or it may suppress urease-producing bacteria, leading to a decrease in production of ammonia.

Copper, fed at a level of 3.5 ppm, produced the smallest increase in growth of the additives studied. Incorporation of copper into the diets resulted in lowered ammonia concentrations and ureolytic activity. Copper is known to inhibit urease in vitro (20). This gives evidence which permits the postulate that growth responses observed with copper may be due to lowered ammonia production in the intestines rather than to a change in the bacterial flora. No change in flora was noted by Smith and Jones (17) in their recent work using copper in their diet. The growth data for all 4 experiments show that chlortetracycline, in combination with either copper or isoniazid, resulted in growth responses which were, or tended to be, additive. However, the addition of copper to diets containing isoniazid resulted in growth differences which were considerably less than additive. The fact that cupric ion increases the concentration of isoniazid required to inhibit tubercle bacilli (21), presumably due to alteration of biological activity of isoniazid, may partly explain this observation.

The evidence brought forward in this paper demonstrates that with a sucrose-casein purified diet, addition of isoniazid, chlortetracycline, and copper resulted in increased growth of chicks concurrently with depressed gastrointestinal ammonia concentration and ureolytic activity.

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