THE JOURNAL OF NUTRITION®

OFFICIAL ORGAN OF THE
AMERICAN INSTITUTE OF NUTRITION

VOLUME 84

SEPTEMBER – DECEMBER 1964

PUBLISHED MONTHLY BY
THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY
PHILADELPHIA, PENNSYLVANIA

THE JOURNAL OF NUTRITION ®
® *Trade mark registered, U. S. Patent Office*

Contents

No. 1 SEPTEMBER 1964

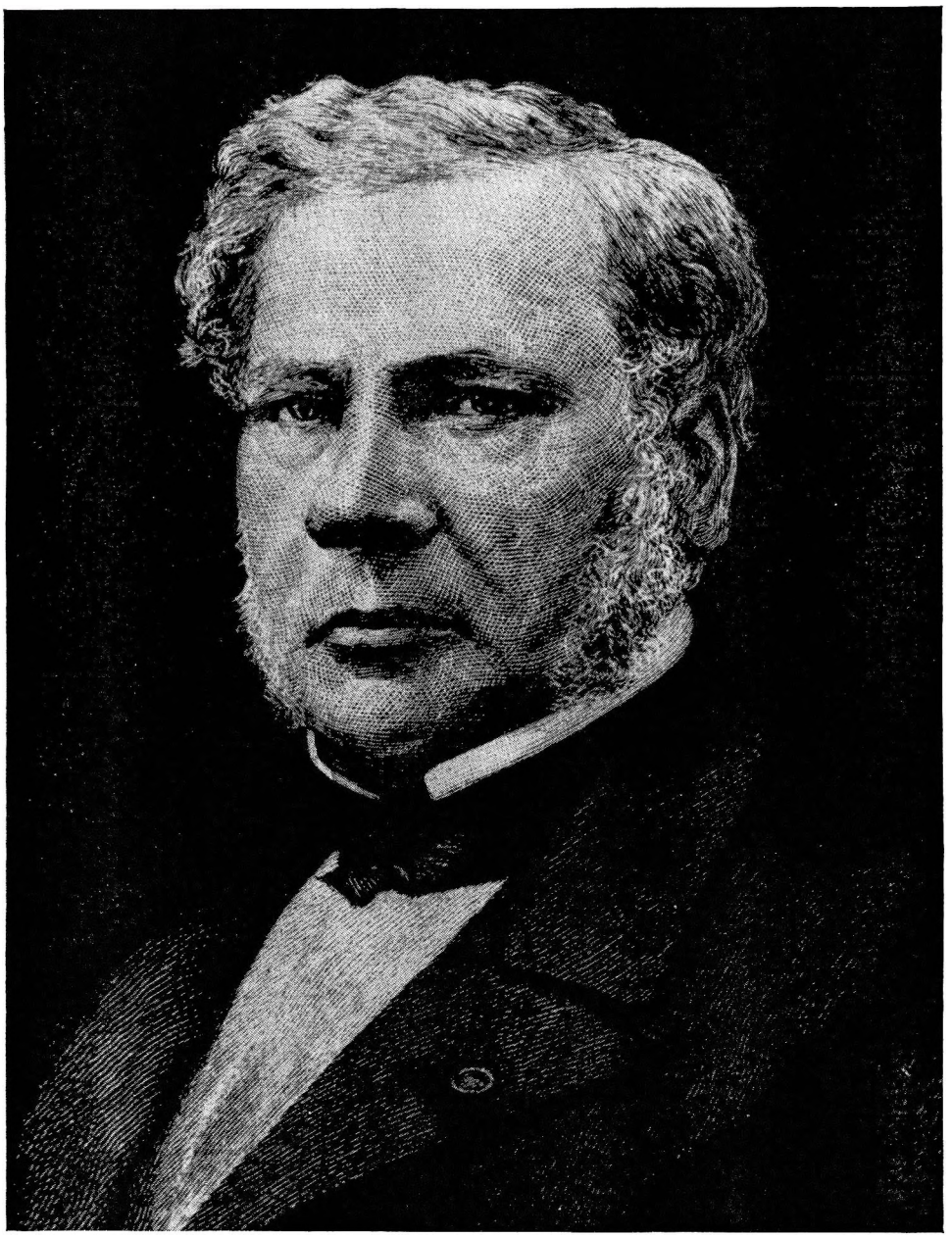
No. 2 OCTOBER 1964

No. 3 NOVEMBER 1964

No. 4 DECEMBER 1964

JEAN BAPTISTE BOUSSINGAULT

(1802 — 1887)



JEAN BAPTISTE BOUSSINGAULT

Jean Baptiste Boussingault — A Biographical Sketch

(February 2, 1802 — May 11, 1887)

A student of nutrition in a tour of Paris would find a visit to the Conservatoire des Arts et Metiers of some historical and scientific interest. In the courtyard is a statue [1] erected as a memorial to the scientist who occupied the chair of agriculture at the Conservatoire for 42 years, from 1845 until his death in 1887. For his studies in nutrition and agricultural chemistry this man occupies an important place in the history not only of these but other scientific disciplines as well.

Jean Baptiste Boussingault was born in Paris on February 2, 1802, the son of a middle-class grocer. His scientific education was obtained at the School of Mines of Saint-Etienne, where, as a student, he was chosen by Thenard to be a preparator in chemistry. Evidently the young student had some talent for research, evidenced by the fact that in the course of his work as a laboratory assistant he made some observations when attempting to melt platinum, and these led to some experiments which were described in a paper entitled, "Note on the Combination of Silicon With Platinum and on its Presence in Steel" (1). This report was published when he was only 19 years of age. On graduating he was called to direct a small asphalt mine in Alsace, and in this work had a close relation with the Director of Mines in Bechelbronn. No doubt this had something to do with his being offered an appointment by the Spanish Government to a professorship at the school of mines in Bogotá, Colombia, South America, which he accepted. During this period the Spanish colonies in South America were in revolt against their mother country. Boussingault joined the rebels and became attached to the staff of the famous Simon Bolivar. When the fighting ceased, Boussingault continued to engage in technical work related to mining, traveling extensively in the region and keeping records of various kinds. During these travels he had the good fortune to become acquainted with the famous German scientist and traveler, Alexander von Humboldt who "warmly praised Boussingault's chemical, meteorological, geographical and astronomical accomplishments" (2). It was during this period that he made his contribution to the problem of iodine supply in relation to goitre which is discussed in some detail later.

On his return to France in 1832, Boussingault was given a doctorate in the sciences and named professor of chemistry on the faculty of Lyon. This position was held for only a short time. He revisited Alsace where he married the sister of a fellow student at St. Etienne. By this marriage Boussingault became, with his brother-in-law, joint proprietor of the estate at Bechelbronn. His brother-in-law was the father of the J. A. Le Bel (1847–1930) who later achieved fame in stereochemistry. On this estate Boussingault established a laboratory so that he could make various studies relating chemistry and physics to problems in agriculture. This laboratory has long been recognized as the first agricultural experiment station in the modern sense. In 1839 the death of Huzard provided an opening in the Academie des Sciences, and Boussingault was elected as the successor. In 1845, Leclerc-Thouin, Professor of Agriculture at the Conservatoire des Arts et Metiers, died. As his successor Boussingault was nominated by the French Academy of Sciences (3). From the time of his marriage Boussingault generally spent about half of the year in Alsace and the other half in Paris.

The revolution of 1848 in France forced him momentarily to change occupations.

[1] The work of the sculptor, Dalou; unveiled in 1895.

He was elected representative of the Department Bas-Rhin to the Constituent Assembly where he sat among the moderate Republicans. He voted against the policies advocated by the distinguished Louis Blanc, against the abolition of the death penalty and in favor of the banning of clubs. He was taken into the Council of State where he participated in drawing up new legislation. Louis Blanc was a publicist, historian and politician who became much interested in the plight of laborers, and his writings were in favor of improving their lot in the socio-economic scene. Blanc is described as having been a forceful orator and an uncompromising idealist who in the Constituent Assembly sat with the extreme "left." From this it would appear that Boussingault did not approve of Blanc's extreme attitude, was more of a realist and was closer to the "middle," but nevertheless somewhat left of the "middle." Regardless of how we may try to classify him now, the government regarded him as being "anti" and this led to his being deprived of his post at the Conservatoire, which act so aroused his professional colleagues that they threatened to resign in a body if he were not reinstated. The imperial government relented and gave him back his post.

The Franco-German Treaty of Frankfort in 1871 forced Boussingault to leave Alsace. He took refuge with his colleague, Holtzer, director of the steel mills near St. Etienne, in whose laboratory he was allowed to continue his studies. This eventually led to his publication in 1875 of "Studies of the Transformation of Iron into Steel by Cementation." (4). The last writing assignment which he gave himself consisted in editing his five-volume Memoirs written for his family and made available to a small circle of readers; they were finally printed posthumously in the years 1892 to 1900.

The scientific contributions of Boussingault are significant to students of nutrition in many ways. Reference has already been made to the fact that his laboratory at Bechelbronn was the first agricultural experiment station in anything like the modern sense. In this connection I cannot do better than to quote McCollum (5, p. 100) who wrote:

> Boussingault deserves the title of founder of scientific agriculture because he studied problems both in the field and in the laboratory. He conducted experiments on soils, crops, fertilizers, the assimilation of atmospheric nitrogen by plants, and changes in composition of seeds during germination, as well as utilization of food by animals, and applied all available knowledge in the planning and conduct of his experiments. He was the friend and collaborator of Dumas, one of the greatest French chemists of all time. All of his studies bear the stamp of philosophic insight.

On examining Boussingault's work on the many topics mentioned by McCollum it is evident that he has many *firsts* to his credit. They deserve some detailed comments.

Nitrogen studies

Among the questions much debated by many of the early workers were whether plants and animals utilize atmospheric nitrogen; whether nitrogen is excreted by the lung; nitrogen balances; and related problems. It had long been known that crops grown on soil where beans had been the preceding crop developed better than on land from which other crops had been harvested. As early as 1806 Vauquelin and Fourcroy (6) showed by numerous analyses of barley, peas and beans that the legumes are richer in "animal matter," by which they meant material partly soluble in acetic acid and consisting in part of glutinous flakes produced on heating. When Boussingault began his work, chemists considered casein of milk, the fibrin of clotted blood, white of egg, gelatin, and the coagulum formed on heating blood serum as distinct chemical substances, but knew nothing of their chemical nature. From his extensive analyses Mulder noticed that these substances contained about the same percentage composition of carbon, hydrogen, nitrogen and oxygen, which suggested the existence of a radical of some sort. For this, in 1839, he proposed the term *protein* (7). Whence came the extra nitrogen found in the legumes: from the soil or the atmosphere?

Boussingault studied this question in the following manner (5, p. 101). Clay soil was ashed to destroy any organic matter present. This soil was placed in porcelain dishes and kept wet with distilled water. Samples of seeds were dried, weighed and

analyzed for carbon, hydrogen and nitrogen by the combustion methods available at that time. Seeds were then planted in the ashed soil and the dishes kept in a glass house that was hermetically sealed but was provided with good illumination by sunlight. During their growth the peas, lucerne (alfalfa) and red clover showed a marked increase in nitrogen content over that of the original seeds; with wheat and oats, however, no nitrogen had been added to what was originally present in the seed. The extra nitrogen in the legumes must have come from the air and not the soil — or so it seemed. The final explanation of these phenomena by the discovery of nitrogen-fixing bacteria in the nodules of the roots of legumes did not come until about 50 years later.

The possible utilization of atmospheric nitrogen was also studied in animals in what was one of the first, if not the first, series of nitrogen balance experiments. Boussingault's early papers on this subject (8) dealt with a 3-day study on a cow and a 24-hour experiment on a horse. The experimental approach was the indirect determination of nitrogen lost through the lungs during a nitrogen balance experiment. The ration and the excreta were analyzed (with the cow, the milk as well). In the 24-hour nitrogen balance period with the horse, 17.2 per cent of the nitrogen taken in could not be found in the excreta. In the 3-day experiment with a cow, 13.5 per cent of the nitrogen was not accounted for in the feces, urine and milk. Boussingault attributed this lost nitrogen to excretion through the lungs. Is it possible that most of it was in excreted ammonia lost by volatilization before the actual analyses?

Three years later he published (9) additional data obtained from a lactating cow and a horse. In the introduction to this paper Boussingault makes a distinction between growth and maintenance; the dietary regimen for the latter he called "la ration d'entretien." A satisfactory food mixture was fed during a preliminary period of one month, the study then made, and a balance sheet set up. He commented that by using the experimental diet for a month previously he was able to conclude that the conditions would be constant during the time the balance data were obtained. As in the earlier experiment, the nitrogen balances showed an appreciable amount of nitrogen unaccounted for. This time, however, he wrote that "it is extremely probable that the nitrogen in the air was not assimilated during the act of respiration;" also, that his results conformed with those of other investigators, particularly those of Dulong who reached the same conclusion on the basis of quite different considerations. He added that he planned other studies to determine whether insects and mollusks can fix atmospheric nitrogen. So far as can be ascertained from the printed records, such additional studies were never made. He did pursue the problem, however, with a turtle dove (10).

The bird was fed millet. One experiment lasted for five days, another for seven. The nitrogen balance sheet showed 35.04 per cent unaccounted for, and this was attributed to loss through the lungs. McCollum suggests that this large loss may have been due to formation of ammonia in the dejecta by bacterial action, and loss of this ammonia by volatilization before the feces were dried for analysis. In this case Boussingault's conclusion of loss of nitrogen via the lungs was erroneous. This error was corrected in the later balance studies reported in 1852 by Bidder and Schmidt (11) on a grown cat fed meat under better-controlled and more exacting experimental conditions; a nearly perfect nitrogen balance was obtained. The slight amount of unaccounted-for nitrogen (only 0.7 per cent of the nitrogen in the meat eaten) was attributed to experimental error.

Rating of foods and feeds by nitrogen content

On the assumption that the nutritive value of foods for formation of blood, muscle, milk and the like might be based on their respective content of nitrogen (an idea held by Mulder and Liebig but not actually tested by them), Boussingault (12) made numerous analyses by the combustion method of Dumas, and from the results drew up a table of equivalents, taking ten pounds of hay as the unit for comparison. He called his table, "Theoreti-

cal Quantities of Different Kinds of Vegetable Feeds Which Will Produce Equal Effects on the Growth of Muscle." Twenty-six items are listed in the table. Interesting groupings of the values are the following: linseed oil cake and vetches, 2; peas, 3; wheat, rye, oats and pea straw, 5; maize, 6; vetch straw, 7; clover hay in flower and lucerne in flower, 8; bran, 9; carrot leaves, 13; potatoes, 28; carrots and green lucerne, 35; potato leaves, 36; white cabbage, 37; old potatoes, 41; wheat straw and barley straw, 52; oat straw, 55; rye straw and turnips, 61.

The balance studies made on cows (13) fed beet roots and potatoes are of particular interest to students of nutrition because, as McCollum has pointed out, these experiments were the first

> to show by chemical analysis, the effects on animals of foods inadequate in quality. Thus he found that when fed *ad libitum* as the sole food potatoes or beet roots, both of which are deficient in protein, were incapable of preventing weight loss in cows. These were the first chemical studies in which animals were restricted to rations derived from a single plant source. From these observations Boussingault drew the conclusion of fundamental importance, that animals could not use nitrogen from the atmosphere to supplement inadequate protein in the rations for synthesis of nitrogenous animal matter (blood, flesh) (5, p. 101).

Digestion and absorption of food

Boussingault performed experiments related to this topic. An excellent example is seen in the study carried out on ducks (14). In one experiment he made a duck swallow 50 grams of gum arabic and after nine hours recovered 46 grams of the gum from the alimentary tract. Using this same technique, he administered different amounts of various foods which could be digested and absorbed within a given time. The ducks were fasted, then fed carefully weighed amounts of the test food. After a stated period the duck was killed. All excreta were collected, as well as the amount of material remaining in the alimentary tract. From these data he calculated the amount of the food that a duck could digest and absorb in one hour. From these data he constructed a table giving comparative values for different foods (5, p. 105). From the one-hour values Boussingault estimated the amounts which could be digested and absorbed in twenty-four hours, and he considered this to represent the duck's capacity to handle that food in one day.

In working out a carbon balance he measured not merely the carbon in the food and dejecta but that in the carbonic acid gas expired in a day. His data led him to two important conclusions: (a) it is impossible for the birds to secure sufficient carbon from "animal matter" and sugars to meet their requirements for the "metamorphosis of matter" within their bodies, and therefore (b) it is necessary that some fat be included in the diet to prevent a deficit of carbon. As he put it:

> The results set forth in this memoir show that albumin, fibrin and casein, although absorbed in considerable amount from the alimentary tract, do not furnish enough combustible elements to the organism, thus explaining why these same substances, although well assimilated, are insufficient if fed as the sole food. For nourishment to be complete there must be enough material to be burned to furnish heat; these nitrogenous bodies need to be in some proportion to non-nitrogenous substances.

Here we see a clear recognition of the importance in nutrition of what the modern worker would call the *calorie* factor as distinguished from the *protein* factor.

Fat formation in the body

Boussingault made interesting contributions to the controversy over the metabolic question whether the animal body can convert carbohydrates to fat. It began about 1843 and came to involve French investigators versus Germans represented chiefly by Liebig and his pupils. The French group endeavored to show by direct experiments that herbivorous animals take as part of their food enough fat to meet their needs for fattening and milk production; and therefore they have no need to form fat, and in fact (?) are unable to do so. An excellent summary of the French ideas of that time is found in the paper presented by Dumas before the French Academy in 1843 (15). The German workers held the opposite view. They cited experiments in which certain animals contain fat and put out in their excreta and milk amounts larger than what was present in their food. In such cases, it seemed obvious that they must have made some fat

from other substances. McCollum points out that the two schools attributed different meanings to the term *fat*. The French chemists considered as fat everything that was extractable by ether. Liebig and his co-workers restricted the meaning to ether-soluble substances that have the general properties of true fat, thus excluding chlorophyll, wax, bile residues in the feces, et cetera. In this connection many workers carried out interesting experiments with bees. Some German workers had claimed that bees can make wax when fed only sugar; but Dumas and Milne-Edwards (16) repeated their work, and upon carefully analyzing the "sugar" fed by these German workers, demonstrated that it contained some wax as an impurity. If pure sugar was fed to bees, they could not survive very long; when given honey which contains some wax (and other things of course), they remained healthy and produced wax. Dumas and Milne-Edwards, in their paper, emphasized the importance of doing good chemical analyses as part of researches of this sort. By numerous experiments with pigs, ducks and cows, Boussingault (17) and Persoz (18) finally obtained data showing the correctness of Liebig's point of view.

In Boussingault's book, *Rural Economy*, (19, p. 562 et seq.) one finds an interesting detailed discussion of this topic in which much literature is cited. One gains a good impression of the kind of critical researcher Boussingault really was from his concluding statement:

> The facts on which that opinion (that body fat merely represents ingested food fat) is based, despite their number, probably are as yet insufficient to constitute a perfectly satisfactory or conclusive theory. New researches are therefore indispensable. It would be requisite to show that a cow kept on a regimen abundant in point of quantity, but as poor as possible in matters analogous to fat, will continue to maintain her condition and yet yield milk abounding in cream; and that it is really possible, as some persons affirm, to fatten animals rapidly on roots and tubers alone.

Distinction between nitrogen of ammonia and urea in urine

Boussingault's extensive analyses of foods, feces, urine, tissues, et cetera, for nitrogen contributed to his making an important discovery of a distinction to be made between the nitrogen of ammonia in urine and that of urea and other substances. Apparently he was the first to do this. He added lime water to the urine until the fluid was alkaline in reaction and then measured the amount of ammonia produced. When this was done with urea solutions, however, ammonia was *not* evolved (20).

Mineral nutrients

Boussingault's interest in mineral substances in nutrition is shown in several papers. In the only experiments he appears to have made on growing organisms as contrasted with adults, he studied the development of mineral substances in the skeleton of the pig (21). Analyses were made of bones of a newborn, a pig 8 months old and a pig 11.5 months old. The analyses were reported as "calcium united to phosphoric acid," "calcium united to carbonic acid," "magnesium" and "phosphoric and carbonic acids." The data reported were the weight of the pig, its dried skeleton and its ash. These appear to be the first estimate of what the diet of the pig must furnish in the way of calcium phosphate in order to provide for normal skeletal development. In his book, *Rural Economy*, (19, p. 553), one finds that he also made some balance studies with a six-month-old calf and a milch cow in calf. The results caused him to emphasize particularly the importance of lime in the ration.

Salt in the ration

Another Boussingault contribution to (22) the subject of mineral nutrients is seen in his experiments with two groups of oxen, three in each group. One was fed the ration with added salt; the other received only the salt present in the ration as such. After one month the appearance and activity of those receiving the added-salt regimen were distinctly superior to those that did not. The latter showed roughness of the hair coat, matting and falling of hair, and abnormal gait, and what Boussingault called a cold temperament ("lente et la froideur de temperament").

Iron

Much later in his career (1872) Boussingault published his data on the iron

content of the bodies of animals (23). To him iron was to be regarded as an essential nutrient. His study was designed to determine the amount of iron which should be in the rations of farm livestock. He reported the iron content of, among other things, a 27-gram mouse, a 32-kilogram sheep, and a fish weighing 182 grams. He gave his estimate of the amount of iron taken in by a cow weighing 600 kilograms and consuming 1.365 grams of iron in the daily ration of hay; and reported that she gave out in 7.52 kilograms of milk 0.135 grams of this element. This same communication to the Academy reported analyses for iron content for thirty-six foods and feeds, several wines and other beverages as well as water from different sources. On the basis of published data on the iron content of blood et cetera, he calculated the iron content provided in the daily rations of French soldiers, English and Irish laborers and others doing hard physical labor; in addition he calculated the iron content of the rations of horses at reserve in the army.

Iodine

The importance of iodine for the prevention and treatment of goitre was appreciated by Boussingault. How he came to be involved in this is of some interest (5, 24).

Iodine was discovered in 1811 by Courtois. Nine years later Coindet in Geneva prescribed it as a remedy for the cure of goitre. As frequently happens, its therapeutic use was excessive by many physicians who prescribed too large doses and thus caused development of symptoms of intoxication. This led to its use falling into disrepute.

> In 1824 Humboldt described the occurrence of goitre in Colombia, South America, and stated that the native Indians knew of a salt deposit which they believed to be remedial for this disease, a property which they did not attribute to other salt sources. A young physician, Dr. Roulin, who had learned in Paris of Coindet's therapeutic use of iodine, went to Colombia, and having learned of the belief in a special curative value for the salt deposit mentioned by Humboldt, secured samples of this and other sources of common salt and requested Boussingault to analyze them (5, p. 106).

He was in Bogotá at this time.

McCollum's account fails to mention any specific reference to a Boussingault publication in this connection. In a long paper which Boussingault published in 1833 after his return to France (24), he reports on his travels into various areas of interest in relation to this subject, the geological formations and types of rocks commonly found there, and speculates as to how iodine came to be present in some salt deposits but not others.

On comparing his analyses of the "good" with the "poor" salts, he noticed that the remedial salts contained appreciably more iodine than many nonremedial ones; or, when compared with certain "poor" salts, they contained iodine in significant amounts, with the "poor" salts lacking it entirely. He attributed the therapeutic value of the salt to its iodine content; and in 1831, just before he returned to France, he advised the Colombian Government to provide for the general distribution of the naturally iodized salts in the interest of the health of the population. Sixty-five years were to pass, however, before Baumann in 1896 was to discover the presence of iodine in the thyroid gland, thus affording a scientific basis for the importance of this element in physiology and medicine; and about one hundred years were to pass before the use of iodized salt for the prevention of goitre was to be placed on a firm foundation and supported by medical authority. According to Para (25), whose paper was published in 1948, the Colombian government at that time was providing for the iodization of salt to the extent of 4 milligrams per 100 grams of salt; in connection with this program the daily intake of salt was estimated to be about 15 grams per person.

Boussingault's successful career was achieved in spite of the fact that he had not enjoyed the university life of a brilliant student. His sole academic experience was that of a student in a school of technology. He mentioned that his vocation was determined by his experience as an assistant chemist in the laboratory of Thenard at the School of Mines. His first publication of research at the early age of nineteen, followed by continued publication of observations made during his sojourn in South America, led to his being granted a doc-

torate and a post as professor of chemistry at Lyon in 1832, although he had only his diploma from the School of Mines as what we would call an undergraduate qualification.

In his later years he presented most of the results of his investigations relating to agriculture in a seven-volume work (26–28), the first of which appeared in 1860, the last in 1884. He was one of the founders of the National Institute of Agronomie. He received many honors from foreign governments and from scientific societies both at home and abroad. In 1878 the Council of the Royal Society (Great Britain) awarded him the Copley Medal for his many and varied contributions to science, especially those related to agriculture. He had as active colleagues in his generation such noted chemists as Thenard, Dumas, Mulder, Liebig, Bidder and Schmidt, and Lawes and Gilbert, to mention but a few. To him all students of agricultural and nutritional biochemistry are indebted for his many contributions made during the nineteenth century (1821 to 1887) when so many fundamental studies were being made in these sciences.

GEORGE R. COWGILL [2]
1225 Charles Street
Pasadena, California

LITERATURE CITED

1. Boussingault, J. B. 1821 Note sur la combinaison du silicium avec le platine, et sur sa presence dans acier. Experiences faites au laboratorie de l'École des Mineurs de Saint-Etienne. Ann. Chim. Phy., *16:* 5.
2. Huntress, E. H. 1952 Biographical digests. IV. Centennials and polycentennials during 1952 with interest for chemists and physicists. Proc. Am. Acad. Arts Sci., *81:* 43.
3. Anonymous 1845 Comp. Rend., *20:* 1371.
4. Boussingault, J. B. 1875 Études sur la transformation du fer in acier par la cementation. Ann. Chim. Phys., *5:* 145.
5. McCollum, E. V. 1957 A History of Nutrition. Houghton Mifflin Company, Boston.
6. Vauquelin, N-L, and A. F. Fourcroy 1806 Annales de Museum d'Histoire Naturell, Tome VII; Phil. Mag., *25:* 176.
7. Mulder, G. J. 1839 Ueber die Proteinverbindungen des Pflanzenreiches. J. Prakt. Chem., *16:* 129.
8. Boussingault, J. B. 1836 Ann. Chim. Phys., *61:* 128.
9. Boussingault, J. B. 1839 Analyses comparées des aliments consommes et des produits rendus par une vache laitiere; recherches entreprises dans le but d'examiner si les animaux herbivores empruntent de l'azote a l'atmosphere. Ann. Chim. Phys., *71:* 113. 128.
10. Boussingault, J. B. 1844 Analyses comparées de l'aliment consommes et des excrements rendus par une tourtelle entreprises pour recherche s'il a exhalation d'azote pendant respiration des granivores. Ann. Chim. Phys., *11:* 433; Compt. Rend., *19:* 73.
11. Bidder, F., and C. Schmidt 1852 Die Verdauungssafte unter der Stoffwechsel. Mitau.
12. Boussingault, J. B. 1844 Economie Rurale. Bechet, Paris, p. 483.
13. Boussingault, J. B. 1844 Experiences sur l'alimentation des vaches avec betteraves et de pommes de terre. Ann. Chim. Phys., *12:* 153.
14. Boussingault, J. B. 1846 Experiences statique sur la digestion. Ann. Chim. Phys., *18:* 444; Compt. Rend., *23:* 569.
15. Dumas, J. B. A., J. B. Boussingault and A. Payen 1843 Recherches sur l'engraissement des bestiaux et la formation du lait. Compt. Rend., *16:* 345.
16. Dumas, J. B. A., and H. Milne-Edwards 1845 Sur la composition de la cire des abeilles. Ann. Chim. Phys., *14:* 400.
17. Boussingault, J. B. 1844 Recherches sur la formation de la graisses chez les animaux. Compt. Rend., *20:* 1726.
18. Persoz, J. F. 1845 Sur la formation de la graisses les oies. Compt. Rend., *21:* 20.
19. Boussingault, J. B. 1845 Rural Economy (translated by Law). B. Bailliére, London.
20. Boussingault, J. B. 1850 Recherche sur la quantité d'ammonique contenue dans urine. Ann. Chim. Phys., *29:* 472.
21. Boussingault, J. B. 1846 Recherches sur le développement de la substance minerale dans le systeme osseux du porc. Ann. Chim. Phys., *16:* 486; Compt. Rend., *22:* 356.
22. Boussingault, J. B. 1848 Suite: Des recherches entreprises pour determiner l'influence que le sel, adjoute a la ration, exerce sur le développement du betail. Ann. Chim. Phys., *22:* 116.
23. Boussingault, J. B. 1872 Du fer contenu dans le sang et dans les ailments. Compt. Rend., *74:* 1353.
24. Boussingault, J. B. 1833 Memoire sur les salines iodiferes des Andes. Ann. Chim. Phys., *54:* 163.
25. Parra, H. 1948 Simple goitre in Colombia. Am. J. Pub. Health, *38:* 820.
26. Bossingault, J. B. 1860–1864 Agronomie, Chimie, Agricole et Physiologie, ed. 2. Revué et Considerablement Augmentée, vols. 1, 2 and 3. Bechet, Paris.
27. Boussingault, J. B. 1869–1870 Agronomie, Chimie, Agricole et Physiologie, ed. 2. Revué et Considerablement Augmentée, vols. 4 and 5. Gauthier-Villars, Paris.
28. Boussingault, J. B. 1878–1884 Agronomie, Chimie, Agricole et Physiologie, ed. 2. Revué et Considerablement Augmentée, vols. 6 and 7. Gauthier-Villars, Paris.

[2] Professor Emeritus of Nutrition, Yale University; Adjunct Professor of Biochemistry and Nutrition, University of Southern California, Los Angeles, California.

Plasma and Liver Lipids of Mice as Influenced by Dietary Protein and Sulfur-containing Amino Acids [1]

G. A. LEVEILLE AND H. E. SAUBERLICH
U. S. Army Medical Research and Nutrition Laboratory, Fitzsimons General Hospital, Denver, Colorado

ABSTRACT The influence of dietary protein level and of sulfur-containing amino acids on plasma and liver lipids was studied in young adult mice. Increasing the level of dietary protein resulted in decreased plasma and liver cholesterol and liver fat levels. A greater hypocholesterolemic effect of dietary protein was observed in mice fed a cholesterol-supplemented diet. The increased level of sulfur-containing amino acids could, in part, account for the hypocholesterolemic effect of dietary protein.

Blood cholesterol levels have been shown to be influenced by a number of dietary variables. In most species studied, dietary protein has been found to be inversely related to circulating cholesterol levels (1–4). The mechanism by which this hypocholesterolemic effect of increased dietary protein is mediated remains obscure. Since growing animals have been used in most studies, it has been difficult to demonstrate a hypocholesterolemic effect of dietary protein unrelated to growth (5). In a number of studies, it has been possible to relate the effect of dietary protein, in part, to its content of sulfur-containing amino acids (5–8).

The influence of dietary protein and amino acids on cholesterol metabolism in the mouse has received relatively little attention. The data presented in this report illustrate the effect of dietary protein and sulfur-containing amino acids on plasma and liver lipid levels of young adult mice.

EXPERIMENTAL

Young adult, male, Swiss-Webster mice were used for all studies. The animals were housed in stainless steel cages having raised wire floors. Four animals were housed per cage and each mouse was identified by ear notches. Temperature and humidity were uniformly maintained for all studies (21°, 50% relative humidity). Food and water were supplied ad libitum and body weight and food consumption were determined at weekly intervals.

The composition of the basal diet used is as follows: (g/100 g of diet) vitamin-free casein, 9; L-cystine, 0.15; salt mixture (USP XIV), 4; non-nutritive fiber, 4; corn oil, 10; vitamin mixture, 0.4,[2] choline chloride, 0.3; glucose, 30; starch, to 100. All additions to the basal diet were made at the expense of starch. Supplemental L-cystine was added to all diets at the rate of 1.67% of the protein. Experiments 1–3 were replicates and their design, as well as that used in experiment 4, are shown in the tables of results.

A 3-week experimental period was used in all experiments except for experiment 3 which was of 2-weeks' duration. At the termination of the experiments, blood was withdrawn by cardiac puncture while the animals were under ether anesthesia. A heparinized syringe was used to prevent coagulation. The liver, after being excised, was frozen and stored at − 20° until analyzed. Plasma cholesterol and lipid phosphorus were determined as previously described (5). Liver fat, cholesterol and lipid phosphorus were also determined as described previously (10).

The data were evaluated statistically by means of regression analysis and the *t* test. In experiments 1, 2 and 3 regression coefficients were calculated and tested to determine significance from zero. In experiment 4, the *t* test was used.

Received for publication April 24, 1964.

[1] The principles of laboratory animal care as promulgated by the National Society for Medical Research were observed.

[2] For composition see Leveille et al. (9).

TABLE 1

Body weight, plasma and liver lipids of mice fed differing levels of protein with or without dietary cholesterol and cholic acid supplementation [1]

Exp. no.	Dietary variables		No. of animals	Final body wt	Plasma		Liver [4]		
	Protein [2]	Cholesterol [3] + cholic acid			Cholesterol	Lipid phosphorus	Fat	Cholesterol	Lipid phosphorus
	% of diet			*g*	*mg/100 ml*	*mg/100 ml*	%	*mg/g*	*mg/g*
1	9	–	9	26.8 ± 3.6 [5]	163 ± 29	—	6.8 ± 0.8	6.2 ± 1.1	1.16 ± 0.09
	18	–	10	27.5 ± 3.0	150 ± 15	—	6.5 ± 1.0	5.5 ± 0.6	1.18 ± 0.10
	27	–	11	28.5 ± 2.0	147 ± 45	—	6.0 ± 1.2	5.9 ± 1.0	1.05 ± 0.21
	36	–	9	28.9 ± 3.6	141 ± 35	—	4.0 ± 1.1	5.3 ± 1.3	0.78 ± 0.21
	9	+	9	27.0 ± 3.0	415 ± 128	—	9.2 ± 3.2	32.9 ± 10.8	0.75 ± 0.17
	18	+	9	28.6 ± 2.2	198 ± 42	—	14.9 ± 3.0	44.5 ± 6.3	1.13 ± 0.08
	27	+	9	28.0 ± 2.9	169 ± 33	—	11.5 ± 2.9	33.6 ± 9.4	1.18 ± 0.32
	36	+	5	28.0 ± 1.5	140 ± 8	—	8.4 ± 1.1	21.5 ± 5.8	1.10 ± 0.07
2	9	–	11	24.6 ± 2.6	151 ± 27	10.98 ± 1.51	5.9 ± 0.9	5.1 ± 0.8	1.14 ± 0.19
	18	–	11	24.6 ± 2.8	133 ± 16	10.11 ± 1.27	6.2 ± 0.7	4.9 ± 0.5	1.11 ± 0.11
	27	–	12	25.9 ± 3.0	145 ± 34	12.02 ± 3.44	5.5 ± 0.9	5.1 ± 0.9	0.96 ± 0.14
	36	–	11	27.1 ± 3.9	141 ± 18	10.93 ± 1.72	5.6 ± 1.0	5.1 ± 0.6	0.94 ± 0.14
	9	+	12	24.7 ± 2.9	473 ± 157	10.12 ± 2.28	17.0 ± 4.1	44.5 ± 10.4	0.77 ± 0.18
	18	+	11	27.6 ± 4.1	349 ± 165	8.96 ± 1.65	13.2 ± 2.9	35.3 ± 9.9	1.02 ± 0.07
	27	+	11	25.2 ± 5.1	253 ± 147	8.62 ± 2.07	14.8 ± 4.5	35.0 ± 17.3	0.99 ± 0.08
	36	+	9	25.1 ± 4.4	189 ± 42	8.23 ± 1.47	8.4 ± 1.6	17.3 ± 7.2	0.89 ± 0.14
3	9	–	15	19.9 ± 3.7	177 ± 33	10.12 ± 1.32	6.2 ± 1.0	5.7 ± 0.9	0.97 ± 0.11
	18	–	15	21.7 ± 2.7	188 ± 44	11.33 ± 2.58	6.8 ± 0.8	6.4 ± 0.6	1.12 ± 0.10
	27	–	15	21.6 ± 2.6	149 ± 44	9.12 ± 2.22	5.8 ± 0.6	5.6 ± 0.5	1.09 ± 0.06
	36	–	15	22.7 ± 2.2	144 ± 19	8.76 ± 1.66	6.0 ± 0.6	5.8 ± 0.5	1.15 ± 0.03
	9	+	15	17.5 ± 2.1	713 ± 231	11.11 ± 2.77	12.8 ± 2.1	46.2 ± 6.3	0.90 ± 0.09
	18	+	15	20.7 ± 3.4	477 ± 149	11.30 ± 2.40	10.2 ± 1.9	39.4 ± 10.2	0.98 ± 0.06
	27	+	15	20.5 ± 3.3	306 ± 82	9.17 ± 1.15	9.3 ± 1.7	34.5 ± 10.3	0.87 ± 0.08
	36	+	13	19.8 ± 3.3	269 ± 94	9.49 ± 2.55	7.7 ± 1.7	24.0 ± 10.2	0.87 ± 0.08

[1] All experiments were of 3-weeks' duration except for experiment 3 which was of 2-weeks' duration.
[2] L-Cystine fed at a level of 1.67% of the protein.
[3] Cholesterol and cholic acid were fed at a level of 1 and 0.2% of the diet, respectively.
[4] All liver values are expressed on a wet-weight basis.
[5] Mean ± SD.

RESULTS

The data presented in table 1 demonstrate the effect of dietary protein on plasma and liver lipids of young adult mice. The regression coefficients of the various parameters on dietary protein level and their significance are shown in table 2.

In general, the observed changes are in the same direction for mice fed a cholesterol-supplemented or unsupplemented diet. However, the magnitude of the differences is considerably greater in the cholesterol-fed mice. The data demonstrate a negative relationship between dietary protein level and plasma cholesterol, liver cholesterol and liver fat. Plasma lipid phosphorus levels are also significantly decreased by increasing dietary protein levels in cholesterol-fed mice. Liver lipid phosphorus levels are not greatly influenced by dietary protein. The agreement between the 3 replicate experiments (table 1) is generally good with the exception of liver lipid phosphorus levels. The response of this parameter to dietary protein is apparently reversed by cholesterol feeding. In experiments 1 and 2, liver lipid phosphorus levels were inversely related to dietary protein level in mice fed a cholesterol-free diet. In these same 2 experiments (1 and 2), in cholesterol-fed mice, liver lipid phosphorus levels were positively related to dietary protein intake. However, the validity of this observation is questionable since this effect of dietary cholesterol was reversed in experiment 3. It may be that the difference in response between these experiments can be related to the differing length of the experimental period (3 vs. 2 weeks), but evidence is lacking to support such a contention.

The influence of sulfur-containing amino acids on plasma and liver lipids is illustrated by the data presented in table 3. The effect of dietary protein is consistent with the results of experiments 1–3 (table 1). A supplement of sulfur-containing amino acids, equivalent to that found in the high protein diet, was without effect when added to a cholesterol-free, low protein diet. In mice fed the cholesterol-containing diet, the supplement of sulfur-containing amino acids depressed plasma and liver cholesterol and liver lipid phosphorus levels, but increased liver fat levels. However, the decrease in lipid levels induced by the amino acid supplement was not as great as that observed as a result of feeding the high protein diet.

DISCUSSION

The data presented in this report are in general agreement with results of similar studies in other species. It has been shown that in the rat and chick, sulfur-containing amino acids can account, in part, but not completely, for the hypocholesterolemic effect of increased dietary protein (5, 7, 11). The data presented in this report are consistent with this observation. Although sulfur amino acid supplements decreased plasma lipid levels and liver cholesterol levels of cholesterol-fed mice, the observed decrease was less than that observed in animals fed a protein-supplemented diet supplying an equivalent amount of sulfur-

TABLE 2

Regression of plasma and liver lipids of mice on level of dietary protein [1]

Exp. no.	Dietary cholesterol + cholic acid	Measurement				
		Plasma		Liver		
		Cholesterol	Lipid phosphorus	Fat	Cholesterol	Lipid phosphorus
1	−	− 0.779	—	−0.101 [2]	−0.026	−0.014 [2]
	+	−10.315 [2]	—	−0.040	−0.429 [3]	+0.014 [2]
2	−	− 0.203	+0.020	−0.050 [2]	+0.002	−0.008 [2]
	+	−10.684 [2]	−0.069 [3]	−0.261 [2]	−0.870 [2]	+0.005
3	−	− 1.538 [2]	−0.069 [3]	−0.016	−0.0002	+0.006 [2]
	+	−19.095 [2]	−0.077 [3]	−0.190 [2]	−0.716 [2]	−0.002

[1] Values in table are regression coefficients.
[2] Significantly different from zero ($P < 0.01$).
[3] Significantly different from zero ($P < 0.05$).

TABLE 3

Body weight, plasma and liver lipids of mice fed different levels of protein and sulfur amino acids in cholesterol-supplemented and unsupplemented diets; probability of differences being significantly different (exp. 4)

Treatment group	Treatment	No. of animals	Final body wt	Plasma		Liver [1]		
				Cholesterol	Lipid phosphorus	Fat	Cholesterol	Lipid phosphorus
			g	*mg/100 ml*	*mg/100 ml*	*%*	*mg/g*	*mg/g*
	No added cholesterol							
1	9% Casein +0.15% L-cystine	14	28.1 ± 4.2 [2]	141 ± 22	10.23 ± 1.45	7.5 ± 0.6	6.4 ± 0.7	1.14 ± 0.08
2	36% Casein +0.60% L-cystine	10	26.6 ± 3.1	128 ± 20	11.33 ± 2.29	7.4 ± 1.2	5.8 ± 0.8	1.20 ± 0.08
3	9% Casein + 0.60% L-cystine + 1.19% DL-methionine	13	25.9 ± 4.3	140 ± 41	11.20 ± 2.55	7.6 ± 2.7	5.6 ± 1.6	1.00 ± 0.10
	1% Cholesterol + 0.2% cholic acid							
4	9% Casein +0.15% L-cystine	16	27.9 ± 2.8	377 ± 109	9.83 ± 1.88	13.6 ± 1.8	45.2 ± 6.6	1.02 ± 0.11
5	36% Casein +0.60% L-cystine	15	29.3 ± 2.3	152 ± 31	8.12 ± 1.33	7.5 ± 2.2	11.0 ± 6.7	0.72 ± 0.28
6	9% Casein + 0.6% L-cystine + 1.19% DL-methionine	15	27.1 ± 4.0	186 ± 24	9.52 ± 3.03	20.2 ± 4.2	32.4 ± 5.6	0.55 ± 0.04
				Statistical analysis [3]				
Comparison								
1 vs. 2		—	—	ns	ns	ns	+	ns
1 vs. 3		—	—	ns	ns	ns	ns	ns
2 vs. 3		—	—	ns	ns	ns	ns	++
4 vs. 5		—	—	++	++	++	++	++
4 vs. 6		—	—	++	ns	++	++	++
5 vs. 6		—	—	++	ns	++	++	++
1 vs. 4		—	—	++	ns	++	++	ns
2 vs. 5		—	—	ns	++	ns	+	++
3 vs. 6		—	—	++	ns	++	++	++

[1] All liver values expressed on a wet-weight basis.
[2] Mean ± SD.
[3] ns = not significant; +, $P < 0.05$; ++, $P < 0.01$.

containing amino acids. Consequently, in the mouse as in other species studied, sulfur amino acids appear to be only partly responsible for the hypocholesterolemic effect of dietary protein.

The mechanism(s) by which either increased dietary protein or sulfur-containing amino acids depress plasma or liver cholesterol levels is not clear. Nishida et al. (12, 13) presented evidence indicating that the serum cholesterol depression observed in chicks fed a high protein diet was the result of a decrease in cholesterol synthesis and an increased conversion of cholesterol to bile acids.

Herrmann (14) has suggested that the sulfur-containing amino acids may depress serum cholesterol levels of cholesterol-fed rats by increasing the amount of taurine available for conjugation with cholic acid. However, the observations of Seidel et al. (7) indicate that such a mechanism cannot account completely for the hypocholesterolemic effect of methionine. Obviously, further study is necessary to elucidate the mechanism(s) by which dietary protein and sulfur-containing amino acids depress serum cholesterol levels.

ACKNOWLEDGMENTS

The authors express their gratitude to Gerhard Isaac for statistical evaluation of the data and to Wayne Goad, Joseph Edelbrock and Harold Schneider for technical assistance.

LITERATURE CITED

1. Moyer, A. W., D. Kritchevsky, J. B. Logan and H. R. Cox 1956 Dietary protein and serum cholesterol in rats. Proc. Soc. Exp. Biol. Med., *92:* 736.
2. Mayer, J., and A. K. Jones 1953 Hypercholesteremia in the hereditary obese-hyperglycemic syndrome of mice. Am. J. Physiol., *175:* 339.
3. Leveille, G. A., and H. E. Sauberlich 1961 The influence of dietary protein level on serum-protein components and cholesterol in the growing chick. J. Nutrition, *74:* 500.
4. Mann, G. V., S. B. Andrus and A. McNally 1953 Experimental atherosclerosis in Cebus monkeys. J. Exp. Med., *98:* 195.
5. Leveille, G. A., J. W. Shockley and H. E. Sauberlich 1962 Influence of dietary protein level and amino acids on plasma cholesterol of the growing chick. J. Nutrition, *76:* 321.
6. Fillios, L. C., and G. V. Mann 1954 Influence of sulfur amino acid deficiency on cholesterol metabolism. Metabolism, *3:* 16.
7. Seidel, J. C., N. Nath and A. E. Harper 1960 Diet and cholesterolemia. V. Effects of sulfur-containing amino acids and protein. J. Lipid Res., *1:* 474.
8. Mann, G. V. 1960 Experimental atherosclerosis. Effects of sulfur compounds on hypercholesterolemia and growth in cysteine-deficient monkeys. Am. J. Clin. Nutrition, *8:* 491.
9. Leveille, G. A., H. E. Sauberlich and J. W. Shockley 1962 Protein value and the amino acid deficiencies of various algae for growth of rats and chicks. J. Nutrition, *76:* 423.
10. Leveille, G. A., and H. E. Sauberlich 1963 Lipid changes in plasma, alpha-lipoproteins, liver and aorta of chicks fed different fats. Proc. Soc. Exp. Biol. Med., *112:* 300.
11. Nishida, T., F. Takenaka and F. A. Kummerow 1958 Effect of dietary protein and heated fat on serum cholesterol and beta-lipoprotein levels and on the incidence of atherosclerosis in chicks. Circulation Res., *6:* 194.
12. Nishida, T., A. Yeno and F. A. Kummerow 1960 Effect of dietary protein on the metabolism of sodium acetate-1-C^{14} in chicks. J. Nutrition, *71:* 379.
13. Nishida, T., A. Yeno and F. A. Kummerow 1960 Metabolism of cholesterol-4-C^{14} in bile duct cannulated chicks and rats. Circulation Res., *8:* 742.
14. Herrmann, R. G. 1959 Effect of taurine, glycine and β-sitosterols on serum and tissue cholesterol in the rat and rabbit. Circulation Res., *7:* 224.

Effect of Linoleic Acid upon the Metabolism of Linolenic Acid [1]

JOSEPH J. RAHM AND RALPH T. HOLMAN
University of Minnesota, The Hormel Institute, Austin, Minnesota

ABSTRACT Weanling rats were fed a fat-free diet supplemented with various ratios of corn and linseed oils to furnish a constant dietary level of linolenate at 1% of calories and levels of linoleate from 0.3 to 17.3% of calories. The fatty acid composition of the total liver lipids was analyzed by gas chromatography. Increasing amounts of dietary linoleate suppressed the levels of the 20:5, 22:5 and 22:6 metabolites of linolenic acid in the liver lipids. The level of dietary vitamin E had no effect upon this phenomenon. When the level of dietary linoleate was increased, the level of 22:4 in the liver lipids, as well as the other metabolites of linoleate, was shown to increase.

Recent investigations (1–6) have shown that dietary linolenate or other polyenoic acids reduce the levels of tissue fatty acids that are derived from linoleic acid.[2] On the other hand, Mohrhauer and Holman (6) observed that, at low levels of diet supplementation, the concentration of linoleate could exceed that of linolenate by a factor of twelve without causing a measurable reduction in the levels of the linolenate metabolites in the tissues. If a competitive inhibition exists between linoleate and linolenate metabolisms, inhibitions should be demonstrable in both directions. The inhibition of linolenate metabolism by linoleate is suggested by a re-examination of studies in which polyunsaturates were measured by alkaline isomerization (7–10).

The purpose of the present experiment was to describe more fully the inhibition of linolenate metabolism by linoleate. The content of specific linolenate metabolites in liver lipids as a function of high levels of dietary linoleate was determined using gas chromatographic analysis (GLC). Feeding increasing amounts of polyunsaturated fatty acids without a corresponding increase in vitamin E could result in the formation of deleterious lipid peroxides (11). The possibility that lipid autoxidation may be responsible for the observed inhibitory effects was studied by determining the fatty acid compositions of the liver lipids from rats fed a high level of polyunsaturates and 2 levels of vitamin E. The level of vitamin E used in all experiments described is considered adequate for diets containing normal amounts of polyunsaturated acids. A level of tocopherol, 4.5 times that of the former, was included in the study to test the possibility that tocopherol is a limiting factor when the high levels of polyunsaturates were fed.

EXPERIMENTAL

Forty-eight weanling, male rats of the Sprague-Dawley strain were fed ad libitum either a fat-free, basic diet described previously (5),[3] or the basic diet with corn oil and linseed oil substituted for equal weights of sucrose. The fatty acid composition of the linseed oil supplement was: palmitic, 7.6; stearic, 3.2; oleic, 20.1; linoleic, 16.4; and linolenic, 52.7; and that of the corn oil was palmitic, 13.4; stearic, 1.9; oleic, 27.7; linoleic, 56.0; and lino-

Received for publication April 25, 1964.

[1] This investigation was supported in part by Public Health Service Research Grant no. AM 04524 from the National Institutes of Health, the Life Insurance Medical Research Fund and the National Dairy Council.

[2] Century, B., and M. K. Horwitt 1963 Relationship of arachidonic acid to nutritional encephalomacia: Implications concerning essential fatty acids in tissues. Federation Proc., 22: 608 (abstract).

[3] The composition of the basic fat-free diet was as follows: (in per cent) vitamin-test casein, 18.0; sucrose, 74.0; salt mixture, 4.0; α-cellulose, 4.0; and vitamin mixture. The vitamin mixture contained: (in mg/kg of diet) vitamin A, 4.00; thiamine, 30.00; riboflavin, 30.00; pyridoxine, 8.00; vitamin B_{12}, 0.05; vitamin D_2, 4.00; vitamin E, 230.00; vitamin K_5, 2.00; DL-Ca pantothenate, 100.00; niacin, 100.00; *i*-inositol, 220.00; *p*-aminobenzoic acid, 75.00; biotin, 0.20; folic acid, 1.00; and choline chloride, 1000.00. Salt mixture: Wesson, L. G., Science, 75: 339, 1932. All components of the basic diet except sucrose were obtained from Nutritional Biochemicals Corporation, Cleveland.

TABLE 1

Fatty acid composition of liver lipids (% of total methyl esters)

Group no.	Vitamin E	Dietary fatty acid 18:2ω6	Dietary fatty acid 18:3ω3	No. days fed supplement	Wt gain	14:0 (14.00) [1]	16:0 (16.00)	16:1ω7 (16.78)	18:0 (18.00)	18.1ω9 (18.67)	18:2ω6 (19.47)	18:3ω3 (20.61)	20:3ω9 (21.85)
	mg/kg diet	*% of calories*			*g*								
1	230	0	0	90	194 ± 12 [2]	1.06 ± 0.08 [2]	27.70 ± 0.81	12.07 ± 0.62	11.71 ± 0.66	30.90 ± 0.73	1.40 ± 0.10	— [3]	9.94 ± 0.75
2	230	0.28	1.10	90	238 ± 14	1.29 ± 0.05	27.74 ± 0.59	9.15 ± 0.61	11.88 ± 0.42	25.04 ± 0.55	2.74 ± 0.07	1.04 ± 0.11	1.07 ± 0.11
3	230	0.97	1.06	90	262 ± 8	1.25 ± 0.15	28.44 ± 0.76	7.55 ± 0.46	11.85 ± 0.60	23.52 ± 1.18	6.65 ± 0.85	0.89 ± 0.03	0.57 ± 0.06
4	230	1.97	1.10	90	247 ± 13	0.83 ± 0.08	25.71 ± 0.29	5.57 ± 0.28	13.37 ± 0.70	20.45 ± 1.17	11.04 ± 0.51	0.88 ± 0.12	0.49 ± 0.09
5	230	3.87	1.09	90	266 ± 11	0.79 ± 0.04	25.78 ± 0.70	5.78 ± 0.33	13.42 ± 0.35	18.41 ± 0.70	12.49 ± 0.51	0.66 ± 0.06	—
6	230	7.57	1.04	90	232 ± 14	0.72 ± 0.07	20.97 ± 0.89	2.90 ± 0.63	14.71 ± 0.85	13.76 ± 0.99	21.05 ± 1.10	0.97 ± 0.05	—
7	230	12.42	1.02	90	242 ± 16	0.53 ± 0.04	19.61 ± 0.65	1.79 ± 0.23	14.62 ± 0.77	13.34 ± 0.58	25.12 ± 0.78	0.94 ± 0.09	—
8	230	17.26	0.91	90	263 ± 13	0.49 ± 0.04	18.31 ± 0.54	1.45 ± 0.02	14.50 ± 1.03	12.45 ± 0.74	28.66 ± 1.65	0.95 ± 0.07	—
9	230	17.26	0.91	6		0.69 ± 0.04	19.93 ± 0.37	2.66 ± 0.36	13.71 ± 0.51	17.82 ± 0.89	25.47 ± 1.09	0.60 ± 0.05	0.46 ± 0.03
10	1035	17.26	0.91	6		0.57 ± 0.05	18.90 ± 1.24	2.87 ± 0.42	12.94 ± 0.45	19.99 ± 1.13	24.20 ± 1.75	0.61 ± 0.07	0.66 ± 0.09

Group no.	Vitamin E	Dietary fatty acid 18:2ω6	Dietary fatty acid 18:3ω3	No. days fed supplement	Wt gain	20:3ω6 (22.14)	20:4ω6 (22.72)	20:5ω3 (23.80)	22:4ω6 (24.55)	22:5ω6 (25.22)	22:5ω3 (25.70)	22:6ω3 (26.32)
	mg/kg diet	*% of calories*			*g*							
1	230	0	0	90	194 ± 12 [2]	—	3.59 ± 0.25	0.27 ± 0.03	—	0.59 ± 0.02	—	0.63 ± 0.05
2	230	0.28	1.10	90	238 ± 14	0.55 ± 0.04	3.04 ± 0.17	4.35 ± 0.15	—	—	2.41 ± 0.27	9.68 ± 0.33
3	230	0.97	1.06	90	262 ± 8	0.88 ± 0.08	5.71 ± 0.26	3.20 ± 0.30	—	—	2.17 ± 0.11	7.27 ± 0.81
4	230	1.97	1.10	90	247 ± 13	1.23 ± 0.09	8.78 ± 0.41	2.17 ± 0.18	—	—	2.40 ± 0.22	6.94 ± 0.67
5	230	3.87	1.09	90	266 ± 11	1.59 ± 0.11	12.98 ± 0.70	1.09 ± 0.05	—	—	1.54 ± 0.15	5.41 ± 0.27
6	230	7.57	1.04	90	232 ± 14	1.18 ± 0.04	16.83 ± 0.54	0.45 ± 0.05	0.43 ± 0.08	0.28 ± 0.03	1.04 ± 0.10	4.66 ± 0.34
7	230	12.42	1.02	90	242 ± 16	0.85 ± 0.12	17.36 ± 0.78	0.22 ± 0.01	0.41 ± 0.02	0.45 ± 0.03	0.77 ± 0.05	4.05 ± 0.38
8	230	17.26	0.91	90	263 ± 13	0.85 ± 0.08	17.04 ± 1.16	—	0.60 ± 0.06	0.36 ± 0.04	0.64 ± 0.12	3.64 ± 0.20
9	230	17.26	0.91	6		1.09 ± 0.11	12.59 ± 0.82	0.16 ± 0.01	0.40 ± 0.03	0.60 ± 0.08	0.59 ± 0.04	3.25 ± 0.35
10	1035	17.26	0.91	6		1.07 ± 0.22	11.82 ± 0.59	0.16 ± 0.01	0.31 ± 0.03	0.50 ± 0.06	0.57 ± 0.05	2.67 ± 0.14

[1] Carbon number (12).

[2] SE.

[3] Content less than sensitivity of analytical method.

lenic, 1.0, according to GLC analyses on ethylene glycol succinate columns. The 2 oils were mixed in various proportions to provide approximately 1% linolenate and 0.3 to 17.3% linoleate, expressed as percentage of total calories of the diet. The animals were divided according to diet into 8 groups of 6 animals each. The actual amounts of linoleic and linolenic acids in the diet of each group, expressed as percentage of calories, are shown in table 1. After 90 days the animals were killed and the livers removed and kept in saline solution at $-20°$ until analysis was performed.

The influence of vitamin E upon the inhibitory effects of high levels of linoleate was studied by subjecting 2 groups of 12 weanling male rats to the following dietary treatment. After being fed the fat-free basic diet for 5 months, the animals received for 6 days a supplement of corn and linseed oils that represented 17.3 and 0.9% of total calories of linoleate and linolenate, respectively. During the 6-day period of supplementation, six of the animals received the amount of vitamin E (α-tocopherol) that was present in the fat-free basic diet (230 mg/kg diet), and 6 animals received 4.5 times that amount of vitamin E (1035 mg/kg diet).

The livers of the experimental animals were homogenized in a mechanical blender and the lipids were extracted with chloroform:methanol (2:1). The lipids were transesterified by refluxing with 30 volumes of 5% HCl in methanol. The resulting methyl esters were analyzed by GLC as described previously (6). The ester peaks on the GLC charts were identified by using internal standards with known structures previously determined by ozonolysis and reduction. Quantification was carried out by triangulation, and the results are reported as area percentage. The fatty acid composition of the liver lipids was determined for each animal and reported as the average of 6 animals with the standard errors.

To distinguish structural isomers and to denote metabolic relationships, the fatty acids are referred to according to the number of carbons that are located after the terminal double bond in the molecule. Thus, ω3 acids are related to linolenate, ω6 acids to linoleate and ω9 acids to oleate.

RESULTS

The average weight gain of the animals that received the lowest level of corn oil–linseed oil mixture in their diet was 56 g higher than that of the animals fed a fat-free diet. The animals fed diets containing higher levels and proportions of corn oil did not exhibit correspondingly increased weight gains. The dermal symptoms of fat deficiency were evident only in the animals fed the fat-free diet.

The fatty acid composition of the liver lipids is shown in table 1. The levels of myristic (14:0), palmitic (16:0), palmitoleic (16:1ω7) and oleic (18:1ω9) acids decreased, and the levels of stearic acid (18:0) increased when the level of linoleate (18:2ω6) in the diet was increased from zero to 17% of total calories. These changes in fatty acid composition of liver lipids correspond to those measured by Tove and Smith (13) in the depot fat of mice that had been fed diets supplemented with safflower oil. Linoleic acid (18:2ω6) and arachidonic acid (20:4ω6) in the liver lipids increased markedly with an increase of linoleate in the diet. An increase in dietary linoleate also caused small but measurable increases in 3 proposed metabolites of 18:2ω6, namely, 20:3ω6 (14), 22:4ω6 and 22:5ω6 (5, 15).

The levels of linolenic acid (18:3ω3) in the tissue lipids were not influenced by increasing 18:2ω6 in the diet. However, the levels of 20:5ω3, 22:5ω3 and 22:6ω3, all metabolites of linolenate (16), were decreased when dietary 18:2ω6 was increased (fig. 1). Different levels of vitamin E in the diet did not influence the inhibitory effect of linoleate upon the metabolism of linolenate (table 1). The contents of polyunsaturated acids in the 2 groups of rats receiving the 2 levels of α-tocopherol were not significantly different.

DISCUSSION

The results of the present study indicate that when linolenate and linoleate are both components of the diet, the levels of the metabolites of linolenate in the tissue lipids are decreased when the level of

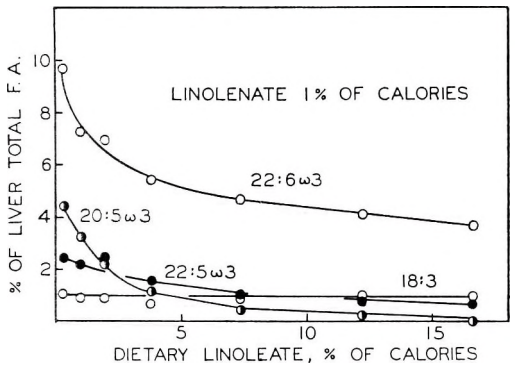


Fig. 1 Effect of varying levels of dietary linoleate upon the levels of linolenate and its metabolites in liver lipids.

dietary linoleate is increased. The constancy of the levels of linolenate in the tissue lipids (fig. 1) precludes dilution as the cause for the depressed content of its higher metabolites. Neither can the inhibitory effects of high levels of dietary linoleate be attributed to the toxic effects of lipid peroxides since increasing the amount of antioxidant in the diet did not influence the degree of inhibition (table 1).

Previous investigations in this laboratory (6) have shown that a similar inhibitory relationship exists between the levels of dietary linolenate and the metabolites of linoleate in tissue lipids. Thus, it appears that the equilibrium of the previously suggested competition (6) between 18:2ω6 and 18:3ω3 can be displaced in either direction, and that the acid which is favored in this competition is dependent upon the relative dietary levels of these acids.

The major changes in the levels of 18:3ω3 metabolites occurred before the levels of 18:2ω6 in the diet reached 8% of calories (fig. 1). Mohrhauer and Holman (6) have shown that when rats were fed increasing levels of 18:3ω3, the major changes in the levels of 18:2ω6 metabolites occurred before the dietary level of 18:3ω3 reached 1% of calories. This observation is in agreement with a suggestion made previously (6) that linolenate is the favored fatty acid in this competition.

The nutritional nature of the present investigation prevents the interpretation of the results in terms of a detailed metabolic mechanism. However, it appears likely that the competition observed here occurs either during the incorporation of the fatty acids into components of the different lipid classes, or during the reactions involved in the interconversion of polyunsaturated fatty acids.

Recently our colleagues in this Institute (17) have shown that in vivo interconversions of polyunsaturated fatty acids can be studied in feeding experiments of relatively short duration. This is evidenced in table 1 which shows that feeding the same fatty acid supplement for 6 days to fat-deficient rats or for 90 days to weanling rats resulted in qualitatively similar fatty acid compositions of liver lipids. The utility of short-term feeding experiments in studying fatty acid interconversions has been further confirmed in this laboratory by several unpublished studies.

Recent studies (5, 15) have indicated that 22:5ω6 is derived from 18:2ω6 via 20:4ω6. During the course of the present study, 22:4ω6 [4] appeared in the tissue lipids in measurable amounts only after feeding 18:2ω6 at levels above 7.5% of calories. This correlation of the levels of 22:4ω6 in the tissue lipids with the levels of dietary linoleate and the similarity of its structure to that of linoleate suggest that 22:4ω6 is an intermediate in the synthesis of 22:5ω6. Thus, if the preferred pathway of linoleate metabolism is reflected by steady state concentrations of intermediates, it is probably as follows:

18:2ω6 → (18:3ω6) → 20:3ω6 → 20:4ω6 → 22:4ω6 → 22:5ω6.

All metabolites in this mechanism, except 18:3ω6, known to be a precursor of arachidonate (18), have been detected in rat liver and were found to increase with the level of dietary linoleate in the present study.

LITERATURE CITED

1. Klenk, E., and K. Oette 1960 The C_{20}- and C_{22}-polyenoic acids of rat liver phosphatides after addition of linoleic or linolenic acid to a fat-free diet. Ztschr. Physiol. Chem., *318*: 86.
2. Marco, G. J., L. F. Machlin, E. Emery and R. S. Gordon 1961 Dietary effects of fats

[4] 22:4 ω 6 was identified by the use of an internal standard that was isolated from beef testis lipids. The standard fatty acid was characterized as 22:4 ω 6 in this laboratory by H. Hofstetter using the method of ozonolysis and reduction.

upon fatty acid composition of the mitochondria. Arch. Biochem. Biophys., *94:* 115.
3. Machlin, L. J. 1962 Effect of dietary linolenate on the proportion of linoleate and arachidonate in liver fat. Nature, *194:* 868.
4. Edwards, H. M., and J. G. Marion 1963 Influence of dietary menhaden oil on growth rate and tissue fatty acids of the chick. J. Nutrition, *81:* 123.
5. Mohrhauer, H., and R. T. Holman 1963 The effects of dose level of essential fatty acids upon the fatty acid composition of the rat liver. J. Lipid Res., *4:* 151.
6. Mohrhauer, H., and R. T. Holman 1963 Effect of linolenic acid upon the metabolism of linoleic acid. J. Nutrition, *81:* 67.
7. Rieckehoff, I. G., R. T. Holman and G. O. Burr 1949 Polyethenoid fatty acid metabolism. Effect of dietary fat on polyethenoid fatty acids of rat tissues. Arch. Biochem., *20:* 331.
8. Widmer, C., and R. T. Holman 1950 Polyethenoid fatty acid metabolism. II. Deposition of polyunsaturated fatty acids in fat-deficient rats upon single fatty acid supplementation. Arch. Biochem., *25:* 1.
9. Holman, R. T., and T. S. Taylor 1950 Polyethenoid fatty acid metabolism. III. Arachidonate supplementation. Arch. Biochem., *29:* 295.
10. Peifer, J. J., F. Janssen, P. Ahn, W. Cox and W. O. Lundberg 1960 Studies on the distribution of lipides in hypercholesteremic rats. I. The effects of feeding palmitate, oleate, linoleate, linolenate, menhaden and tuna oils. Arch. Biochem. Biophys., *86:* 302.
11. Machlin, L. J. 1963 The biological consequences of feeding polyunsaturated fatty acids to antioxidant-deficient animals. J. Am. Oil Chemists' Soc., *40:* 368.
12. Woodford, F. P., and C. M. van Gent 1960 Gas-liquid chromatography of fatty acid methyl esters: the "carbon number" as a parameter for comparison of columns. J. Lipid Res., *1:* 188.
13. Tove, S. B., and F. H. Smith 1960 Changes in the fatty acid composition of the depot fat of mice induced by feeding oleate and linoleate. J. Nutrition, *71:* 264.
14. Howton, D. R., and J. Mead 1960 Conversion of 8,11,14-eicosatrienoic acid to arachidonic acid in the rat. J. Biol. Chem., *235:* 3385.
15. Rahm, J. J., and R. T. Holman 1964 The relationship of single dietary polyunsaturated fatty acids to fatty acid composition of lipids from subcellular particles of liver. J. Lipid Res., *5:* 169.
16. Klenk, E., and H. Mohrhauer 1960 Investigations about the biosynthesis of polyunsaturated fatty acids in the rat. Ztschr. Physiol. Chem., *320:* 218.
17. Schlenk, H., N. Sen and D. M. Sand 1963 Interconversion of long-chain fatty acids in the rat. Biochem. Biophys. Acta, *70:* 708.
18. Mead, J., and D. R. Howton 1957 Conversion of γ-linolenic acid to arachidonic acid. J. Biol. Chem., *229:* 575.

Calcium Metabolism and Skeletal Dynamics of Laying Pullets [1]

WERNER J. MUELLER, ROSEMARY SCHRAER AND HARALD SCHRAER
Departments of Poultry Science and Biophysics, The Pennsylvania State University, University Park, Pennsylvania

ABSTRACT Twenty-four pullets were fed a constant specific activity ration for 26 days, after which an identical but Ca^{45}-free ration was fed for 29 days. The specific activity of the egg shell calcium was used to determine calcium balance and parameters of skeletal metabolism. Of the calcium intake 78% was absorbed, 8% was excreted as endogenous calcium and 70% was retained. Shell formation caused a significant increase in calcium retention and a shift of calcium from apposition in bone and endogenous excretion to mobilization from bone and shell deposition. From 4.3 to 4.9 g of the skeletal calcium participated in egg shell formation, of which 1 g was turned over daily. The size of the exchangeable bone calcium pool was related to the quantity of shell produced and was larger in pullets with a negative calcium balance than in pullets with a positive balance. The quantity of calcium removed from the skeleton per day was positively correlated with the size of the exchangeable calcium pool, the quantity of calcium absorbed per day and shell mass. The correlation coefficient between the specific activity of medullary bone and egg shell calcium was 0.845, confirming the important role of medullary bone in shell formation.

The principal pathways and biokinetic aspects of calcium metabolism have been investigated by Aubert and Milhaud (1), Bauer and Carlsson (2), Bauer et al. (3) in humans and rats and by Luick et al. (4) in dairy cows. From the available literature, including a review by Simkiss (5), it was concluded that no reported attempts have been made to obtain quantitative data on the excretion of endogenous calcium, the rates of bone apposition and mobilization or the size of the exchangeable calcium pool in the skeleton of laying hens.

The laying hen offers an unusual opportunity for basic studies of calcium metabolism. Simkiss (5) estimated that the domestic fowl uses calcium about 20 times as rapidly as a mammal in the latter term of gestation. Comar and Driggers (6) calculated from tracer experiments that from 25 to 40% of the egg shell calcium comes from the skeleton and the remainder is derived directly from ingested calcium. Furthermore, in tracer experiments with other animals frequent blood sampling is needed to quantitate the different aspects of calcium metabolism. Egg shell formation, on the other hand, represents an "automatic" sampling mechanism, since the specific activity of the shell calcium reflects the specific activity of the blood calcium during the preceding 18 to 20 hours.

The objectives of the experiment presented here were: (a) to investigate whether the specific activity of the egg shell calcium could be used to determine the parameters of skeletal metabolism in laying hens; (b) to compare calcium balance and skeletal metabolism during periods of shell and no shell formation; and (c) to study the relationships among different parameters of calcium metabolism.

EXPERIMENTAL

Twenty-four 311-day-old Single Comb White Leghorn pullets were used for this study. For 2 weeks prior to the experiment and for its entire duration, the pullets were housed in individual cages located in a room where temperature was maintained between 20 and 22° and relative humidity at 70% ± 5%. Artificial light was provided for 14 hours each day.

Received for publication June 6, 1964.

[1] Authorized as paper no. 2753 in the Journal Series of the Pennsylvania Agricultural Experiment Station. This investigation was supported in part by U. S. Public Health Service Research Grant A-4362 from the National Institutes of Health.

The experiment consisted of 2 parts: In the first part, which lasted 26 days, a ration containing 5 μc of Ca^{45}/100 g feed at the time of mixing, was fed until the specific activities of feed and egg shell calcium were equal. The second part of the experiment lasted from the evening of the 26th day, when the Ca^{45}-containing ration was replaced with a similar but nonradioactive diet, to the 56th day.

The radioactive ration was prepared as follows: 5 mc of Ca^{45} (Oak Ridge National Laboratory preparation P-2) were diluted to 5000 ml with distilled water and mixed with 10 kg of the ration described in table 1. This Ca^{45}-containing premix was dried at 70°, ground, passed through a 5-mm sieve and mixed with 90 kg of ration.

The nonradioactive ration was identical with that shown in table 1, except that 1% of the ground yellow corn was replaced with chromic oxide which served as an indicator of calcium retention (7). The calcium content of both the radioactive and nonradioactive ration was 1.95% by analysis. This relatively low calcium content was a compromise used to insure high enough specific activities for experimental purposes and to avoid the use of large quantities of Ca^{45}. The adequacy of the ration with respect to calcium is substantiated by the balance data reported in table 2.

TABLE 1

Composition of the experimental ration

	kg/100 kg
Ground yellow corn	70.02
Soybean oil meal (44%)	16.50
Fish meal (60%)	2.50
Alfalfa meal, dehydrated	2.50
Dried brewer's yeast	2.50
Ground limestone	3.00
Steamed bonemeal	2.50
Iodized salt	0.40
Manganese sulfate	0.02
Riboflavin concentrate (8 mg/g)	0.02
Stabilized vitamin A concentrate (10,000 IU/g)	0.02
Vitamin D-activated animal sterol (3000 ICU/g)	0.02

Representative samples of the 24-hour excreta voided by each pullet were obtained on days 35, 37, 39, 40, 44 and 45 of the experiment. Individual feed consumption was determined for the period from the thirty-fifth to the forty-fifth day to obtain the average calcium intake per day. The percentage of calcium retention was calculated for each pullet and each collection period from the ratios of chromic oxide in feed and excreta (7). The average calcium retention in grams per day from the thirty-fifth to the forty-fifth day could then be obtained from the average percentage of calcium retention and the respective calcium intake per day. Endogenous calcium excretion as the percentage of ingested calcium was obtained according to the method of Comar (8), who assumed that the specific activities of the blood and endogenous calcium were equal. In the present experiment it was assumed that the specific activity of the endogenous calcium was equal to the specific activity of the egg shell calcium secreted during approximately the same period. For days

TABLE 2

Calcium balance

	All pullets	Shell formation [1]	No shell formation [1]	Standard deviation (all pullets)
Number of days	144	76	32	—
Absorption, % [2]	78	78	76	—
Endogenous excretion, % [2]	8	7	12	—
Retention, % [2]	70	71	64	—
Calcium intake, g/pullet/24 hours	2.32	2.32	2.32	0.17
Calcium absorbed, g/pullet/24 hours	1.80	1.81	1.76	0.17
Endogenous calcium, g/pullet/24 hours	0.18	0.16	0.28	0.04
Calcium retained, g/pullet/24 hours	1.62	1.65	1.48	0.15
Egg shell calcium, g/pullet/24 hours	1.64	2.09	0	0.17
Balance ($Ca_{b+} - Ca_{b-}$) g/pullet/24 hours	−0.02	−0.44	1.48	0.16

[1] See text for assumption.
[2] In percentage of calcium intake.

when no shell calcium was secreted, the specific activity of the endogenous calcium was obtained from the 2 closest eggs by interpolation. The percentage of calcium absorbed and grams calcium absorbed per day were obtained from the respective sums of calcium retention and endogenous excretion.

Fifty-six days after the beginning of the experiment the pullets were killed, the right femur was removed and cleaned of adhering tissue. The diaphyseal portion of the femur was split longitudinally and the medullary bone was removed by scraping.

The shells, including shell membranes, of all eggs laid during the 56 days of the experiment were air-dried and saved for total calcium and Ca^{45} analysis. The calcium in the remainder of the egg was neglected since it was calculated from data given by Romanoff and Romanoff (9) that the shell and shell membranes contain 99% of the egg calcium.

The calcium content of medullary and cortical bone, feed, excreta and egg shells was determined according to the method of Gehrke et al. (10). Ca^{45} in these substances was determined with the method of Comar (8) and the chromic oxide content of feed and excreta according to Hill and Anderson (11).

ANALYSIS OF DATA AND RESULTS

In the following the symbol Ca designates the average quantity of total calcium ($Ca^{40} + Ca^{45}$) in grams per pullet per 24 hours. The symbol *Ca designates analogous quantities of Ca^{45} in counts per minute.

Ca_{abs} = calcium absorbed
Ca_{end} = endogenous calcium excretion
Ca_{shell} = calcium used for egg shell formation
Ca_{b+} = calcium deposited in bone
Ca_{b-} = calcium removed from bone
U = calcium content of exchangeable bone
*U = Ca^{45} content of exchangeable bone.

Calcium balance

The average calcium balance of the 24 pullets during the 6 days of excreta collection is shown in the first column of table 2. The data indicate that for the group of 24 pullets the difference between apposition and mobilization of skeletal calcium (Ca_{b+}–Ca_{b-}) was approximately zero. Thus there was no net loss of calcium from the skeleton during the balance experiment despite the low calcium content of the feed and the relatively high average egg production of 45.3 eggs/pullet during the 55 days of the experiment. This was achieved by high absorption and retention of feed calcium (table 2).

Since the percentage of calcium absorption, endogenous excretion and retention were calculated for each day of excreta collection, the effect of shell formation on these parameters could be studied. Eighteen of the 24 pullets formed no shell during at least one of the excreta collection periods. The average balance data for these 18 pullets on days of shell and no shell formation are shown in columns 2 and 3 of table 2. A comparison of the 2 columns indicates that shell formation had no significant effect on the percentage of calcium absorption, but reduced the percentage of endogenous excretion and increased percentage retention significantly (1% level). Since feed consumption records were not kept for each day of the experiment the percentage figures can only be translated into quantities of calcium if it is assumed that feed intake was not affected by shell formation. If this assumption is made, table 2 indicates that shell formation was characterized by a shift of calcium from deposition in bone (Ca_{b+}) and from endogenous excretion to mobilization from bone (Ca_{b-}) and shell deposition.

Skeletal dynamics

The curve for the specific activity of the egg shell calcium (fig. 1) can be divided into 3 phases for purposes of this experiment. Specific activity increased rapidly from days 1 through 3 and then rose slowly until day 16. During this period Ca^{45} was accumulated in the skeleton. During the second phase from the eighteenth to the twenty-sixth day, the specific activities of the egg shell and feed calcium were equal within the error of analysis. Comar and Driggers (6) have reported that from 25 to 40% of the shell calcium is supplied by the skeleton; therefore, the specific activities of the exchangeable bone calcium and the feed calcium were approximately equal. In the final phase of

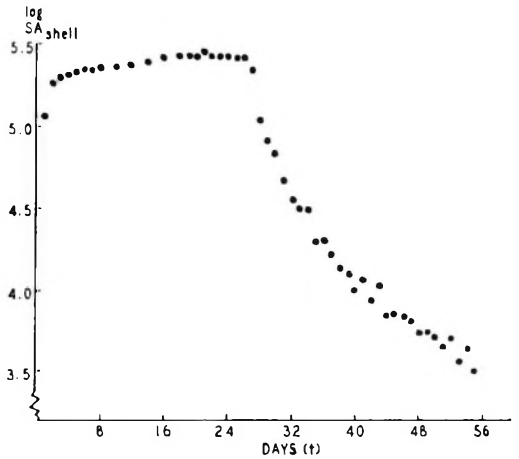


Fig. 1 Specific activity of egg shell calcium.

the curve, which started on the evening of the twenty-sixth day with the withdrawal of the ration containing Ca^{45}, there was a curvilinear decline of the specific activity from the twenty-seventh to the thirty-fifth day, after which the decline was approximately linear.

In the following the parameters of skeletal metabolism will be estimated from the increasing and decreasing parts of the specific activity curve. In addition to the usual assumptions of tracer work (12), it will be assumed that (a) the calcium balance data obtained between days 35 and 45 applied to the entire experiment, and (b) the specific activities of the egg shell calcium, the endogenous calcium and the calcium deposited in the skeleton were equal.

Analysis of the increasing part of the specific activity curve from days 1 to 16. From the calcium balance it follows that:

$$*Ca_{b+} - *Ca_{b-} = *Ca_{abs} - *Ca_{shell} - *Ca_{end}. \quad (1)$$

Using this equation, the quantity $*Ca_{b+} - *Ca_{b-}$, which will be designated as A in the following, can be calculated from the results of the balance experiment, the specific activity of the feed and the specific activity of the egg shell calcium. As shown in figure 2, the relationship between log A and time was linear from day 2 to 14. Thus:

$$A = A_o e^{-at} = 188{,}000\ e^{-0.134t}. \quad (2)$$

The Ca^{45} content of the exchangeable bone at the end of the first phase of the experiment ($*U_{eq}$) is equal to the sum of all the A's, which can be obtained by integrating equation (2). Since the specific activities of feed (SA_{feed}) and exchangeable bone were equal at the end of this phase:

$$U = \frac{*U_{eq}}{SA_{feed}}. \quad (3)$$

There is some uncertainty as to the time when equilibrium is reached. According to equation (2) t should be taken as infinite, whereas figure 2 shows that A falls rapidly after the fourteenth day. U for $t = 16$ was 4.31 g; for $t = \infty$, 4.88 g.

Ca_{b+} can be calculated from the following equation which is based on the assumptions outlined above:

$$A = Ca_{b+} \cdot SA_{shell} - Ca_{b-} \cdot SA_{bone}. \quad (4)$$

At time zero the specific activity of the exchangeable bone is zero and equation (4) becomes:

$$A_o = Ca_{b+} \cdot SA_{shell_o}. \quad (5)$$

From equation (5):

$$Ca_{b+} = 1.02 \text{ g},$$

and since on the basis of the balance experiment:

$$Ca_{b+} - Ca_{b-} = -0.02 \text{ g} \quad (6)$$
$$Ca_{b-} = 1.04 \text{ g}.$$

Analysis of the decreasing phase of the specific activity curve from days 26 to 55. After the withdrawal of the Ca^{45} contain-

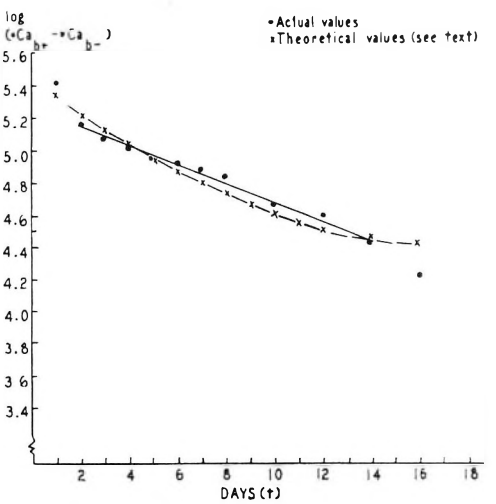


Fig. 2 Ca^{45} accumulation in skeleton during first 18 days with constant specific activity diet.

ing ration, $*Ca_{abs}$ soon becomes approximately zero and equation (1) becomes:

$$*Ca_{b-} - *Ca_{b+} = *Ca_{shell} + *Ca_{end}. \quad (7)$$

The relationship between log ($*Ca_{b-} - *Ca_{b+}$) and time was curvilinear and a graphical analysis (12) was used to derive the following equation:

$$*Ca_{b-} - *Ca_{b+} = 157{,}800\ e^{-0.292t} + 58{,}500e^{-0.0797t}. \quad (8)$$

The Ca^{45} content of the exchangeable bone at the time the radioactive diet was withdrawn can be obtained by integrating equation (8) and its calcium content from equation (3). Thus:

$$U = 4.42 \text{ g.}$$

The quantity of calcium removed from the slow bone compartment (Ca_{s-}) and the fast bone compartment (Ca_{f-}) can be obtained from the 2 exponentials in equation (8) as follows: $*Ca_{f-} - *Ca_{f+}$ is at a minimum when the specific activities of the fast bone compartment and the egg shell calcium are equal. Then analogously to equation (4):

$$58{,}500 = Ca_{s-}SA_{feed} + Ca_{f-}SA_{shell_0} - Ca_{b+}SA_{shell_0}. \quad (9)$$

Since:

$$Ca_{b-} = Ca_{s-} + Ca_{f-} \quad (10)$$

it follows from equation (6) that:

$$Ca_{f-} - Ca_{b+} = 0.02 \text{ g} - Ca_{s-} \quad (11)$$

substituting equation (11) in equation (9):

$$58{,}500 = Ca_{s-}SA_{feed} + SA_{shell_0}(0.02 - Ca_{s-}) \quad (12)$$

and:

$$Ca_{s-} = 0.23 \text{ g.}$$

Using the same reasoning for the net loss of Ca^{45} from the fast bone compartment at zero time:

$$157{,}800 = Ca_{f-}SA_{feed} + SA_{shell_0}(0.02 - Ca_{f-}) \quad (13)$$

and:

$$Ca_{f-} = 0.79 \text{ g.}$$

A summary of the parameters of skeletal metabolism is given in table 3.

Relationships among different parameters of calcium metabolism

The correlation coefficients given in table 4 show that pullets that produced a large shell mass (number of eggs during experiment × average shell weight) had larger pools of exchangeable bone calcium than pullets with a small shell mass. Furthermore, the size of this pool was larger for pullets with a negative calcium balance than for pullets with a positive balance. Correlation analysis showed that shell mass and calcium balance combined accounted for 75% of the variability in the size of the exchangeable calcium pool.

The quantity of calcium removed from the skeleton per day (Ca_{b-}) was positively correlated with the size of the exchangeable calcium pool, the quantity of calcium absorbed per day and shell mass. The first 2 factors were the most important and together accounted for 52% of the variability in Ca_{b-}.

Table 5 shows that the specific activity of the shell calcium of the eggs laid on the last day of the experiment was about one-half as great as the specific activity of the cortical and medullary calcium obtained from the diaphyseal portion of the femur. The specific activity of the cortical calcium varied considerably less than that of either the medullary or shell calcium.

The correlation coefficient between the specific activity of the egg shell calcium on the 56th day and that of the corre-

TABLE 3

Parameters of skeletal metabolism

	Ca content of exchangeable bone (U)	Removed from bone/ 24 hours (Ca_{b-})	
	g	*g*	%
From decreasing part of specific activity curve			
Slowly mobilized bone	2.55	0.23	9.0
Rapidly mobilized bone	1.87	0.79	42.2
Sum	4.42	1.02	23.1
From increasing part of specific activity curve			
Exchangeable bone for: $t = 16$ [1]	4.31	1.04	24.1
$t =$ infinite [1]	4.88	1.04	21.3

[1] See text.

TABLE 4

Correlation coefficients among certain parameters of calcium metabolism

	Ca_{b-}	Shell mass	Ca absorbed	Ca balance ($Ca_{b+} - Ca_{b-}$)
Exchangeable calcium pool (U)	0.601 [1]	0.750 [1]	0.279	−0.558 [1]
Calcium removed from skeleton/day (Ca_{b-})		0.410 [2]	0.556 [1]	−0.141

[1] Significant at 1% level.
[2] Significant at 5% level.

TABLE 5

Specific activity of cortical bone, medullary bone and egg shell calcium

	Cortical Ca	Medullary Ca	Shell Ca (day 56)
Specific activity, count/min/g Ca	7387	7994	4080
Standard deviation, count/min/g Ca	1757	5758	2413
Coefficient of variability, %	23.8	72.0	59.1

sponding medullary bone calcium was 0.845 (significant at the 1% level), whereas the correlation coefficients between these 2 characteristics and the specific activity of the cortical calcium were 0.199 and 0.139, respectively (both not statistically significant).

DISCUSSION

The experiment reported here shows that the parameters of skeletal metabolism of laying hens can be obtained from the increase of the specific activity of the egg shell calcium with a constant specific activity diet or the decrease of specific activity after this diet has been withdrawn. The mathematical analyses used are relatively simple and the estimates obtained in the 2 phases of the experiment agreed closely.

However, there was one major discrepancy between the 2 analyses. Although a function with one exponential was sufficient to describe the accumulation of Ca^{45} in bone with the constant specific activity diet, a function with 2 exponentials was needed to simulate the release of Ca^{45} from bone with the nonradioactive diet. To explore this difference further, the accumulation of Ca^{45} during the first phase of the experiment was reconstructed from the parameters of skeletal metabolism obtained in the third phase and the calcium balance data. Figure 2 shows that the plot of these theoretical values was only slightly curvilinear and agreed well with the actual values. This suggests that the mathematical functions derived from the increasing and decreasing parts of the specific activity curve may represent different levels of approximation of the skeletal system in which calcium is exchanged at a number of different rates because of differences in accessibility.

The parameters of calcium metabolism reported here apply only to the group of layers used and the conditions of the experiment. The calcium balance of chickens is influenced by many factors, such as calcium intake (13, 14) and environmental temperature (15). The effect of hormones, particularly estrogen, on medullary bone of chickens has been amply demonstrated (5). Nevertheless, it is interesting to compare the results reported here for pullets which contain approximately 20 to 25 g of calcium (16) with those obtained by Aubert and Milhaud (1) for a 22-year-old man weighing 70 kg and containing approximately 1100 to 1500 g calcium (17). In man the quantity of calcium taken up by bone (Ca_{b+}) was 0.88 g/day; in the average pullet it was 1.02 g. In man the size of the exchangeable calcium pool, including blood and soft tissue, was 6.57 g; in the pullet 4.42 g of the skeletal calcium participated in egg shell formation. The importance of

the skeleton in the calcium metabolism of laying hens is further illustrated by the fact that of the 2.82 g of calcium which entered the blood pool per day ($Ca_{abs} + Ca_{b-}$) 1.02 g or 36% came from the skeleton. This estimate is within the range given by Comar and Driggers (6) for the percentage of egg shell calcium supplied by the skeleton.

The estimates discussed above are averages for an extended period including days of shell deposition and no shell deposition. As shown in table 2 active shell formation caused a considerable shift in calcium balance. The reduction in the excretion of endogenous calcium on days of shell formation, although relatively small, is in line with the hypothesis made in an earlier paper (18) that excretion and shell formation may be competing processes.

The results presented here also indicate that the size of the exchangeable calcium pool is related to the quantity of shell produced ($r = 0.750$) and is larger in pullets with a negative calcium balance than in pullets with a positive balance. The latter observation is in agreement with a report of Benoit and Clavert (19) who observed in experiments with ducks that with calcium-deficient diets medullary bone was formed at the expense of other parts of the skeleton.

The important role of medullary bone in egg shell formation is confirmed by the highly significant correlation between the specific activities of the last egg shell and medullary bone from the femur. Cortical bone as a whole, on the other hand, did not appear to participate significantly in egg shell formation. This, however, does not exclude the possibility that a portion of the cortical bone was in equilibrium with the medullary bone or contributed directly to shell formation.

LITERATURE CITED

1. Aubert, J. P., and G. Milhaud 1960 Méthodes de mesure des principales voies du métabolisme calcique chez l'homme. Biochim. Biophys. Acta, *39:* 122.
2. Bauer, G. C. H., and A. Carlsson 1955 Metabolism of bone salt investigated by simultaneous administration of ^{45}Ca and ^{32}P to rats. J. Bone Joint Surg., *37B:* 658.
3. Bauer, G. C. H., A. Carlsson and B. Lindquist 1958 Use of isotopes in clinical studies of skeletal metabolism. Radioaktive Isotope Klinik Forschung, *3:* 25.
4. Luick, J. R., J. M. Boda and M. Kleiber 1957 Some biokinetic aspects of calcium metabolism in dairy cows. Am. J. Physiol., *189:* 483.
5. Simkiss, K. 1961 Calcium metabolism and avian reproduction. Biol. Rev., *36:* 321.
6. Comar, C. L., and J. C. Driggers 1949 Secretion of radioactive calcium in the hen's egg. Science, *109:* 282.
7. Mueller, W. J., R. V. Boucher and E. W. Callenbach 1956 Influence of age and sex on the utilization of proximate nutrients and energy by chickens. J. Nutrition, *58:* 37.
8. Comar, C. L. 1955 Radioisotopes in biology and agriculture. McGraw-Hill Book Company, Inc., New York.
9. Romanoff, A. L., and A. J. Romanoff 1949 The Avian Egg. J. Wiley and Sons, Inc., New York.
10. Gehrke, C. W., H. E. Affsprung and Y. C. Lee 1954 Direct ethylene diaminetetraacetate titration procedure for calcium in biological substances. Anion exchange separation of phosphate. Missouri Agricultural Experiment Station Research Bull. 570. Columbia.
11. Hill, F. W., and D. L. Anderson 1958 Comparison of metabolizable energy and productive energy determination with growing chicks. J. Nutrition, *64:* 587.
12. Solomon, A. K. 1960 Compartmental methods of kinetic analysis. In Mineral Metabolism, vol. 1A, eds., C. L. Comar and F. Bronner. Academic Press, New York, p. 119.
13. Common, R. H. 1943 Observations on the mineral metabolism of pullets. III. The calcium requirements of the laying bird. J. Agr. Sci., *33:* 213.
14. Tyler, C., and J. S. Wilcox 1942 Calcium and phosphorus balances with laying birds. J. Agr. Sci., *32:* 43.
15. Mueller, W. J. 1959 The effect of environmental temperature and humidity on the calcium balance and serum calcium of laying pullets. Poultry Sci., *38:* 1296.
16. Taylor, T. G., and J. H. Moore 1958 The effect of high and low levels of dietary inorganic phosphate on the pre-laying storage of calcium and phosphorus and on the composition of medullary and cortical bone in pullets. Brit. J. Nutrition, *12:* 35.
17. Sherman, H. C. 1947 Calcium and phosphorus in foods and nutrition. Columbia University Press, New York.
18. Mueller, W. J. 1962 Carbonic anhydrase, diuretics and egg shell formation. Poultry Sci., *41:* 1792.
19. Benoit, J., and J. Clavert 1945 Hypércalcémie et ostogénèse medullaire folliculiniques chez le canard domestique soumis a un régime acalcique. C. R. Soc. Biol. (Paris), *139:* 737.

Effect of Magnesium, Fluoride, and Ascorbic Acid on Metabolism of Connective Tissue [1]

DAVID J. THOMPSON, JUDITH F. HEINTZ AND PAUL H. PHILLIPS
Department of Biochemistry, University of Wisconsin, Madison, Wisconsin

ABSTRACT Experiments were conducted in which young growing guinea pigs were fed diets which were deficient in ascorbic acid or magnesium or contained supplements of fluoride. The effect of these dietary factors upon connective tissues was measured by hexosamine, uronic acid, and hydroxyproline analyses. Blood serum hexosamine content was normal in fluorosis and magnesium deficiency when compared with the control group. Hexosamine values were uniformly elevated and hydroxyproline values depressed in animals fed diets low in ascorbic acid, diets high in fluoride (450 ppm) and low in magnesium (96 ppm), and in those animals fed low magnesium-low ascorbic acid diets. Blood serum levels of magnesium underwent a marked and characteristic reduction from normal ranges of 2 to 3 mg/100 ml to values less than 1 mg/100 ml in those groups receiving low magnesium diets. Growth rates were retarded by low magnesium, low ascorbic acid or the addition of 450 ppm fluoride. Results of hexosamine and hexuronic acid analysis on heart, aorta, kidney and selected connective tissue samples showed no significant differences as a result of dietary test diets fed for 17 days. A dyschondroplasia was observed in the hind leg bones of animals fed 450 ppm F with a diet containing 96 ppm magnesium.

Observations of nutritional experiments concerning Mg and F indicate that these elements influence or alter the calcification process by inadequate or excessive amounts in the diet (1–3). The important role of connective tissues in the calcification of the skeletal structures and soft tissues led us to study the effects of dietary levels of Mg, F, and ascorbic acid upon the metabolism of connective tissues.

EXPERIMENTAL

Seven- to nine-day-old male guinea pigs weighing 140 to 160 g were allotted to groups of 6 animals each. The basal diet was composed of: (in per cent) ethanol-extracted casein, 30; sucrose, 43; roughage,[2] 14; alfalfa meal, 1; corn oil, 5; potassium acetate, 2.5; salts, 4; and vitamins. The salts contained: (in per cent) $CaHPO_4 \cdot 2H_2O$, 39.8; KCl, 25.8; NaCl, 22.7; $CaCO_3$, 10.8; $Fe_2(SO_4)_3$, 0.818; $CuSO_4 \cdot 5H_2O$, 0.062; $MnSO_4 \cdot H_2O$, 0.035; $ZnCl_2$, 0.047; $CoCl_2 \cdot 6H_2O$, 0.002; and KIO_3, 0.00045. The diet contained by analysis 96 ppm magnesium, 0.55% phosphorus, and 0.56% calcium. The vitamin mixture supplied 2 mg menadione, 16 mg each of thiamine, riboflavin and pyridoxine, 200 mg niacin, 40 mg calcium pantothenate, 10 mg folic acid, 0.6 mg biotin, 0.04 mg of vitamin B_{12}, 3000 mg choline chloride, 200 mg inositol, 20,000 IU of vitamin A,[3] and 3200 IU of vitamin D_2[4]/kg of diet. In addition 18 mg of α-tocopherol/kg of diet were added to the corn oil and 5 mg ascorbic acid/kg of body weight were added daily by pipette as a supplement where indicated. The diets were pelleted by moistening with distilled water and running them through a meat grinder and drying in a forced-air oven.

The dietary regimen was as follows:

- Lot 1 Basal diet with 0.18% magnesium added as magnesium oxide and 5 mg of ascorbic acid/kg of body weight given by dropper daily.
- Lot 2 Basal as in lot 1 without magnesium supplement.
- Lot 3 Basal as in lot 1 without ascorbic acid supplement.
- Lot 4 Basal as in lot 1 plus 0.1% sodium fluoride.
- Lot 5 Basal as in lot 1 without magnesium supplement, plus 0.1% sodium fluoride.
- Lot 6 Basal as in lot 1 without magnesium supplement and ascorbic acid.

Received for publication April 10, 1964.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

[2] Cellu Flour, Chicago Dietetic Supply House, Chicago.

[3] Gelatin coated, obtained from Nutritional Biochemicals Corporation, Cleveland.

[4] See footnote 3.

The animals were housed by lots in wire-mesh cages with food and distilled water given ad libitum. They were killed on the 17th day. Samples of kidney, heart, aorta, femur, costal and xiphoid cartilage processes were immediately removed and frozen on dry ice. Blood was collected and allowed to clot. Serum was analyzed for magnesium with the atomic absorption spectrophotometer.[5] Samples of tissues were freeze-dried and then hydrolyzed by Dowex-50 resin and dilute hydrochloric acid according to the method of Anastassiades and Common (4), prior to determining the hexosamines by the method of Anastassiades and Common (5), uronic acids by the method of Dische (6), and hydroxyproline according to the method of Woessner (7) as modified by Goll.[6] The results were subjected to analyses of variance according to the procedure of Snedecor (8) and the significance between means determined by Duncan's multiple range test (9).

RESULTS

A marked depression in growth rate resulted due to low dietary magnesium (96 ppm), high fluoride (450 ppm), or low ascorbic acid. The growth rate was retarded within the first week by low magnesium, low ascorbic acid, low magnesium-low ascorbic acid, or supplemental fluoride. A very marked reduction in growth rate occurred by the 14th day in those animals fed low magnesium (96 ppm) diets. Animals fed the low magnesium diet developed the characteristic symptoms of the magnesium-deficient animal, namely, tetany, vasodilitation, and in marked contrast to the symptoms in the dog (10), no marked effect on the muscle tonus in the phalanges. The scorbutic guinea pigs showed extensive subcutaneous hemorrhages in traumatic areas of the body. Their bones were brittle and easily fractured.

The period of time involved in these experiments did not permit full development of the fluorosis syndrome in the guinea pig, but there was a marked depression of growth, and the hair coat was rough and dull. This condition was worsened by a high fluoride (450 ppm), low magnesium (96 ppm) diet. The animals exhibited stiffness similar to that of scorbutic animals and reluctance in all voluntary movement. Their legs had a swollen appearance and were painful to the touch. A large amount of connective tissue overgrowth was observed on the rear legs and this completely enveloped the bone, joints, and major tendons. Blood serum levels of magnesium underwent a marked ($P < 0.01$) and characteristic reduction from normal ranges of 2 to 3 mg/100 ml to values less than 1 mg/100 ml (table 1). The deficiency of ascorbic acid or toxic dietary levels of fluoride did not significantly lower serum magnesium levels. Blood serum hexosamine content was normal in fluorotic and low magnesium animals when compared with the controls.

[5] Perkin Elmer Corporation, model 214, Norwalk, Connecticut.

[6] Goll, D. E., Ph.D. Thesis, University of Wisconsin, 1962.

TABLE 1

Summary data of the effects of Mg, F, and ascorbic acid on growth, serum Mg, serum hexosamine, and aorta hydroxyproline in guinea pigs [1]

Lot	Avg gain/week		Serum Mg [2]	Serum hexosamine	Aorta hydroxyproline
	Week 1	Week 2			
	g	*g*	*mg/100 ml*	*mg/100 ml*	*mg/g*
Control	46	36	3.17 ± 0.44 [3]	100 ± 12 [3]	27.7
Low Mg, 96 ppm	35	4	0.55 ± 0.29	102 ± 13	27.2
Low ascorbic acid	36	23	2.59 ± 0.57	144 ± 7 [4]	22.9 [4]
High F, 450 ppm	27	11	2.88 ± 0.60	106 ± 13	26.7
High F and low Mg	27	7	1.25 ± 0.29	144 ± 7 [4]	21.9 [4]
Low ascorbic acid and low Mg	39	6	0.70 ± 0.28	134 ± 17 [4]	20.7 [4]

[1] Means of 6 animals/lot.

[2] Result of Duncan's multiple range test on serum Mg means: 0.50 0.70 1.25 2.59 2.88 3.17. Any 2 means not underscored by the same line are significantly different ($P < 0.01$).

[3] SD.

[4] Significantly different ($P < 0.01$) from control.

Hexosamine values were uniformly increased by diets low in ascorbic acid and by diets high in fluoride-low in magnesium ($P < 0.01$). Hydroxyproline levels in the aorta (table 1) were lowered by diets low in ascorbic acid and by diets high in fluoride-low in magnesium. Similar results were obtained on the femur and costal cartilage. No differences in hydroxyproline levels were observed in the kidney and heart. The results of the hexosamine and uronic acid analysis on the heart, kidney, aorta, bone, costal and xiphoid cartilage did not show abnormal changes in these tissues at 17 days. Perhaps a longer experimental period might cause alterations.

Because it was believed that the time factor in the development of scurvy was highly important, the experiment was terminated at a period when the animals were showing early symptoms of scurvy but which had not developed to the point of classical symptoms of the disease. Examination of the data indicates that chemical alterations had appeared during this short experimental period. A reduction in growth rate was evident at the end of 7 days and a weight loss was noted by the end of the second week. Blood serum levels of magnesium were insignificantly reduced in scurvy and in fluorosis in contrast with the marked loss of serum magnesium when the diet contained insufficient magnesium. When the diet was not supplemented with ascorbic acid the serum hexosamine was significantly increased. A similar response was obtained when the diet contained high fluoride-low magnesium, or low ascorbic acid-low magnesium. The interpretation of these data are not clear at present. It has been reported that the synthesis of mucopolysaccharides is decreased in the scorbutic animal (11). The elevated serum hexosamine indicates that either an increased amount of hexosamines are produced or synthesized or their incorporation in the form of new connective tissue may have been blocked. When the diet was not supplemented with ascorbic acid the aorta hydroxyproline levels were decreased. A similar response was obtained when the diet contained high fluoride and low magnesium or low ascorbic acid and low magnesium. Fluoride, magnesium, and ascorbic acid affect constituents which are known to be involved with normal formation of bone and connective tissue.

A problem which complicated a study of this type is that many times it is difficult to distinguish between the direct effects of a nutrient being studied and those effects that are the consequence of decreased food consumption. The food intake values in these experiments parallel the growth responses shown in table 1; however, the changes in hexosamine or hydroxyproline values do not correlate with growth.

The growth responses reported have been observed many times and studies on the serum Mg values have been repeated, not only on the guinea pig, but on the white rat and the dog with similar results. The serum hexosamine response has been repeated in the guinea pig.[7]

ACKNOWLEDGMENT

The authors wish to acknowledge the competent technical assistance given by Helen Burcalow.

LITERATURE CITED

1. National Research Council, Committee on Animal Nutrition 1960 The fluorosis problem in livestock production, pub. 824. National Academy of Sciences — National Research Council, Washington, D. C.
2. Chiemchaisri, Y., and P. H. Phillips 1963 Effect of dietary fluoride upon the magnesium calcinosis syndrome. J. Nutrition, *81:* 307.
3. Bunce, G. E., Y. Chiemchaisri and P. H. Phillips 1962 The mineral requirements of the dog. IV. Effect of certain dietary and physiologic factors upon the magnesium deficiency syndrome. J. Nutrition, *76:* 23.
4. Anastassiadis, P. A., and R. H. Common 1958 Liberation of hexosamine, hexuronic acid, and hydroxyproline from tissues by resin hydrolysis. Canad. J. Biochem. Physiol., *36:* 413.
5. Anastassiadis, P. A., and R. H. Common 1953 Studies on the glycoproteins of the domestic fowl. I. A modification of the method of Elson and Morgan for the determination of hexosamine and its applicability to tissue hydrolyzates. Canad. J. Chem., *31:* 1093.
6. Dische, Z. 1947 A new specific color reaction of hexuronic acids. J. Biol. Chem., *167:* 189.
7. Woessner, J. F., Jr. 1961 The determination of hydroxyproline in tissue and protein

[7] Unpublished results.

samples containing small proportions of this amino acid. Arch. Biochem. Biophys., 93: 440.
8. Snedecor, G. W. 1956 Statistical Methods. Iowa State College Press, Ames, Iowa.
9. Duncan, D. B. 1955 Multiple range and multiple F tests. Biometrics, *11*: 1.
10. Bunce, G. E., K. J. Jenkins and P. H. Phillips 1962 The mineral requirements of the dog III. The magnesium requirement. J. Nutrition, 76: 17.
11. Kent, P. W., and M. W. Whitehouse 1955 Biochemistry of the Amino Sugars. Academic Press, Inc., New York.

Effect of the Copper Status of the Rat on the Copper-Molybdenum-Sulfate Interaction

LOUISE F. GRAY AND LOUISE J. DANIEL

U. S. Plant, Soil and Nutrition Laboratory, ARS, USDA, Ithaca, New York, and Department of Biochemistry, Cornell University, Ithaca, New York

ABSTRACT The effect of the copper status and dietary copper intake of the animal on its response to molybdenum and sulfate was studied. When the rat's copper stores were low and a copper-deficient diet was fed, small amounts of molybdenum produced toxic symptoms which were *intensified* by the simultaneous addition of sulfate. However, when the copper stores and dietary copper intake were adequate, larger amounts of molybdenum were required to produce molybdenosis, and sulfate completely *prevented* the harmful effects of molybdenum. In the first instance, the copper deficiency syndrome was evident and dietary copper prevented all toxicity symptoms. In the second case, the corrective effect of copper was negligible, but sulfate prevented the marked growth retardation produced by molybdenum. These observations are discussed in relation to several hypotheses concerned with the molybdenum-copper-sulfate interaction. An analysis of the liver-copper stores of these animals revealed that the suggested direct relationship between copper intake and copper storage in the liver does not always obtain. The copper status of the animal and the dietary levels of molybdenum and sulfate, as well as copper, significantly influence the level of copper in the liver.

An understanding of the copper nutrition of the animal is not possible until more information is available concerning several other dietary constituents which have been shown to influence the animal's copper requirement. Both molybdenum and sulfate are known to affect the copper metabolism of the animal, but the mechanism of action is unknown.

The first evidence of this interference by molybdenum was the recognition by Ferguson et al. (1) that the forage of the "teart" pastures in England contained unusually high levels of molybdenum, and that the scouring disease of cattle associated with these areas could be corrected or prevented by feeding copper. The copper content of the "teart" forage was "normal" or "above normal"; nearby non-teart pastures, where no scouring occurred, were lower in copper. The molybdenum-copper antagonism, as a result of which a physiological copper deficiency occurred, has since been reported in ruminants grazing pastures in widely diverse areas of the world (2–4).

The outstanding discovery by Dick (5) that dietary sulfate intensified the harmful action of molybdenum in the copper nutrition of sheep, introduced another factor into this already puzzling copper-molybdenum interrelationship.

At about the time that this observation was made with ruminants, Gray and Daniel (6) reported that methionine alleviated molybdenum toxicity in the rat. Subsequent work by Van Reen and Williams (7) confirmed the methionine effect and included the additional observation that sulfate was as effective. The beneficial effect of sulfate on molybdenum-fed rats was also shown by Miller et al. (8). This paradox of sulfate intensifying the toxic effect of molybdenum in the ruminant and alleviating molybdenum toxicity in the rat could possibly be explained as a species difference, but such diametrically opposite effects are difficult to dismiss so lightly.

In an effort to reconcile these contradictory observations and to learn more about this interaction, studies were undertaken to test the hypothesis that the apparently anomalous effects of dietary sulfate on the copper-molybdenum interaction could be due to differences in the copper status of the animals.

EXPERIMENTAL

Methods. Weanling male rats from the laboratory colony (Sprague-Dawley strain) were used in these studies. When copper-depleted animals were needed, the young

Received for publication April 2, 1964.

were weaned at 21 days of age and fed unsupplemented whole milk powder.[1] The length of the depletion period varied with the time of weaning and the external evidence of anemia. At the beginning of the experiment, the rats were randomized into replications of 6 animals according to weight and to length of depletion period. The animals were housed on wire screens with 3 animals/cage in an air conditioned room. Both the cages and screens were coated with aluminum paint. Feed and redistilled water were supplied ad libitum. The glass water bottles and porcelain feeding dishes were cleaned with hot HNO_3 before the start of the experiment. The copper-adequate animals were housed under the same conditions, but no precautions were taken to insure copper-free glassware, and tap water was supplied instead of redistilled water.

The copper-deficient basal diet was whole milk powder (< 1 ppm Cu) supplemented with carbonyl iron and $MnSO_4 \cdot H_2O$ to furnish the rat approximately 1 mg Fe and 0.3 mg Mn/day. The basal diet fed the copper-adequate animals was whole milk powder supplemented with copper, iron and manganese to supply about 0.06 mg Cu, 0.4 mg Fe and 0.3 mg Mn/day. The copper was added as $CuSO_4 \cdot 5H_2O$ and the iron as $FeSO_4 \cdot 7H_2O$, both reagent grade salts. When molybdenum and sulfate were used, they were added as $Na_2MoO_4 \cdot 2H_2O$ and Na_2SO_4, respectively. Percomorph liver oil [2] was administered once a week during the 4-week experimental period.

Weights were recorded weekly. Hemoglobin determinations (9) were made at the end of the depletion period for the depleted rats, and at the end of the experiment for all animals. Copper analyses were performed on the livers of rats in experiment 2, using a modification of the method of Parks et al. (10). The principal modification was the omission of the dithizone extraction step. A small sample of each liver was taken for a dry weight determination. The rest of the liver was ashed by heating with repeated additions of distilled HNO_3 and a final treatment with a small quantity of 70% $HClO_4$ (reagent grade).

Analyses of variance were made and the *t* test for significance between the treatment means was applied to the data (11).

Experiment 1. This experiment was planned to investigate the effect of sulfate on molybdenum toxicity in copper-deficient rats. The rats were depleted for 9 to 12 days, at which time the mean hemoglobin level was 6.3 g/100 ml, compared with a mean value for the stock animals of the same age of 9.0 g/100 ml. The depleted rats were deficient in copper and iron at the start of the experiment. Using the copper-deficient basal diet, the effect of 3 levels of molybdenum with and without added sulfate was studied.

Experiment 2. The purpose of this experiment was to study the effect of sulfate and methionine on molybdenosis in copper-depleted and in copper-adequate rats under identical experimental conditions. Two groups of animals from one breeding of the stock colony were used. One group was depleted for 16 to 17 days, after which time it was fed the diets shown in table 1. The second group was fed the experimental diets at weaning (21 to 23 days of age). These rats had adequate copper stores and were fed a basal diet containing sufficient copper to meet the needs of the growing rat. Since previous work at this laboratory had shown that a level of 800 ppm molybdenum retarded growth with copper-adequate rats (6), this level was again used. The treatments studied are presented in table 2.

RESULTS AND DISCUSSION

Experiment 1. The results of the first experiment are summarized in table 3. The rats fed the basal diet (< 1 ppm Cu) were obviously copper-deficient, as evidenced by the effect of 3.0 ppm Cu on growth and hemoglobin formation. Low levels of molybdenum were extremely toxic, 50 ppm markedly reducing growth, and 10 ppm significantly lowering the hemoglobin level. These molybdenum levels are of similar magnitude to those observed in forage which produced molyb-

[1] Klim Powdered Whole Milk, The Borden Company, New York. Trade names and company names are included for the benefit of the reader and do not infer any endorsement or preferential treatment of the product listed by the U. S. Department of Agriculture.

[2] A-D Percomorph Liver Oil, Abbott Laboratories, Inc., North Chicago, Illinois.

TABLE 1

Effect of molybdenum, copper and sulfur compounds on growth and hemoglobin formation in copper-depleted rats [1]

Supplement	Mean weight change		Mean hemoglobin	
	Copper added, ppm		Copper added, ppm	
	None	3.0	None	3.0
	g	*g*	*g/100 ml blood*	
None	61 [2]	112	6.6	11.5
10 ppm Mo	42	123	5.8	13.1
100 ppm Mo	−20(2)	115	3.6(2)	13.5
2.7% SO_4	43(5)	101	6.2(5)	13.4
1% DL-Methionine	14		6.6	
10 ppm Mo + SO_4	−2(5)	116	5.0(5)	14.0
100 ppm Mo + SO_4	all dead	87(5)	all dead	13.0(5)
100 ppm Mo + DL-methionine	24(3)	88	3.8(3)	13.0
L.S.D.$_{0.01}$ [3]	20	20	2.1	2.1
L.S.D.$_{0.05}$	14	14	1.5	1.5

[1] The copper-depleted rats were fed unsupplemented whole milk powder for 16 to 17 days, after which they were fed the copper-deficient diet described under Methods. The rats averaged 97 g when placed on experiment.
[2] Each figure represents the mean for 6 male rats, unless otherwise indicated by numbers in parentheses. The experimental period was 4 weeks.
[3] Least significant difference at specified probability levels.

TABLE 2

Effect of molybdenum, copper and sulfur compounds on growth and hemoglobin formation in copper-adequate rats [1]

Supplement	Mean weight change		Mean hemoglobin	
	Copper added, ppm		Copper added, ppm	
	None	300	None	300
	g	*g*	*g/100 ml blood*	
None	149 [2]	151	14.4	14.7
800 ppm Mo	73	92	14.0	14.5
0.645% SO_4	155		14.8	
2.7% SO_4	143		14.3	
1% DL-Methionine	133		14.6	
Mo + 0.645% SO_4	151	144	14.7	14.6
Mo + 2.7% SO_4	138	148	14.7	14.6
Mo + DL-Methionine	118	118	14.1	15.0
L.S.D.$_{0.01}$ [3]	26	26		
L.S.D.$_{0.05}$	18	18		

[1] The copper-adequate rats were placed on experiment at weaning with an average weight of 60 g. They were fed a basal diet of whole milk powder supplemented with 5.33 ppm Cu, making the total copper content approximately 6.3 ppm.
[2] Each figure represents the mean for 6 male rats for 4-week experimental period.
[3] Least significant difference at specified probability levels. No statistical analysis was made on hemoglobin values, since it was evident that differences were negligible.

denosis in cattle in England (1) and New Zealand (12). In these copper-depleted rats, the addition of sulfate potentiated the deleterious effect of molybdenum. At the 2 higher levels of molybdenum, the addition of sulfate resulted in the death of all animals. Sulfate alone caused a reduction in growth and hemoglobin concentration. Diarrhea, a frequent symptom of molybdenosis in cattle, was a common observation in the molybdenum-toxic rats. The addition of 3.0 ppm Cu, a level found to be adequate for these rats, prevented the adverse effects of molybdenum and sulfate. These results indicate that the rat with low copper stores responds to dietary molybdenum and sulfate as does the ruminant.

Experiment 2. The results for the 2 groups of animals compared in this experiment — copper-depleted and copper-adequate — are presented in tables 1 and 2, respectively.

The depleted animals were more copper-deficient than those in experiment 1. This

TABLE 3

Effect of molybdenum and sulfate on growth and hemoglobin formation in copper-depleted rats [1]

Supplement	Mean weight change		Mean hemoglobin	
	Copper added, ppm		Copper added, ppm	
	None	3.0	None	3.0
	g	*g*	*g/100 ml blood*	
None [2]	91	140	9.6	14.5
10 ppm Mo [3]	80	143	7.2	14.4
50 ppm Mo	23(5)	146	4.9(5)	14.1
100 ppm Mo	24(5)	140	5.6(5)	14.8
2.7% SO_4 [4]	65(5)		7.2(5)	
10 ppm Mo + SO_4	13(5)	133	5.4(5)	13.7
50 ppm Mo + SO_4	all dead	117	all dead	13.4
100 ppm Mo + SO_4	all dead	138	all dead	14.8
L.S.D.$_{0.01}$ [5]	36	36	2.0	2.0
L.S.D.$_{0.05}$	26	26	1.4	1.4

[1] All animals were depleted by feeding whole milk powder for 9 to 12 days before the start of the experiment. Experimental period was 4 weeks. All values are the mean for 6 male rats unless otherwise indicated by numbers in parentheses.

[2] The basal diet was whole milk powder supplemented with iron and manganese as described under Methods.

[3] Molybdenum added as $Na_2MoO_4 \cdot 2H_2O$.

[4] Sulfate added as Na_2SO_4.

[5] Least significant difference at specified probability levels.

can be seen by a comparison of the responses of the animals in the 2 experiments to the basal diets of the same composition, and to diets with the same levels of molybdenum and sulfate. The adverse effect of sulfate added to the molybdenum diets is again apparent in experiment 2; its effect on growth when combined with 10 ppm Mo is especially striking. The inclusion of methionine in the diets produced results which are difficult to interpret. Methionine alone depressed growth, but the animals fed 100 ppm Mo + methionine grew better than those fed 100 ppm Mo alone. There was an unusual amount of variation within all groups fed methionine. With 3.0 ppm Cu added, the toxic effect of molybdenum or sulfate alone, or 10 ppm Mo + sulfate was completely prevented. This level of copper did not prevent completely the harmful effects of 100 ppm Mo + sulfate or methionine. The more severe depletion of these rats may explain the difference in these results from those obtained in experiment 1. All rats receiving 3.0 ppm Cu, except those fed the basal diet, had normal hemoglobin levels. With the exception of those fed sulfate alone, they also received molybdenum. This observation is reminiscent of the more rapid response of rats with a milk-induced anemia to molybdenized iron than to iron per se (13).

The results obtained with the copper-adequate rats are presented in table 2. Molybdenum (800 ppm) reduced growth very significantly, but had no effect on hemoglobin formation. Diarrhea did not occur. The deleterious effect of molybdenum on growth was completely prevented by either level of sulfate fed. As in previous work at this laboratory, methionine partially alleviated the toxic effect of molybdenum (6), and copper was much less effective than either methionine or sulfate. Since 0.645% sulfate and 1% methionine contain equivalent amounts of sulfur, not as much of the sulfur in methionine was available in the form needed to prevent the harmful effect of molybdenum. It seems probable that some of the beneficial effect of methionine under these conditions is due to its conversion to sulfate. However, since retardation of growth is the principal effect of toxic amounts of molybdenum fed to animals with an adequate copper status, molybdenum may be interfering with some aspect of protein metabolism in which methionine is concerned. Johnson and Miller (14) observed aminoaciduria in rats fed toxic levels of molybdenum, and suggested that molybdenum interfered in some manner with protein synthesis.

The liver is the principal storage organ for copper, and its copper content has gen-

erally been considered a reasonably reliable index of the copper status of the animal (15). The liver-copper levels of all animals in this experiment were determined to learn the effect of the copper status of the animal and the interaction of different dietary levels of molybdenum, copper and sulfate (table 4). The copper concentration in the livers of all copper-deficient rats receiving no added copper was low. No treatment except Mo + methionine significantly affected it. The level of copper in the livers of the copper-deficient animals fed 3.0 ppm Cu and copper-adequate animals fed 5.33 ppm Cu was within the normal range for the rat (15).

When copper-adequate rats were fed 5.33 ppm Cu, molybdenum increased the copper concentration in the liver, an observation made previously by others (8, 16, 17). Methionine and 0.645% sulfate prevented the accumulation of liver copper attributable to molybdenum; however, the higher level of sulfate had no effect. On the other hand, rats receiving 300 ppm Cu had a lower level of liver copper when molybdenum was fed. Sulfate prevented the decrease in liver copper due to molybdenum and methionine intensified it. There was a large variation in the individual values for the high copper diets, as indicated by the L.S.D. values. The patterns of copper storage have been found to vary with different species (18). In most species, except for the sheep and the duck, moderate increases in dietary copper above the normal intake will result in little if any increase in liver copper. Not until a rather high level is fed does the liver copper increase greatly. It may be that there is considerable variability in this threshold for different individuals within a species, which could account for the results obtained here.

The total liver copper values were calculated and analyzed statistically, but they are not presented since they show essentially the same trends as noted for copper concentration.

Molybdenum appears to have had 2 different actions in this study. In the copper-depleted rats consuming a copper-deficient diet, molybdenum produced a greater copper deficiency which was exacerbated by sulfate and prevented by copper. In the rats with normal copper stores receiving adequate dietary copper, molybdenum did not induce a copper deficiency, but caused some other dysfunction in metabolism which was prevented partially by methionine and completely by sulfate. Dick (19) has proposed that sulfate causes a decrease in both the absorption of molybdenum from the gut and its reabsorption from the

TABLE 4

Effect of molybdenum, copper and sulfur compounds on liver-copper levels of rats in depleted and adequate copper status [1]

Supplement	Cu-depleted		Cu-adequate	
	Copper added, ppm		Copper added, ppm	
	None	3.0	5.33	300
	μg/g dry weight		*μg/g dry weight*	
None	3.9	16.0	15.0	258
10 ppm Mo	2.8	13.4		
100 ppm Mo	3.4(2)	15.0		
800 ppm Mo			24.5	174
0.645% SO_4			16.9	
2.7% SO_4	3.5(5)	13.9	16.4	
1% DL-Methionine	2.3		14.5	
10 ppm Mo + 2.7% SO_4	2.9(5)	14.8		
100 ppm Mo + 2.7% SO_4	all dead	14.6(5)		
100 ppm Mo + DL-methionine	1.6(3)	10.0		
800 ppm Mo + 0.645% SO_4			16.2	357
800 ppm Mo + 2.7% SO_4			26.9	262
800 ppm Mo + DL-methionine			16.2	79
$L.S.D._{.01}$ [2]	2.4	2.4	7.3	121
$L.S.D._{.05}$	1.7	1.7	5.2	85

[1] Experimental conditions described in footnotes of tables 1 and 2. All values are the mean for 6 male rats unless otherwise indicated by numbers in parentheses.

[2] Least significant difference at specified probability levels.

kidney tubules, which results in a lower level of tissue molybdenum. At certain ratios of copper, molybdenum and sulfate, copper is also removed from the tissues, and high levels of molybdenum and sulfate may impede the passage of copper through tissue membranes. If the effect of methionine is dependent on the conversion of its sulfur to sulfate, then methionine would only affect the excretion of molybdenum in the urine, since the catabolism of methionine to sulfate occurs mainly in the tissues. This could account for the greater effectiveness of sulfate in the copper-adequate rats. On the other hand, methionine may also be counteracting some abnormality in protein metabolism (14).

A decrease in liver sulfide oxidase activity, resulting in the accumulation of sulfide and the precipitation of copper has been suggested as the mode of action of molybdenum (20–22). This appears to be an unlikely explanation, at least for the effect of molybdenum on the copper-adequate animals, since copper did not prevent molybdenosis and methionine was very effective. If methionine was being catabolized via cysteine and the cysteine desulfhydrase reaction, it would have supplied more sulfide and hence have aggravated the effect of molybdenum. On the other hand, if methionine is metabolized to sulfate by way of cysteine sulfinic acid, a major metabolite of cysteine catabolism (23), sulfide is not an intermediate. Certainly, in the copper-adequate rats, any decrease in sulfide oxidase activity was unimportant, since added copper had no appreciable beneficial effect. A reduction of sulfide oxidase activity could have more importance in copper-deficient rats, since the removal of a small amount of copper might reduce the already critical level of tissue copper.

An adequate explanation for the copper-molybdenum-sulfate interaction is not yet available. However, the data obtained in this study clearly demonstrate that the contradictory results reported in the literature on the effect of sulfate on molybdenosis in ruminant and nonruminant animals may be explained on the basis of the copper status of the animals and their dietary copper intake.

LITERATURE CITED

1. Ferguson, W. S., A. H. Lewis and S. J. Watson 1943 The teart pastures of Somerset. I. The cause and cure of teartness. J. Agr. Sci., *33:* 44.
2. Cunningham, I. J. 1946 Copper deficiency in cattle and sheep on peat lands. New Zealand J. Sci. Tech., 27 (A): 381.
3. Britten, J. W., and H. Goss 1946 Chronic molbydenum poisoning in cattle. J. Am. Vet. Med. A., *108:* 176.
4. Comar, C. L., G. K. Davis and L. Singer 1948 The fate of radioactive copper administered to the bovine. J. Biol. Chem., *174:* 905.
5. Dick, A. T. 1953 The effect of inorganic sulphate on the excretion of molybdenum in the sheep. Australian Vet. J., *29:* 18.
6. Gray, L. F., and L. J. Daniel 1954 Some effects of excess molybdenum on the nutrition of the rat. J. Nutrition, *53:* 43.
7. Van Reen, R., and M. A. Williams 1956 Studies on the influence of sulfur compounds on molybdenum toxicity in rats. Arch. Biochem. Biophys., *63:* 1.
8. Miller, R. F., N. O. Price and R. W. Engel 1956 Added dietary inorganic sulfate and its effect upon rats fed molybdenum. J. Nutrition, *60:* 539.
9. Sheard, C., and A. H. Sanford 1929 A photo-electric hemoglobinometer. Clinical applications of the principles of photo-electric photometry to the measurement of hemoglobin. J. Lab. Clin. Med., *14:* 558.
10. Parks, R. Q., S. L. Hood, C. Hurwitz and G. H. Ellis 1943 Quantitative chemical microdetermination of twelve elements in plant tissue. Ind. Eng. Chem. (Anal. ed.), *15:* 527.
11. Snedecor, G. W. 1946 Statistical Methods, ed. 4. The Iowa State College Press, Ames.
12. Cunningham, I. J. 1954 Copper deficiency in cattle and sheep. New Zealand J. Agr., *88:* 369.
13. Neary, E. R. 1946 The use of molybdenized ferrous sulfate in the treatment of true iron deficiency anemia in pregnancy. Am. J. Med. Sci., *212:* 76.
14. Johnson, H. L., and R. F. Miller 1963 Possible mechanism for dietary molybdenum toxicity in the rat. J. Nutrition, *81:* 271.
15. Underwood, E. J. 1962 Trace Elements in Human and Animal Nutrition, ed. 2. Academic Press, Inc., New York.
16 Marston, H. R. 1952 Cobalt, copper and molybdenum in the nutrition of animals and plants. Physiol. Rev., *32:* 66.
17. Kulwich, R., S. L. Hansard, C. L. Comar and G. K. Davis 1953 Copper, molybdenum and zinc interrelationships in rats and swine. Proc. Soc. Exp. Biol. Med., *84:* 487.
18. Beck, A. B. 1963 The copper metabolism of warm-blooded animals with special reference to the rabbit and the sheep. Australian J. Agr. Res., *14:* 129.

19. Dick, A. T. 1956 Molybdenum and copper relationships in animal nutrition. Symposium on Inorganic Nitrogen Metabolism, eds., W. D. McElroy and B. Glass. The Johns Hopkins Press, Baltimore, Maryland, p. 445.
20. Mills, C. F., K. J. Monty, A. Ichihara and P. B. Pearson 1958 Metabolic effects of molybdenum toxicity in the rat. J. Nutrition, 65: 129.
21. Halverson, A. W., J. H. Phifer and K. J. Monty 1960 A mechanism for the copper-molybdenum interrelationship. J. Nutrition, 71: 95.
22. Siegel, L. M., and K. J. Monty 1961 A mechanism for the copper-molybdenum interrelationships. II. Response of liver sulfide oxidase activity to nutritional factors. J. Nutrition, 74: 167.
23. Meister, A. 1957 Biochemistry of the Amino Acids. Academic Press, Inc., New York.

Effect of Protein Deficiency during Gestation and Lactation on Body Weight and Composition of Offspring

P. S. VENKATACHALAM AND K. S. RAMANATHAN
Nutrition Research Laboratories, Indian Council of Medical Research, Tarnaka, Hyderabad-7, India

ABSTRACT The effects of feeding a 7% wheat protein diet either during gestation or lactation, or both, on body weight and composition of offspring of rats at birth and weaning and the lactation performance of dams were studied. The difference between litter size, birth weight and body composition of the progeny of rats receiving the 18% mixed protein and the 7% wheat protein diet was negligible. The 7% wheat protein diet fed during gestation and lactation severely affected the quantity of milk produced but not the concentration of protein in the milk. Pups born to and suckled by mothers receiving the 7% wheat protein diet suffered 100% mortality. Pups born to rats receiving the 18% mixed protein diet but suckled by mothers receiving the 7% wheat protein diet showed very high mortality and the lowest weaning weight. Among those born to mothers receiving the 7% wheat protein diet but suckled by rats receiving the 18% mixed protein diet, the mortality was less and the weaning weight higher than in those of the above group. The highest weaning weight was shown in rats born to and suckled by mothers receiving the 18% mixed protein diet. The body fat, protein and ash content at the time of weaning in those rats stressed by protein deficiency during the prenatal or preweaning period was lower than those of the control rats.

Investigations on experimental animals and human subjects have demonstrated the importance of adequate protein intake during pregnancy for the normal growth and development of the fetus and the successful termination of pregnancy. The amount of protein in the maternal diet during pregnancy appeared to influence the birth weight, birth length and the physical condition of the human newborn (1). Adverse effects on reproduction such as a high percentage of resorption, stillbirths and low birth weight, have been recorded in rats fed a 5% protein (casein) diet during gestation (2). It was found that a level of 5% protein in the diet was critical for normal reproductive performance in rats and that at a level below this, reproduction was severely disturbed. On the other hand, there is no information on the effect of feeding during gestation a low protein diet with protein content above the 5% critical level on the preweaning growth of the offspring. Shaw and Griffiths (3) reported that although birth weight was not strikingly different, the preweaning growth of offspring born to and nursed by rats fed 8% protein (casein) was poor compared with that of offspring born to and nursed by rats receiving an adequate protein diet.

In the present investigation the effect of feeding a 7% wheat protein diet to adult female rats during gestation and lactation on the body weight and body composition of the offspring at birth and weaning was studied. The experiment was designed in such a way that the offspring were subjected to the stress of a protein-deficient diet being fed the dams during gestation or during lactation, or both. The 7% wheat protein diet was chosen to simulate in general the quality and level of protein in the diet of the average Indian.

EXPERIMENTAL

Female albino rats weighing between 150 to 200 g were used. The fertility of the animals and their capacity to rear a litter were established by preliminary mating and observation. The investigation was conducted in 4 experiments, each lasting 6 weeks, 3 weeks during gestation and lactation, respectively.

Received for publication March 17, 1964.

Prior to mating, all of the animals received the normal stock colony diet. From the day of successful mating (judged by the presence of a vaginal plug, or spermatozoa in the vaginal smear) one-half of the animals were fed a low protein diet (7% wheat protein) and the other half continued to be fed the stock colony diet (18% mixed protein). The composition of the diets is shown in table 1. The experimental diet was mixed with water, steam-cooked and fed to the rats in individual cups.

The experimental and control rats were kept in individual cages and no measures were taken to prevent coprophagy.

Experiment 1. Seventeen female rats were used, 9 being fed the low protein diet and 8, the normal protein diet. After birth the number of pups in each litter and the total litter weight at birth were recorded. The newborn from each litter were killed immediately for determination of body composition.

Experiment 2. Sixty female rats were used. Thirty unselected pups consisting of an equal number of males and females from 5 litters born to dams receiving the low protein diet during gestation were transferred within 24 hours of birth to 5 lactating dams (3 males and 3 females per dam) receiving the normal protein diet. The offspring of the latter, less than 24 hours old, had been removed earlier. During the course of the first few days after the transfer, some of the pups from each of the 5 litters died. Whenever such deaths occurred those pups were replaced within 24 hours by others from the 5 litters so that on the 21st day there were 3 dams suckling 6 pups each. It was not possible to maintain the sex ratio of 1:1 in any of these 3 litters. This group of pups was thus exposed to prenatal protein deficiency stress, but was suckled by "normal" dams after birth.

Similarly 108 pups from 14 litters born to rats receiving the normal protein diet during gestation were transferred on the day of birth to 18 lactating dams (3 males and 3 females per mother) receiving the low protein diet. A large number of the offspring died throughout the 21 days and were replaced within 24 hours of death from among the 18 litters so that on the 21st day, there were 4 dams suckling 6 pups each. The sex ratio of 1:1 was not maintained. This group of progeny was thus not exposed to prenatal protein deficiency stress but was suckled by "protein-deficient" dams after birth.

TABLE 1

Composition of diets

	Stock diet 18% protein	Experimental diet 7% protein
	%	%
Whole wheat flour	33.3	60
Skim milk powder	25	—
Chick pea (*Cicer arietenum*)	16	—
Lucerne grass	20	—
Peanut oil	5	5
$CaPO_4$	0.7	—
Cornstarch	—	30
Salt mixture [1]	—	4
Vitamin mixture [2]	—	1
Total	100	100
Supplements to diets		
Vitamin A/week/rat	800 IU	800 IU
Vitamin D/week/rat	—	80 IU
Vitamin E/week/rat	—	5 mg
Choline chloride (added to daily diet before cooking)	—	0.1%

[1] Salt mixture contained: (in grams) calcium lactate, 39; calcium phosphate, 16.2; K_2HPO_4, 28.62; ferrous sulphate, 3.54; NaCl, 5.19; $MgSO_4$, 7.98; sodium phosphate, 10.41; and KI, 0.5.

[2] Vitamin mixture contained: (in grams) thiamine, 0.5; riboflavin, 0.8; niacin, 4.0; pyridoxine, 0.5; Ca pantothenate, 4.0; folic acid, 0.2; menadione, 0.5; inositol, 10.0; *p*-aminobenzoic acid, 10.0; and glucose to make 1000.

Experiment 3. Forty-eight pups — an equal number of males and females — from 8 litters born to rats receiving the low protein diet during gestation were allowed to be suckled by these dams. All except 4 animals died during the first few days of suckling. The four managed to live for a longer time and died between the 17th and 19th day after birth. These pups were thus exposed to protein deficiency stress during both the prenatal and preweaning periods.

Four litters of 6 pups each (3 males and 3 females) from rats receiving the normal protein diet were suckled by the same rats until the 21st day. All animals in this group survived. These pups were thus not exposed to any protein deficiency stress either in utero or postnatally and served as controls.

Weanling weights were recorded on the 21st day and the rats were killed for determination of body composition. The intestinal contents were removed and the carcasses of the weanling rats from each litter were pooled, cut into small pieces and dried in a hot-air oven at 100° until constant weight. The following determinations were carried out; moisture, fat by Soxhlet extraction, and protein (N × 6.25) by Kjeldahal method and ash.

Experiment 4. Forty female rats, one-half receiving the low protein diet and the others, the normal protein diet during gestation, were allowed to give birth and to suckle pups. On the 5th day (1st week), 11th and 14th day (2nd week) and 18th and 21st day (3rd week) after birth samples of breast milk were expressed from 4 dams at a time from each group. The milk was collected without anesthetizing the rats by the method of Ezekiel and Morgan (4). During the 2nd and 3rd week after birth it was not possible to express more than a drop or two of milk from the breasts of rats receiving low protein diet. The total solids, protein, fat and ash content of the pooled samples of milk from 4 animals were determined in duplicate using standard methods.

RESULTS

The results are presented in tables 2, 3, and 4.

The litter size and birth weight of offspring of rats receiving the normal or the low protein diet during gestation showed practically no difference. Also, the moisture, fat, protein and ash content showed no difference between the 2 groups at the time of birth.

On the other hand, weaning weight showed differences between the groups. None of the rats subjected to protein deficiency stress both during prenatal and preweaning periods survived. The four that lived longest weighed 9.4, 9.7, 11.0 and 12.0 g, respectively, a day prior to death. Rats subjected to protein deficiency stress only during the preweaning period showed 78% mortality and low weaning weight. Animals exposed to protein deficiency stress in utero but not during suckling showed 40% mortality and significantly higher weaning weight than the previous group. The highest weaning weight comparable to that obtained in the stock colony was shown by those born to and suckled by dams receiving the normal protein diet.

TABLE 2

Litter size, weight and body composition at birth

Diet of dams during gestation	No. of rats	Litter size	Birth weight	Body composition			
				Moisture	Fat	Protein (N × 6.25)	Ash
			g	% of body wt	% of body wt	% of body wt	% of body wt
18% Protein	8	7.1 ± 0.73 [1]	5.01 ± 0.123 [2]	86.3	1.3	9.9	1.6
7% Protein	9	6.2 ± 0.68	5.04 ± 0.113 [2]	86.6	1.1	9.8	1.6

[1] Mean ± SE.

[2] $\frac{\text{Total litter weight at birth}}{\text{Total no. of offspring}}$.

TABLE 3

Body weight and composition at weaning

Diet fed dams		No. weaned	Mortality	Wt at weaning	Body composition			
During gestation	During lactation				Moisture	Fat	Protein (N × 6.25)	Ash
			%	g	% of body wt	% of body wt	% of body wt	% of body wt
7% Protein	7% protein	0	100	—	—	—	—	—
18% Protein	7% protein	24	78	13.8 ± 0.23 [1]	75.9	4.2	16.4	2.5
7% Protein	18% protein	18	40	18.2 ± 0.32	74.7	3.3	17.3	3.1
18% Protein	18% protein	24	0	35.3 ± 0.50	70.7	9.7	16.8	2.5

[1] Mean ± SE.

The total moisture content in relation to body weight was reduced in all the groups at the time of weaning compared with the values at birth shown in table 2. The moisture content in relation to body weight of weanlings not exposed to any stress (controls) was lower than that of the other 2 groups. However, the moisture content expressed as percentage of lean body mass was nearly the same in all groups.

The carcasses of rats subjected to either prenatal or preweaning protein deficiency contained less fat compared with that of control animals. The protein and ash content also was lower in the 2 former groups compared with the controls.

The milk output was satisfactory in dams receiving the normal protein diet. During one expression of milk, nearly 0.5 to 1.5 ml could be obtained from a single rat. In the later weeks, the output of milk was even greater. On the other hand, the output of milk of dams receiving the low protein diet was negligible. Although barely 0.5 to 0.75 ml of milk was obtained from 4 animals during the first week, it was not possible to obtain even this quantity in the subsequent weeks even though sufficient sucking stimulus was provided by replacing the dying pups immediately. The quality of the breast milk with respect to total solids, protein, fat and ash did not change.

DISCUSSION

Nelson and Evans (2) showed that as the level of protein (casein) in the diet fell below 5%, the reproductive performance was affected markedly. Ambegaokar and Chandran (5) demonstrated that poor fertility, small litter size and low average birth weight of offspring were the result of feeding female rats a 5% rice protein ration from a very young age.

On the basis of evidence presented, it is concluded that the rats' consumption of a 7% wheat protein diet ad libitum during gestation caused no adverse effects on reproduction. The animals were apparently normal, did not lose body weight and delivered a normal-size litter with offspring of average birth weight of over 5 g and of normal body composition. The low protein diet did not affect the appetite of the gestating rats and the food intake was adequate.

The results of the present investigation showed, however, that although the reproductive performance was not affected, the growth performance of the newborn rats following birth was markedly influenced by feeding the dams a 7% wheat protein diet during gestation or lactation, or both.

The pups subjected to the stress of protein deficiency during both the prenatal and preweaning periods showed the poorest results. The 100% mortality in this group was largely the result of poor lactation by the dams. Even though the output of milk in dams receiving the 7% wheat protein diet was reduced severely, the quality of milk with respect to protein showed no difference compared with that of the controls fed the normal protein ration. This observation was somewhat similar to results obtained with human mothers in whom protein supplementation caused increased milk output but a slight decrease in protein concentration (6), and confirmed the results obtained in rats by other workers (7). It appears that the stress of protein deficiency on the lactating

TABLE 4

Composition of breast milk of rats

Constituents	Diet fed dams during lactation			
	18% Protein diet			7% Protein diet
	Week 1	Week 2	Week 3	Week 1
	g/100 ml	*g/100 ml*	*g/100 ml*	*g/100 ml*
Total solids	21.15	22.07 [1]	25.43 [1]	21.60
Protein	9.19	9.22	9.30	8.84
Fat	8.67	8.76	10.75	8.70
Ash	1.20	1.16	1.30	0.92

[1] Average of 2 values which closely agreed with each other.

dam was manifested as reduced output of milk, resulting in starvation during suckling.

The high mortality and the low weaning weight of rats subjected to preweaning protein deficiency stress could be attributed largely to the inadequate breast milk available in the foster mothers. The weight gain of the animals was uniformly slow throughout the preweaning period and the young died at regular intervals. However, the weaning performance in this group was somewhat better than that of those subjected to protein deficiency both in utero and after birth. It is possible that the low protein diet fed during gestation might have aggravated the deleterious influence on growth brought about by insufficient milk during the early weeks of life. This is of great significance because the average birth weight of the offspring in the 2 groups of mothers receiving 7% wheat and 18% mixed protein diets during gestation showed no difference (table 1).

The weight at weaning of rats exposed to protein deficiency stress prenatally but not after birth was distinctly superior to that of rats fed the protein-deficient ration only after birth. However, the weaning weight of these animals was only one-half that of the controls and the mortality among them was nearly 40%. The poor preweaning growth and high mortality was obviously not due to lack of breast milk since the young were suckled by "normal" dams. It appears, therefore, that protein deficiency during gestation, even if it is not reflected in lowered body weight of offspring at birth, can impair the growth of the offspring, and that such impairment would be evident even if adequate protein was supplied in the postnatal period.

It is necessary, however, to exclude the possibility that foster mothers had failed to suckle the offspring. In our rat colony, it has been our experience that in nearly 80 to 90% of the cases, the dams will suckle the pups of another litter without any difficulty if transferred within 48 hours. If the dams do not accept the litter, they destroy the pups; there is nothing like partial acceptance where foster mothers provide lesser amounts of milk to the suckling rats. In the present study the adult female rats were used for the experiment only after their capacity to rear offspring had been established previously.

Weanlings suckled by dams receiving the stock diet, after a period of time (usually 2 weeks after birth) had free access to the high protein stock diet. It cannot be stated that better performance of these offspring is due to consuming the stock diet, because the weanlings receiving the protein-deficient diet prenatally had equal opportunities to consume the stock diet. The poor growth performance of the latter might be due to the inability of the offspring, though born with normal birth weight, to obtain sufficient milk while suckling. The other possibility is that the rats were not able to utilize the consumed milk for body growth.

ACKNOWLEDGMENT

The authors express their thanks to Dr. C. Gopalan, Director, Nutrition Research Laboratories, for his interest and encouragement.

LITERATURE CITED

1. Burke, B. S., S. S. Stevenson, J. Worcester and H. C. Stuart 1949 Nutrition studies during pregnancy. V. Relation of maternal nutrition to condition of infant at birth: Study of siblings. J. Nutrition, *38:* 453.
2. Nelson, M. M., and N. M. Evans 1953 Relation of dietary protein levels to reproduction in rat. J. Nutrition, *51:* 71.
3. Shaw, J. H., and D. Griffiths 1963 Dental abnormalities in rats attributable to protein deficiency during reproduction. J. Nutrition, *80:* 123.
4. Ezekiel, E., and E. H. Morgan 1963 Milk iron and its metabolism in the lactating rat. J. Physiol. (London), *165:* 336.
5. Ambegaokar, S. D., and K. Chandran 1959 Physiological effects of low protein diets. I. Studies on nitrogen balance, growth, reproduction and lactation and basal metabolism in rat. Ind. J. Med. Res., *47:* 539.
6. Gopalan, C. 1958 Effect of protein supplementation and some so-called "galactogogues" on lactation of poor Indian women. Ind. J. Med. Res., *46:* 317.
7. Mueller, A. J., and W. M. Cox 1946 The effect of changes in diet on the volume and composition of rat milk. J. Nutrition, *31:* 249.

Effect of Feeding Colostral and Milk Components on the Cessation of Intestinal Absorption of Large Molecules (Closure) in Neonatal Pigs [1]

J. G. LECCE, D. O. MORGAN AND G. MATRONE
Department of Animal Science, North Carolina State of the University of North Carolina, Raleigh, North Carolina

ABSTRACT For approximately the first 36 hours of life, neonates of some species, e.g., piglets, are able to absorb large molecules from their intestines. The time when the piglet ceased absorbing large molecules (closure) was a function of feeding regimen. Closure activity was in sow's and cow's colostrum. A diet containing protein in a simple salt solution (either egg albumin, porcine albumin or porcine γ-globulin) did not render the piglet's gut impermeable to large molecules. Nor was milk fat needed in the diet for closure to occur. A diet of boiled (essentially protein- and fat-free) cow's colostral whey engendered closure. Also, closure activity was in a dialyzate of cow's colostrum and in a dialyzate of non-fat dried milk solids. No activity was associated with synthetic milk salts, vitamins or sugars. Thus, closure is possible with a heat-stable, low molecular weight, protein- and fat-free fraction of milk; and activity is not dependent on the absorption of large molecules.

Neonates of many species such as pigs, ruminants and horses are able to absorb large protein molecules through their intestines without prior digestion or alteration of the molecules. This special capacity for absorption ceases when the neonate is approximately 36 hours old (1). Recently, Lecce and Morgan (2) showed that the time interval for the cessation of absorption of large molecules (closure) was a function of the feeding regimen. For example, neonatal pigs that were starved from the time of birth remained open or could absorb polyvinylpyrrolidone (PVP) for at least 86 hours, whereas nursing piglets were closed or were unable to absorb this large polymer when 24 to 36 hours old. It was possible to mimic the closure time (24 hours) observed in nursing piglets by feeding them at least 300 to 400 ml of cow's colostrum. Comparable results were obtained with lambs (2).

Since cow's colostrum profoundly affected closure time, it seemed worthwhile to define further the factor(s) in colostrum responsible for closure. Grossly, colostrum can be thought of as water containing fats, carbohydrates, proteins, minerals, and vitamins. The experiments reported herein were intended to test which of these major groups per se were required for closure.

MATERIALS AND METHODS

Experimental animals. Piglets farrowed in an isolation unit were caught at birth in sterile towels, transferred to another isolation unit and individually caged. Here, they were fed from a pan a total of 600 ml of the randomly assigned diet. The first feeding of 100 ml occurred within 3 hours of birth. The piglets were fed again 150 ml when 8 hours old, 175 ml at 12 hours, and 175 ml at 15 hours of age. Piglets were permitted no food after 20 hours. When 24 hours old they were tested for absorbing capacity.

A total of 105 pigs from 14 litters was used. The number of pigs fed each diet is listed in table 1.

Closure testing system. The procedure used for determining whether piglets could absorb large molecules when 24 hours old was described previously (2). Essentially, it consisted of administering to 24-hour-old piglets a test dose of 20 ml of 21% PVP-K30[2] via a stomach tube. Six hours later the piglets were bled from the anterior

Received for publication April 13, 1964.

[1] Contribution from the Animal Science Department, North Carolina Agricultural Expermient Station, Raleigh, North Carolina. Published with the approval of the Director of Research as Paper no. 1770 of the Journal Series.

[2] Average molecular weight of 40,000. Antara Chemicals, New York.

TABLE 1

Effect of dietary components on closure in piglets

	Experiment 1			
	Boiled colostral whey	Simple salt	Simple salt + 1.5% porcine protein [1]	Simple salt + 8% egg white
Number closed [2]	6 [3]	1	0	2
Number open	0	5	6	5

	Experiment 2		
	Cow's colostrum	Dialyzed cow's colostrum	Dialyzate cow's colostrum
Number closed	3	3	3
Number open	0	0	0

	Experiment 3		
	Skim milk	Dialyzed skim milk	Dialyzate skim milk
Number closed	5	2	16
Number open	1	4	0

	Experiment 4		
	Complex salt	Complex salt + sugar [4,5]	Complex salt + vitamins
Number closed	2	2	1
Number open	16 [5]	7	9

[1] Three pigs were fed 1.5% porcine albumin and none were closed; 3 pigs were fed 1.5% porcine γ-globulin and none were closed.
[2] Did not absorb measurable amounts of polyvinylpyrrolidone (PVP-K30) from the gut when 24 hours old.
[3] Three of these pigs were tested for absorbing capacity with cow's colostrum.
[4] Either 2% lactose or 3% glucose.
[5] Seven pigs were fed 3% glucose and 2 were closed; 2 pigs were fed 2% lactose and none were closed.

vena cava and the serum analyzed for PVP using agar electrophoresis (2, 3). To guard against the possibility that the results with PVP were artifacts of unnatural molecules, on occasion, cow's colostrum, a more natural system, was substituted for PVP. In these instances, at 24 hours of age piglets were given 40 to 50 ml of cow's colostrum via stomach tube. The piglet's serum, obtained at 30 hours, was analyzed for bovine globulin using agar electrophoresis; and unless the β_2-γ-globulin fraction increased more than 3%, the piglet was considered closed or negative for absorption.

Diets. Boiled colostral whey: Commercial rennett dialyzed free of salt was used in a 1:2000 final concentration to precipitate the casein in skimmed cow's colostrum. The casein-free fraction, brought to pH 5.4 with concentrated HCl, was boiled for ten minutes and the coagulated whey proteins removed by filtration. The filtrate was adjusted to pH 7.0 with an equal molar mixture of NaOH and KOH and centrifuged at 16,000 × *g* at 4° for 30 minutes to remove the remaining fat. There was less than 1.0% protein left in these preparations.

Simple salt diet: This basal diet had 77 mEq/liter Na^+, 42 K^+, 79 Cl^-, and 60 $PO_4^{\equiv}$. This Na-to-K ratio is closer to that found in colostrum than in serum. A final concentration of either 1.5% porcine albumin,[3] 1.5% porcine γ-globulin, or 8% fresh egg white also was added to this basal salt solution. Since the additives may have salt contaminants, they were dialyzed against the simple salt solution before being added.

Complex salt diet: This solution contained all the major and trace salts in amounts equivalent to that of cow's colos-

[3] Porcine albumin and γ-globulin obtained from Pentex Incorporated, Kankakee, Illinois.

trum; i.e., 64 mEq/liter Ca^{++}, 29.1 Na^{+}, 40.9 K^{+}, 12.0 Mg^{++}, 7.2 Zn^{++}, 2.4 Fe^{++}, 0.12 Co^{++}, 0.42 Cu^{++}, 1.2 Mn^{++}, 0.28 Mo^{+}, 122 $PO_4^{\equiv}$, 42 Cl^{-}, 16 $SO_4^{\equiv}$; in some instances lactose at a final concentration of 2% or glucose at a concentration of 3% was added. Also some of the piglets were fed the salt mixture to which vitamins were added (table 2).

Dialyzate from cow's colostrum: This was prepared by dialyzing colostrum against 5 times its volume of distilled water at 4°. The distilled water was changed and saved 4 times over a period of 5 days. The dialyzate was concentrated to the original volume of colostrum by boiling. Na^{+} and K^{+} determination on the dialyzate indicated that at least these 2 ionic species had been recovered and concentrated to the same amount as originally found in colostrum.

Dialyzate from dried non-fat cow's milk: This was prepared similarly to the dialyzate from colostrum except that prior to dialysis non-fat milk solids (powdered milk) were resuspended in distilled water so that the concentration was 2.5 times that of milk. After dialysis the volume of the dialyzate was readjusted to that found in milk and again Na^{+} and K^{+} determinanation indicated almost complete recovery in the dialyzate of these 2 ions.

Earlier experiments showed that piglets fed salt-free diets would consume only approximately 300 ml in 15 to 20 hours. If more was forced into the piglet, he became comatose and died. Because of this, the simple salt mixture was added to the dialyzed colostrum and milk before feeding them to piglets.

TABLE 2

Vitamins in complex salt solution

	mg/600 ml diet
Inositol	109
Vitamin B_{12}	45
Ca pantothenate	14
Niacin	10.8
p-Aminobenzoic acid	10.8
Riboflavin	8.2
Pyridoxine	5.5
Thiamine	3.1
Menadione	0.1
Folic acid	0.5
Biotin	0.01
α-Tocopherol	5.5
Choline chloride	1090

RESULTS

Experiment 1. The first experiment was designed to determine whether fat and protein were necessary for closure. For this purpose, piglets were fed fat- and protein-free colostrum (boiled colostral whey). Our preparations of boiled colostral whey still contained a trace amount of proteinaceous material (less than 1%) that migrated electrophoretically more rapidly than the original milk proteins. However, there were no proteins left in boiled colostral whey that were recognizable as milk proteins by immunoelectrophoresis. Since there was the possibility of trace protein left in boiled whey, to help evaluate the effect of small amounts of protein, a control diet consisting of either 1.5% porcine γ-globulin or 1.5% porcine albumin in the simple salt solution (Na^{+}, K^{+}, $PO_4^{\equiv}$, Cl^{-}) also was fed. Results of this experiment (table 1, exp. 1) indicated that protein and fat as they exist in colostrum were not necessary for closure activity since piglets fed the boiled fat- and protein-free colostral whey were closed when tested with PVP or cow's colostrum when 24 hours old. The absorption of small amounts of protein appeared to have little effect on closure as piglets fed the salt diet with added 1.5% porcine proteins were able to absorb at the end of 24 hours just as well as the piglets fed the salt diet alone.

Further evidence that closure and absorption were independent phenomena was obtained by feeding piglets the simple salt diet to which fresh egg white was added. In this instance the concentration of protein was approximately 8%, similar to the concentration of soluble protein in colostrum. Piglets readily absorbed egg albumin from this diet (fig. 1). This showed that the absorption mechanism had been stimulated and in operation, yet 5 out of 7 of these pigs still were open to the test at 24 hours of age (table 1, exp. 1).

Experiment 2. As the experiment above indicated that closure activity was associated with a heat-stable, non-protein, non-fat fraction, it was of interest to exclude other high molecular weight material that might be left in boiled colostral whey. Accordingly, piglets were fed dialyzate from cow's colostrum, dialyzed cow's colostrum, and the cow's colostrum. Results of

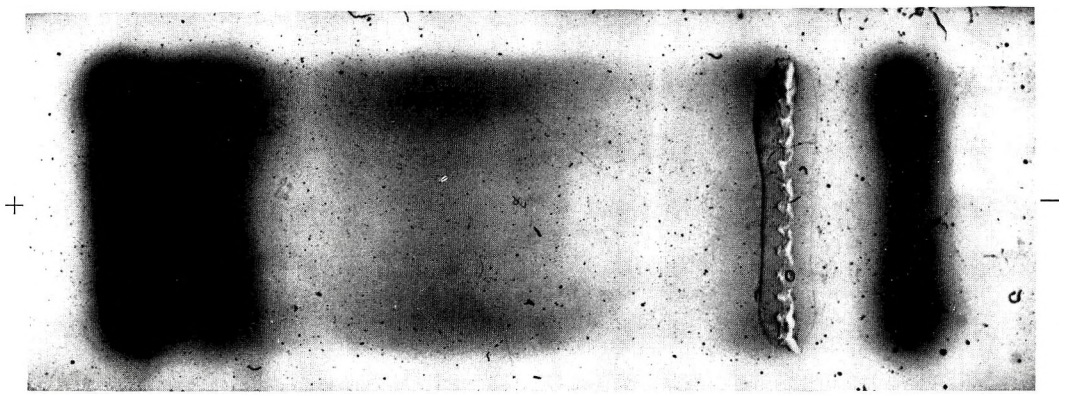


Fig. 1 Agar electrophoresis of serum from pig fed egg white in simple salt solution, showing the absorption of the egg protein, conalbumin. Veronal buffer pH 8.6, 0.075 ionic strength, (+) indicates direction of protein migration.

this experiment indicated that the closure activity could pass through a dialysis bag that excludes material over an approximate molecular weight of 10,000 to 20,000 (table 1, exp. 2). Dialyzed colostrum still had closure activity which could have been caused by the binding of some of the active material to large molecules, thereby resulting in inefficient dialysis (4).

Experiment 3. For future work it would be desirable to have a more readily available source of the active closure principal than cow's colostrum. Thus, in this experiment the activity of dried non-fat milk was tested. Here, piglets were fed skim milk, dialyzed skim milk, and the dialyzate from skim milk. This experiment showed that closure activity was present in the dialyzate as all 16 piglets were unable to absorb the test dose at 24 hours of age. It appeared that the dialysis was more complete with the skim milk in comparison with the cow's colostrum as 4 of the 6 piglets fed dialyzed skim milk were still open (table 1, exp. 3).

Experiment 4. In the last experiment of this series the major and minor salts that are found in colostrum were tested for closure activity. Piglets fed diets containing these salts readily absorbed the test dose at 24 hours of age. These results indicated that the salts were not of primary importance for closure (table 1, exp. 4); nor did the addition of either lactose, glucose, or vitamin have any obvious effect. Unexpectedly, piglets fed this complex salt diet ate it readily and appeared extremely content and vigorous for the first 3 days. From then on the piglets became weak and died.

DISCUSSION

Pinocytosis (cell drinking) is regarded as the mechanism for the absorption of large molecules, including the temporary specialized absorption of large amounts of protein by some neonates (5, 6). Briefly, it is thought that charged nutrients are adsorbed to the cell surface, thereby stimulating indentation and folding-in of the surface membrane resulting in a pinching off of the membrane and vesiculation. Thus the nutrients, surrounded by the membrane, are passed into the cell. Protein and salt solutions are potent pinocytotic inducers (7, 8); and in the neonatal pig, calf, kitten, lamb, and goat, colostrum strongly stimulates this vesiculation of the intestinal epithelium (9–11). Clark (12), studying the neonatal rat and mouse gut with the electron microscope, showed that when the animals were approximately 20 days old they were no longer able to form vesicles when fed pinocytotic stimulators such as protein. This time of 20 days also was coincident with closure time (the period after which the mouse or rat cannot absorb large molecules).

Since proteins are potent stimulators of pinocytosis and since they are so intimately involved in the aborption per se, we and

others (13, 14) were biased in feeling that protein must be somehow associated with the cell for closure to occur — perhaps by exhausting pinocytotic sites on the cell membrane. However, this does not appear to be so. Our results indicate that absorption of large molecules and closure are 2 independent phenomena. For example, in experiment 2, piglets fed salt solutions containing high molecular weight materials such as porcine and avian proteins readily absorbed these polymers, without resulting in closure, whereas in some of the other experiments, protein-free diets (such as the boiled colostral whey and dialyzate from milk and colostrum) did engender closure.

In addition to demonstrating that absorption of protein per se was not necessary for closure, our experiments were able to exclude other obvious groups of colostral components such as carbohydrates, vitamins, and minerals as being responsible by themselves for closure. The factor(s) responsible for closure appears to be a heat-stable, low molecular weight (less than 10,000 to 20,000) compound that is a part of the dialyzate of colstrum or milk. However, the importance of an interaction between the component parts of the dialyzate has not yet been ruled out. And, closure-diets thus far are rather complete nutritionally.

Closure, mediated by diet, appears a basic enough phenomenon that no doubt plays an important role in the economy of the piglet in nature. In this respect, our past and present results suggest that the piglet is born fetal-like in at least 2 aspects. He is born with an immature serum protein profile (15), and also he is born with a primitive-functing intestinal epithelium. In nature, the sow's colostrum rapidly alters both these immature physiologic states by supplying soluble proteins that contribute to the maturation of the serum protein profile (15) and also by supplying factors that induce maturation of the intestinal epithelium (closure). These 2 states are in a delicate and synchronous balance; that is, at about the time the piglet has absorbed adequate protein, his epithelial cell is mature and can no longer absorb large molecules (approximately 36 hours).

Perhaps in part, the difficulty in raising piglets artificially results from not reckoning with these 2 immature states, and as a consequence diarrhea, bacteremia and death ensue (16). To illustrate this point, neo-natal piglets are most unhealthy when fed diets tnat permit uninhibited growth of intestinal microbes at the time the piglet is open and probably most susceptible to damage by bacteria. On the other hand, neonatal piglets are healthiest when fed diets containing large amounts of γ-globulin (carrying antibodies or bacterial inhibitors) (17). Likely, this healthy state results in part from inhibition of microbial growth and invasion in the gut by excess γ-globulin during the time the piglet is open and is in the process of absorbing needed serum proteins. This time period coincides with the time the piglets would receive colostrum naturally (3). It is suggested then that piglets fed antibody-devoid diets must be therapeutically protected while open and, in addition, fed diets that promote rapid closure. It follows that once closed the intestinal microbes will be of little moment (17).

Possibly, the phenomenon of closure may be complicating the health of other animals raised artificially. Recently, a number of papers have been published indicating that many unthrifty human infants, particularly in their first year, have antibodies to cow's milk (18–26). These antibodies result, presumably, from the absorption of bovine proteins which in turn behave as foreign antigens engendering via antibody production a hypersensitive or allergic state. The question arises whether the alleged absorption of bovine proteins in the infant results from transient local defects in the intestinal epithelium or whether it results from a remnant (some infants have more of a remnant than others) of a fetal or primitive absorption mechanism found so exaggerated in the piglets. If the latter was the case, then possibly the non-allergenic factors responsible for the maturation of the piglet's intestinal epithelium could well serve the same purpose in infants.

LITERATURE CITED

1. Brambell, F. W. R. 1958 The passive immunity of the young mammal. Biol. Rev., 33: 488.

2. Lecce, J. G., and D. O. Morgan 1962 Effect of dietary regimen on cessation of intestinal absorption of large molecules (closure) in the neonatal pig and lamb. J. Nutrition, *78:* 265.
3. Morgan, D. O., and J. G. Lecce 1964 Electrophoretic and immunoelectrophoretic analysis of the proteins in sow's mammary secretions throughout lactation. Res. Vet. Sci., *5:* 332.
4. Dupree, L. T., K. K. Sanford, B. B. Westfall and A. B. Covalesky 1962 Influence of serum proteins on determination of nutritional requirements of cells in culture. Exp. Cell Res., *28:* 381.
5. Holter, H. 1959 Pinocytosis. Intern. Rev. Cytol., *8:* 481.
6. Wilson, T. H. 1962 Intestinal Absorption. W. B. Saunders Company, Philadelphia.
7. Mast, S. O., and W. L. Doyle 1934 Ingestion of food by amoeba. Protoplasma, *20:* 555.
8. Chapman-Andresen, C., and D. M. Prescott 1956 Studies on pinocytosis in the amoebae *Chaos chaos* and *Amoeba proteus*. C. R. Trav. Lab., Carlsberg, Ser. Chim., *30:* 57.
9. Smith, T. 1925 Hydropic stages in the intestinal epithelium of new-born calves. J. Exp. Med., *41:* 81.
10. Comline, R. S., R. W. Pomeroy and D. A. Titchen 1953 Histological changes in the intestines during colostrum absorption. J. Physiol., *122:* 6.
11. Hill, K. J., and W. S. Hardy 1956 Histological and histochemical observations in the intestinal cells of lambs and kids absorbing colostrum. Nature, *178:* 1353.
12. Clark, S. L. 1959 The ingestion of proteins and colloidal materials by columnar absorptive cells of the small intestine in suckling rats and mice. J. Biophys. Biochem. Cytol., *5:* 41.
13. Payne, L. C., and C. L. Marsh 1962 Gamma globulin absorption in the baby pig: the non-selective absorption of heterologous globulins and factors influencing absorption time. J. Nutrition, *76:* 151.
14. Payne, L. C., and C. L. Marsh 1962 Absorption of gamma globulin by the small intestine. Federation Proc., *21:* 909.
15. Lecce, J. G., and G. Matrone 1960 Porcine neonatal nutrition: the effect of diet on the blood serum proteins and performance of the baby pig. J. Nutrition, *70:* 13.
16. Lecce, J. G., G. Matrone and D. O. Morgan 1961 The effect of diet on the maturation of the neonatal piglet's serum protein profile and resistance to disease. Ann. N. Y. Acad. Sci., *94:* 250.
17. Lecce, J. G., and B. R. Reep 1962 *Escherichia coli* associated with colostrum-free neonatal pigs raised in isolation. J. Exp. Med., *115:* 491.
18. Parish, W. E., A. M. Barrett, R. R. A. Coombs, M. Gunther and F. E. Camps 1960 Hypersensitivity to milk and sudden death in infancy. Lancet, *2:* 1106.
19. Gunther, M., R. Aschaffenburg, R. H. Matthews, W. E. Parish and R. R. Coombs 1960 The level of antibodies to the proteins of cow's milk in the serum of normal human infants. Immunology, *3:* 296.
20. Gunther, M., E. Cheek, R. H. Matthews and R. R. A. Coombs 1962 Immune esponses in infants to cow's milk proteins taken by mouth. Intern. Arch. Allergy, *21:* 257.
21. Collins-Williams, C. 1962 Cow's milk allergy in infants and children. Intern. Arch. Allergy, *20:* 38.
22. Heiner, D. C., J. W. Sears and W. T. Kniker 1962 Multiple precipitins to cow's milk in chronic respiratory disease. Am. J. Dis. Child., *103:* 634.
23. Holland, N. H., R. Hong, N. C. Davis and C. D. West 1962 Significance of precipitating antibodies to milk proteins in the serum of infants and children. J. Pediatrics, *61:* 181.
24. Kaufmann, H. J., J. Lantz and A. Burgin-Wolff 1963 Anaphylactic shock of the lungs triggered by microaspiration of cow's milk: a form of sudden unexpected death in early infancy. Ann. Paediat., *200:* 20.
25. Peterson, R. D. A., and R. A. Good 1963 Antibodies to cow's milk proteins — their presence and significance. Pediatrics, *31:* 209.
26. Anonymous 1963 Milk antibodies in human serum. Nutrition Rev., *21:* 80.

Selenium-75 Metabolism in the Gestating Ewe and Fetal Lamb: Effects of Dietary α-Tocopherol and Selenium [1]

P. L. WRIGHT AND M. C. BELL
Agricultural Research Laboratory of the University of Tennessee Oak Ridge, Tennessee [2]

ABSTRACT Forty-eight pregnant ewes were fed a purified ration containing urea as the sole nitrogen source. Selenium and α-tocopherol were added separately and in combination to the basal ration. Serum glutamic oxaloacetic transaminase (SGO-T) in young pregnant ewes was increased after the animals had received the α-tocopherol-deficient ration for 9 weeks; selenium delayed but did not prevent this increase. No changes in SGO-T values were observed in the aged ewes during the 18-week experimental period. Selenium supplementation reduced the apparent absorption of an oral dose of Se^{75} and increased the urinary Se^{75} excretion. Very high concentrations of radioselenium were noted in kidney and spleen samples from the selenium-deficient ewes, implicating these tissues as prime areas of importance in selenium metabolism. Intracellular particulate matter separations revealed an increase in Se^{75} content of the liver microsomes and kidney nuclei in tissues from selenium-deficient ewes. The radioselenium distribution pattern in fetal tissues was similar to that in maternal tissues. A definite placental barrier was observed, with single fetuses having twice the concentration of Se^{75} as twin fetuses. The expected severe gross pathological lesions of α-tocopherol and selenium deficiency were not observed in these studies based on a ration which was not highly auto-oxidizable.

The methods used in the experimental production of dietary-induced myopathies were divided between 2 general stratagems. The first consisted of feeding rations to which copious amounts of fats containing highly unsaturated fatty acids were added or, similar in mechanism, the use of torula yeast as a protein and unsaturated fatty acid source. The second was the use of feedstuffs from areas where muscular dystrophy is enzootic. Results from such studies indicate that myopathies induced by addition of unsaturated fats to the ration respond most readily to α-tocopherol (1) or synthetic antioxidant (2) therapy, whereas those resulting from use of rations from certain areas affected with enzootic dystrophies are more responsive to selenium supplementation (3). Numerous reports demonstrating the beneficial effects of selenium therapy in farm animals have been reviewed (4–6).

Limited information is available on the influence of low dietary intake of selenium upon the selenium content of animal tissues. Cousins and Cairney (7) and Burton et al. (8) reported that the selenium levels of kidneys or livers of healthy lambs or lambs from flocks not affected with white muscle disease were approximately twofold greater than similar values for affected lambs or lambs from affected flocks. Lindberg and Sirén (9) reported similar observations in the pig.

In a recent review, McConnell (10) pointed out that selenium readily traverses cellular membranes, that it is readily incorporated into a variety of tissue proteins, and that once selenium enters the animal it is retained for extended periods of time. Relatively few detailed studies have been conducted on the metabolic fate of dietary selenium in animals on low dietary selenium or low α-tocopherol intakes. Wright and Bell (11) demonstrated that selenium as selenite moved into ovine blood cells in vitro via an oxygen-dependent transport mechanism and that the magnitude of this influx was inversely proportional to the dietary intake of selenium.

Received for publication April 24, 1964.
[1] Published with the permission of the Director of the University of Tennessee Agricultural Experiment Station, Knoxville, Tennessee.
[2] Operated by the Tennessee Agricultural Experiment Station for the U. S. Atomic Energy Commission under Contract no. AT-40-1-GEN-242.

The present study was designed to separate the effects of dietary selenium and of α-tocopherol on the metabolism of selenium in the gestating ewe and fetal lamb. A purified ration utilizing urea as the sole source of nitrogen was used in an effort to surmount the confounding effects of using a preformed protein source which would contribute significant amounts of selenium or unsaturated fats. Lard subjected to molecular distillation was fed as the only source of fat in order to maintain an α-tocopherol-free diet.

MATERIALS AND METHODS

The basal ration used in these experiments is shown in table 1. The mineral mixture, essentially that of Oltjen et al. (12), consisted of reagent grade chemicals. The basal ration contained 0.01 ppm selenium as determined by neutron activation analysis at Oak Ridge National Laboratory; no α-tocopherol was detectable in 5-g aliquots (13). Vitamin D_2 was added to the diet to provide 400 IU[3] per ewe per day. Vitamin A was injected intramuscularly at weekly intervals to provide 2500 IU[4] per ewe per day. The animals were housed in a barn open on one side, with concrete floors. No bedding was provided.

During a 2-year period, 48 ewes (24, 4- to 5-year-old ewes and 24, 9-month-old ewe lambs) were placed on the experimental ration at time of mating. These ewes were uniformly divided into 4 groups representing a factorial arrangement of the following variables: zero or 0.5 ppm selenium as sodium selenite in the ration, and zero or 100 IU of vitamin E/day as the acetate[5] in a drench. The rations were fed ad libitum.

TABLE 1

Composition of basal diet

	%
Cellulose [1]	30.0
Cornstarch	29.7
Glucose monohydrate	29.7
Urea	4.0
Stripped lard [2]	2.0
Choline chloride	0.1
Ground plastic [3]	1.0
Mineral mixture [4]	3.5

[1] Solka Floc, BW-40, Brown Company, Berlin, New Hampshire.
[2] Molecular distillation, donated by Distillation Products Industries, Rochester, New York.
[3] Polyethylene, M-700. E. I. du Pont de Nemours Company, Wilmington, Delaware.
[4] Contained: (in per cent) $CaHPO_4$, 48.84; K_2CO_3, 31.55; $MgSO_4$, 10.75; NaCl, 7.43; $FeSO_4$, 0.91; $Na_2B_4O_7$, 0.19; $ZnSO_4$, 0.14; $MnSO_4$, 0.10; KI, 0.03; $CuCO_3$, 0.02; MoO_3, 0.0008; $CoCl_2$, 0.0003.

Serum glutamic oxaloacetic transaminase (SGO-T) was determined [6] at 3-week intervals. On approximately the 135th day of gestation, 8 ewes from each treatment were placed in individual metabolism stalls and dosed orally with 250 μc of Se^{75} (H_2SeO_3, 40 mc/mg Se) adsorbed on cellulose in a gelatin capsule. Urine was collected by means of a retained foley catheter [7] placed directly into the bladder via the urethra, and the feces were collected in a tray.

Forty-eight hours postdosing, the ewes were exsanguinated. The kidneys, liver, lungs, heart, and spleen were removed intact and weighed. Samples of the longissimus dorsi, biceps femoris, and blood were also obtained. The whole blood mass was calculated as 5.8% of body weight (14). The gastrointestinal tract of the ewes was removed and the total contents collected, pooled, weighed and sampled for Se^{75}. The fetal lambs were removed from the uteri and similar tissue samples collected; where twin fetuses were present they were sampled individually. A 2-g sample of each tissue was placed in plastic tubes for Se^{75} determination in a well-type scintillation counter.

Immediately after removal, aliquots of the kidney cortex and liver from the ewe lambs were placed in ice-cold 0.25 M sucrose. Cell particulate matter fractions were separated from duplicate samples of each tissue by differential centrifugation (15).

Statistical evaluation of the data was by the method of least squares analysis of variance.

RESULTS AND DISCUSSION

Experimental rations were consumed sufficiently well that body weight gains by the ewes (table 2) were large enough (16) to allow normal development of fetal lambs. Ewes fed the basal ration gained less ($P < 0.05$) than ewes fed the basal

[3] Super Drex-95, Nopco Chemical Company, Newark, New Jersey.
[4] Injectable Vitamin A, Nopco Chemical Company.
[5] Type F-50, Distillation Products Industries, Rochester, New York.
[6] Sigma Chemical Company 1961 Technical Bulletin no. 508, St. Louis.
[7] Bardex 120 p, 10 French.

TABLE 2

Feed consumption and body weight gains of ewes fed a purified ration during gestation

	Treatment [1]			
	E + Se	Se	O	E
	kg	*kg*	*kg*	*kg*
Avg feed consumption/day	1.01	1.12	0.88	0.99
Avg gain [2]	5.08	5.40	2.45 [3]	5.0

[1] E represents 100 IU α-tocopherol/day, Se represents 0.5 ppm dietary selenium, O represents basal ration only.
[2] Average total gain/ewe during first 135 days of gestation.
[3] Less than other values within row ($P < 0.05$).

ration supplemented with α-tocopherol or selenium, or with both. In previous studies involving α-tocopherol and selenium suppementations to rations for gestating ewes, no body weight changes for the ewes were reported. Increases in growth rate of lambs following selenium supplementation have been reported by numerous workers (6).

The SGO-T values determined throughout the experiment are presented in figure 1. Elevated transaminase levels were observed in the ewe lambs receiving the basal ration or the selenium supplement only. These increased levels were first observed on the 9th and 12th weeks of the experiment, respectively. Thus selenium supplementation delayed the increase but did not prevent it. Increases in transaminase concentration occurred slightly later than the increases in in vitro Se^{75} uptake by the erythrocytes from ewes

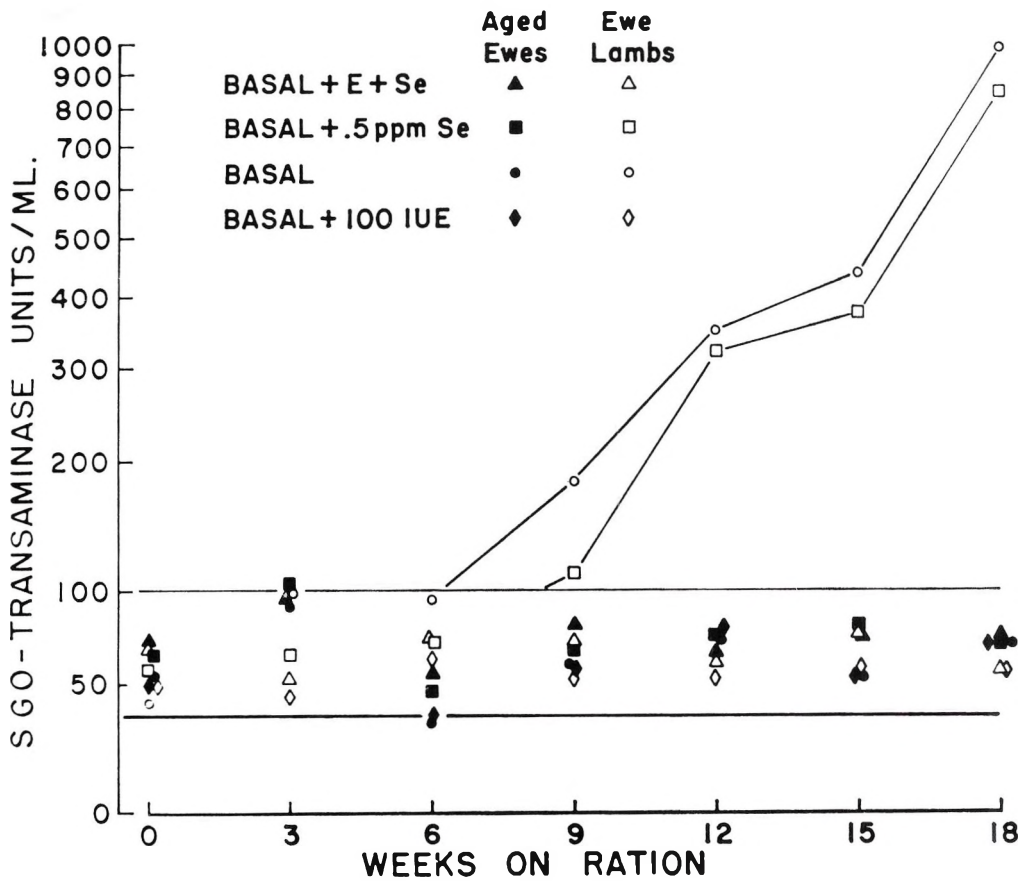


Fig. 1 Changes in serum glutamic-oxaloacetic transaminase of gestating aged ewes and gestating ewe lambs fed a purified basal ration with and without α-tocopherol (E) or selenium (Se) supplementation. Parallel lines represent plus or minus 2 standard deviations from average of individual group means.

fed the low selenium rations (11). These results, with gestating ewes, are similar to those in which a synthetic antioxidant (2) and α-tocopherol [8] (17) were effective in maintaining normal serum transaminase concentrations in lambs fed dystrophogenic rations, but selenium alone was not. The normal transaminase levels throughout the experiment in the aged ewes (fig. 1) receiving the rations not supplemented with vitamin E may be indicative of greater body reserve of α-tocopherol at the beginning of the experiment.

Attempts to produce dialuric acid induced hemolysis of the erythrocytes, a classical symptom of α-tocopherol deficiency in the rat, were unsuccessful even with cells from ewes which showed elevated serum transaminase levels.

Because of the oral route of radioselenium administration and a minimal amount (≈ 6 μg) of stable selenium, these data should reflect the effects of dietary α-tocopherol and selenium upon the fate of ingested selenium in the gestating ewe. No effect of age on Se^{75} metabolism was noted; hence the results obtained with the aged ewes and with the ewe lambs were pooled.

The entire Se^{75} content of the gastrointestinal tract was added to the fecal excretion of Se^{75} during the 48-hour collection period (table 3) in order to eliminate individual differences in rate of passage. The combined fecal and residual gastrointestinal Se^{75} values were greater ($P < 0.05$) for those ewes which had received 0.5 ppm selenium in the ration (67%) than those for ewes which had received the basal ration (61%) or the basal ration plus α-tocopherol (52%). Jensen et al. (18) reported a similar effect of dietary selenium level upon the retention of an oral dose of Se^{75} in the chick. Supplementation of the low selenium (0.01 ppm) ration with α-tocopherol tended to reduce the apparent nonabsorbed Se^{75}. At the higher selenium intake the α-tocopherol effect was not so evident. These results, along with the previous report (11) in which dietary α-tocopherol resulted in a consistent but nonsignificant increase in the erythrocyte accumulation of Se^{75} in vitro suggest that α-tocopherol may increase the transference of selenium across cellular membranes. This may be through an indirect effect of α-tocopherol upon the integrity of cellular membranes.

A greater ($P < 0.01$) urinary Se^{75} excretion (11.5%) was observed in ewes which received the dietary selenium supplement, than in those which did not (1.9%). A mechanism allowing for renal concentration of selenium in the sheep was thus demonstrated. The magnitude of this action was dependent upon the dietary selenium intake. No effect of α-tocopherol upon urinary excretion of selenium was observed.

Peterson and Spedding (19) observed that 54% of an oral dose of Se^{75} (6.1 μg selenium) from an organic source was excreted in the feces within 99 hours after dosing. Urinary excretion during the same period was about 2% of the dose. Cousins and Cairney (7) report that slightly over 30% of a 5-mg dose of selenium as sodium selenite was excreted in the feces and 10% in the urine, during a 72-hour

[8] Hopkins, L. L., A. L. Pope and C. A. Baumann 1961 Contrasting responses of lambs to selenium and vitamin E on natural and semipurified dystrophogenic rations. J. Animal Sci., 20: 936 (abstract).

TABLE 3

Effect of dietary selenium and α-tocopherol upon the apparent absorption and excretion of Se^{75}

	Treatment [1]			
	E + Se	Se	O	E
	% of dose	*% of dose*	*% of dose*	*% of dose*
Gastrointestinal contents plus feces	66.2 ± 2.0 [2,3]	68.5 ± 2.7 [3]	61.0 ± 8.4	52.1 ± 6.3
Urine	11.9 ± 2.5 [4]	11.2 ± 1.6 [4]	1.6 ± 2.5	2.2 ± 0.3
Total	78.1 ± 2.9 [3]	79.7 ± 4.0 [3]	62.6 ± 7.8	54.3 ± 5.2

[1] E represents 100 IU α-tocopherol/day, Se represents 0.5 ppm dietary selenium, O represents basal ration only.
[2] Mean ± SE.
[3] Values within row greater than unmarked values ($P < 0.05$).
[4] Values within row greater than unmarked values ($P < 0.01$).

period after dosing. In both investigations the selenium contained in the feces was highly insoluble, possibly as a result of a reduction of dietary selenium in the rumen to an insoluble, unavailable form. The differences observed in urinary excretion of selenium may have been due to differences in dietary selenium intake of the sheep (7, 19).

The distribution of Se^{75} in the maternal tissues 48 hours after a single oral dose of Se^{75} is summarized in table 4. The kidney contained the greatest concentration of radioselenium by at least threefold. The liver was second, followed by the spleen, plasma, and whole blood. The concentration of Se^{75} in the kidney and spleen from the ewes fed the low selenium diet were two- to threefold greater ($P < 0.01$) than corresponding tissues from ewes in the selenium-supplemented groups. The whole blood and plasma concentrations were also greater ($P < 0.01$). Conversely, the Se^{75} concentration of the biceps femoris and longissimus dorsi was highest ($P < 0.01$) in those ewes which had received the selenium supplementation. Concentrations in the liver, lung, and heart were not affected by dietary selenium intake when these tissues were sampled 48 hours postdosing. The Se^{75} concentration in the spleen from ewes fed the low selenium diet was significantly increased ($P < 0.05$) by α-tocopherol supplementation. No other effects of dietary α-tocopherol were observed. Jensen et al. (18) reported that α-tocopherol had no effect on the distribution of selenium, but that selenium itself had a marked effect on the uptake and retention of radioselenium in all tissues of the chick.

The total radioselenium content of several organs is shown in table 5. The kidneys, spleen, and whole blood from ewes fed the basal ration or basal plus α-tocopherol contained much more ($P < 0.01$) and the lungs slightly more ($P < 0.05$) of the single oral dose of radioselenium than did corresponding tissues from the ewes fed the selenium supplement. There were no significant effects upon total organ content due to α-tocopherol supplementation.

The increased radioselenium content of the kidneys from ewes fed the low selenium ration may have been a result of reduced urinary selenium excretion. Gross autoradiographs showed that the Se^{75} is concentrated almost exclusively in the cortex of the kidney where filtration and reabsorption take place. The slight increase in the lungs could be a result of increased Se^{75} content of the blood remaining in the lungs of the ewes fed the low selenium ration. However, the increased content of the spleen is greater than that which could be explained on the basis of higher blood Se^{75} levels in those ewes fed the low selenium diet.

The kidney has been demonstrated to be a potent source of "Factor 3 selenium" (20), whereas the importance of the spleen in selenium metabolism has previously been associated only with selenium toxicity (21); however, selenium may be incorporated into certain blood constituents during hematopoiesis in the spleen.

TABLE 4

Concentration of Se^{75} in ewe tissues: influence of dietary α-tocopherol and selenium

Tissue	Treatment [1]			
	E + Se	Se	O	E
	10^{-4}% of dose/ g fresh tissue		*10^{-4}% of dose/ g fresh tissue*	
Kidney	244.1 ± 13.8 [2]	224.8 ± 20.6	632.6 ± 85.6 [3]	699.3 ± 107.3 [3]
Liver	82.0 ± 10.0	81.1 ± 7.9	86.8 ± 7.4	108.0 ± 12.6
Lung	27.9 ± 3.7	25.2 ± 1.3	31.9 ± 4.4	27.9 ± 2.9
Heart	16.6 ± 1.2	14.9 ± 0.8	18.1 ± 2.8	15.0 ± 1.6
Spleen	32.2 ± 3.6	28.5 ± 1.8	62.6 ± 13.1 [3]	75.0 ± 7.4 [3,4]
Biceps femoris	3.4 ± 0.4 [3]	3.8 ± 0.6 [3]	2.7 ± 0.1	2.3 ± 0.3
Longissimus dorsi	4.0 ± 0.8 [3]	3.4 ± 0.4 [3]	2.3 ± 0.1	2.6 ± 0.3
Whole blood	23.2 ± 2.1	25.4 ± 2.4	35.4 ± 2.8 [3]	35.5 ± 4.2 [3]
Plasma	32.6 ± 2.5	35.7 ± 3.4	52.1 ± 3.1 [3]	51.3 ± 5.8 [3]

[1] E represents 100 IU α-tocopherol/day; Se, 0.5 ppm dietary selenium; O, basal ration only.
[2] Mean ± SE.
[3] Values within row greater than unmarked values ($P < 0.01$).
[4] Basal plus E greater than basal ($P < 0.05$).

The present data suggest that dietary selenium may be first concentrated in those tissues which have the greatest capacity for utilizing inorganic selinium in the biosynthesis of organoselenium compounds which may then be redistributed throughout the organism, notably to the muscle where gross manifestations of selenium deficiency in sheep are observed (4–6).

The intracellular distribution of radioselenium in the liver and kidney (table 6) demonstrates a characteristic pattern of distribution for each tissue. In the liver samples from ewes fed the low selenium basal, a greater ($P < 0.01$) proportion of the Se^{75} was contained in the microsomal fraction than in the corresponding fraction of liver samples from the ewes fed the selenium-supplemented ration. The increase in concentration in the microsomes occurred at the expense of each of the other cellular components. The greater microsomal burden may have been indicative of increased selenium incorporation into selenoproteins within the microsomes of the liver (22). Alterations in compartmentalization within the kidney were less distinct. The nuclear fraction in kidney samples from the selenium-deficient ewes contained a greater ($P < 0.01$) proportion of the radioselenium than did the corresponding fraction from selenium-supplemented ewes. This increase occurred with corresponding decreases in Se^{75} content of the mitochondria ($P < 0.05$) and the soluble fraction ($P < 0.01$). These shifts in intracellular particulate matter distribution of Se^{75} resulting from a reduced die-

TABLE 5

Total tissue content of Se^{75} in ewes: influence of dietary α-tocopherol and selenium

Tissue	Treatment [1]			
	E + Se	Se	O	E
	% of dose	*% of dose*	*% of dose*	*% of dose*
Kidney	2.30 ± 0.18 [2]	2.17 ± 0.22	6.14 ± 0.92 [3]	6.12 ± 0.52 [3]
Liver	5.84 ± 0.89	5.80 ± 0.75	5.67 ± 0.63	6.01 ± 0.44
Lung	1.18 ± 0.11	1.05 ± 0.08	1.41 ± 0.15 [4]	1.36 ± 0.25 [4]
Heart	0.32 ± 0.04	0.30 ± 0.04	0.40 ± 0.09	0.28 ± 0.02
Spleen	0.20 ± 0.02	0.18 ± 0.03	0.49 ± 0.04 [3]	0.56 ± 0.08 [3]
Whole blood	4.26 ± 0.26	4.79 ± 0.57	6.17 ± 0.99 [3]	7.38 ± 0.86 [3]

[1] E represents 100 IU α-tocopherol/day; Se, 0.5 ppm dietary selenium; O, basal ration only.
[2] Mean ± SE.
[3] Values within row greater than unmarked values ($P < 0.01$).
[4] Values within row greater than unmarked values ($P < 0.05$).

TABLE 6

Intracellular particulate matter distribution of Se^{75} in livers and kidneys of ewes

Cellular fraction	Treatment [1]			
	E + Se	Se	O	E
	%	%	%	%
	Liver			
Homogenate	100	100	100	100
Nuclear fraction	30.1 ± 2.3 [2,3]	25.8 ± 3.7 [3]	20.8 ± 2.7	19.2 ± 1.9
Mitochondria	15.7 ± 2.6 [3]	14.1 ± 1.2 [3]	10.9 ± 0.7	11.8 ± 1.0
Microsomes	20.4 ± 1.5	22.7 ± 1.8	35.8 ± 4.9 [4]	39.4 ± 3.5 [4]
Soluble fraction	33.8 ± 3.5	37.4 ± 2.1	32.5 ± 2.6	29.6 ± 0.8
	Kidney			
Homogenate	100	100	100	100
Nuclear fraction	75.5 ± 1.1 [2]	73.9 ± 1.4	77.1 ± 1.6 [4]	82.4 ± 1.0 [4]
Mitochondria	5.1 ± 0.8 [3]	5.5 ± 0.7 [3]	4.0 ± 0.6	2.9 ± 0.1
Microsomes	7.0 ± 0.5	7.3 ± 0.6	9.4 ± 1.0	7.1 ± 0.5
Soluble fraction	12.4 ± 0.5 [4]	13.3 ± 0.5 [4]	9.5 ± 0.4	7.6 ± 0.5

[1] E represents 100 IU α-tocopherol/day; Se, 0.5 ppm dietary selenium; O, basal ration only.
[2] Mean of 4 ewes/treatment ± SE.
[3] Values within row greater than unmarked values ($P < 0.05$).
[4] Values within row greater than unmarked values ($P < 0.01$).

tary selenium intake are essentially the reverse of those observed by Wright and Mraz (23) in livers and kidneys from chickens consuming toxic levels of selenium. The effects of dietary α-tocopherol were not pronounced but were most evident in the tissues from the ewes fed the low selenium ration, where α-tocopherol supplementation tended to increase the proportion of the Se^{75} contained within the particulates and reduce the amount in the soluble fraction.

No gross congential abnormalities were observed in any of the lambs removed from the ewes that had been fed the experimental ration from the time of mating until they were killed at approximately the one-mindred and thirty-fifth day of gestation.

The radioselenium distribution pattern (table 7) observed in the fetal lamb tissues was similar to that noted in the maternal tissues; the kidney, liver, and spleen had the highest concentrations. Only the Se^{75} concentration in the muscle samples from the fetal lambs approached the levels noted in the maternal tissues. The whole blood and plasma concentrations of Se^{75} were highest ($P < 0.01$) in the lambs from ewes fed the low selenium ration. The ratio of maternal plasma to fetal plasma was not affected by dietary treatment of the dams. In the fetal tissues, as in the maternal tissues, the total kidney Se^{75} content (table 8) in the fetuses from dams consuming the low selenium ration was higher ($P < 0.05$) than that of fetuses from dams receiving the selenium supplement. The liver content of fetal lambs from the nonselenium-supplemented ewes was lower ($P < 0.05$) than that of fetuses from the selenium-supplemented ewes. Addition of α-tocopherol to the ewes' ration did not affect the passage of radiose-

TABLE 7

Concentration of Se^{75} in fetal lamb tissues: influence of α-tocopherol and selenium in the dam's ration

Tissue	Treatment [1]			
	E + Se	Se	O	E
	10^{-4}% of dose/ g fresh tissue		*10^{-4}% of dose/ g fresh tissue*	
Kidney	23.7 ± 1.4 [2,3]	16.7 ± 1.3	19.6 ± 1.5	24.3 ± 2.4 [3]
Liver	13.2 ± 1.6	10.7 ± 1.1	9.0 ± 1.4	10.1 ± 1.4
Lung	4.8 ± 1.0	3.7 ± 0.5	2.7 ± 0.4	3.8 ± 0.9
Heart	5.6 ± 0.8	4.3 ± 0.5	4.6 ± 0.7	4.5 ± 0.5
Spleen	6.1 ± 0.9	6.4 ± 0.7	5.4 ± 0.8	8.5 ± 1.2
Biceps femoris	1.3 ± 0.1	1.9 ± 0.4	1.4 ± 0.1	1.6 ± 0.2
Longissimus dorsi	1.3 ± 0.1	1.9 ± 0.4	1.4 ± 0.1	1.6 ± 0.2
Whole blood	2.1 ± 0.2	2.0 ± 0.2	2.7 ± 0.2 [4]	2.4 ± 0.1 [4]
Plasma	3.1 ± 0.3	3.1 ± 0.4	4.5 ± 0.3 [4]	4.0 ± 0.3 [4]

[1] E represents 100 IU α-tocopherol/day; Se, 0.5 ppm dietary selenium; O, basal ration only.
[2] Mean for single lambs ± SE.
[3] Values within row greater than unmarked values ($P < 0.05$).
[4] Values within row greater than unmarked values ($P < 0.01$).

TABLE 8

Total tissue content of Se^{75} in fetal lambs: influence of α-tocopherol and selenium in the dam's ration

Tissue	Treatment [1]			
	E + Se	Se	O	E
	10^{-2}% of dose	*10^{-2}% of dose*	*10^{-2}% of dose*	*10^{-2}% of dose*
Kidney	3.08 ± 0.3 [2]	3.21 ± 0.2	3.47 ± 0.1 [3]	4.06 ± 0.3 [3]
Liver	9.55 ± 1.4 [3]	9.66 ± 1.3 [3]	6.91 ± 0.6	7.18 ± 0.9
Lung	2.94 ± 0.8	3.46 ± 0.3	2.34 ± 0.4	2.76 ± 0.3
Heart	1.04 ± 0.1	1.25 ± 0.2	1.09 ± 0.1	1.00 ± 0.2
Spleen	0.26 ± 0.1	0.38 ± 0.1	0.24 ± 0.1	0.33 ± 0.1

[1] E represents 100 IU α-tocopherol/day; Se, 0.5 ppm dietary selenium; O, basal ration only.
[2] Mean for single lambs ± SE.
[3] Values within row greater than unmarked values ($P < 0.05$).

lenium across the placental membranes. Slightly more of the total Se^{75} dose given to the ewe was contained in the fetal organs (table 8) when the ewes' ration contained the 0.5-ppm selenium level.

Burton et al. (8) reported that selenium content of muscle and liver from fetal lambs 100 or 122 days of age was influenced by dietary selenium intake of the dams. Their studies showed that the concentration of selenium in the maternal liver was nearly twice that in the fetal lamb. The differences between maternal and fetal muscle selenium content were slightly less.

Tissues from twin fetuses contained approximately one-half as much radioselenium as tissues from single fetuses (table 9). The ratio of the concentration in the maternal plasma to the concentration in the fetal plasma was 11.7:1 for the single fetuses and 21.8:1 for the twin fetuses, demonstrating a marked placental barrier. Furthermore, these data suggest that only a limited fraction of the selenium in the maternal tissues was in a form which could traverse the placental membrane. This amount was divided equally between twin fetuses *in utero*, and the sum of the radioselenium in tissues of twin pairs was equal to that of single fetuses.

These experiments have demonstrated that normal fetal lamb development could proceed when urea was the sole source of dietary nitrogen and in the absence of dietary B-complex, C, and K vitamins.

The absorption and tissue retention data demonstrate that, in the sheep, mechanisms were present which allowed an increased tissue accumulation of radioselenium when the dietary selenium intake was low. The individual organ content as well as the intracellular particulate matter distribution were also influenced by dietary intake. Furthermore, the data suggest that α-tocopherol may directly or indirectly affect the passage of selenium across cellular and subcellular membranes. These results support the hypothesis that selenium is activity metabolized and that it may have specific metabolic functions within the cell.

TABLE 9

Influence of geminal fetation upon tissue content of Se^{75} in fetal lambs

Tissue	Twin fetuses	Single fetuses
	10^{-2}% of dose	*10^{-2}% of dose*
Kidney	1.74	3.45
Liver	4.15	8.32
Lung	1.60	2.88
Heart	0.62	1.09
Spleen	0.15	0.30
Plasma [1]	1.98	3.67

[1] 10^{-4}%/g.

ACKNOWLEDGMENTS

Our appreciation to W. A. Lyke for competent technical assistance and to Dr. L. L. Christian for advice concerning the statistical analysis of the data.

LITERATURE CITED

1. Welch, J. G., W. G. Hoekstra, A. L. Pope and P. H. Phillips 1960 Effects of feeding fish liver oil, vitamin E and selenium to ewes upon the occurrence of muscular dystrophy in their lambs. J. Animal Sci., *19:* 620.
2. Erwin, E. S., W. Sterner, R. S. Gordon, L. J. Machlin and L. L. Tureen 1961 Etiology of muscular dystrophy in the lamb and chick. J. Nutrition, *75:* 45.
3. Muth, O. H., J. E. Oldfield, J. R. Schubert and L. F. Remmert 1959 White muscle disease (myopathy) in lambs and calves. VI. Effects of selenium and vitamin E on lambs. Am. J. Vet. Res., 20: 231.
4. Blaxter, K. L. 1962 Muscular dystrophy in farm animals: its cause and prevention. Proc. Nutrition Soc., *21:* 211.
5. Oldfield, J. E., J. R. Schubert and O. H. Muth 1963 Implications of selenium in large animal nutrition. J. Agr. Food Chem., *11:* 388.
6. Wolf, E., V. Kollonitsch and C. H. Kline 1963 A survey of selenium treatment in livestock production. J. Agr. Food Chem., *11:* 355.
7. Cousins, F. B., and I. M. Cairney 1961 Some aspects of selenium metabolism in sheep. Australian J. Agr. Res., *12:* 927.
8. Burton, V., R. F. Keeler, K. F. Swingle and S. Young 1962 Nutritional muscular dystrophy in lambs — selenium analysis of maternal, fetal, and juvenile tissues. Am. J. Vet. Res., *23:* 962.
9. Lindberg, P., and M. Sirén 1963 Selenium concentrations in kidneys of normal pigs and pigs affected with nutritional muscular dystrophy and liver dystrophy (hepatosis Dialtetica). Life Sci., *5:* 326.
10. McConnell, K. P. 1963 Metabolism of selenium in the mammalian organism. J. Agr. Food Chem., *11:* 385.
11. Wright, P. L., and M. C. Bell 1963 Selenium and vitamin E influence upon the *in vitro* uptake of Se^{75} by ovine blood cells. Proc. Soc. Exp. Biol. Med., *114:* 379.

12. Oltjen, R. R., R. J. Sirny and A. D. Tillman 1962 Purified diet studies with sheep. J. Animal Sci., *21:* 277.
13. Bieri, J. G., C. J. Pollard, I. Prange and H. Dam 1961 The determination of α-tocopherol in animal tissues by column chromatography. Acta Chem. Scand., *15:* 783.
14. Schalm, O. W. 1961 Veterinary Hematology. Lea and Febiger, Philadelphia.
15. Hogeboom, G. H. 1955 Methods of Enzymology, eds., S. P. Colowick and N. O. Kaplan. Academic Press, Inc., New York.
16. Wright, P. L., A. L. Pope and P. H. Phillips 1962 Effect of protein and energy intake on lamb production and certain blood constituents of ewes. J. Animal Sci., *21:* 602.
17. Lagacé, A., D. S. Bell, A. L. Moxon and W. D. Pounden 1961 Serum transaminase in the blood of lambs given preventive treatments for white muscle disease. Am. J. Vet. Res., 22: 686.
18. Jensen, L. S., E. D. Walter and J. S. Dunlap 1963 Influence of dietary vitamin E and selenium on distribution of Se^{75} in the chick. Proc. Soc. Biol. Med., *112:* 899.
19. Peterson, P. J., and D. J. Spedding 1963 The excretion by sheep of ^{75} selenium incorporated into red clover (*Trifolium pratense* L.): The chemical nature of the excreted selenium and its uptake by three plant species. New Zealand J. Agr. Res., 6: 13.
20. Schwarz, K. 1961 Development and status of experimental work on factor 3-selenium. Federation Proc., *20:* 666.
21. Ganther, H. E., and C. A. Baumann 1962 Selenium metabolism. II. Modifying effects of sulfate. J. Nutrition, *77:* 408.
22. McConnell, K. P., and D. M. Roth 1962 Se^{75} in rat intracellular liver fractions. Biochim. Biophys. Acta, 62: 503.
23. Wright, P. L., and F. R. Mraz 1964 Influence of dietary selenium and age upon the metabolism of selenium-75 by chicks. Poultry Sci., 43: 947.

Calcium, Strontium and Phosphorus Utilization by Chicks as Influenced by Nutritional and Endocrine Variations [1]

PAUL E. WAIBEL AND FRANK R. MRAZ

Agricultural Research Laboratory of the University of Tennessee, Oak Ridge,[2] Tennessee, and Department of Poultry Science, University of Minnesota, St. Paul, Minnesota

ABSTRACT The effects of vitamin D, various energy sources, and hormone treatments on tibia deposition of orally and intramuscularly administered Ca^{45}, Sr^{89}, and P^{32}, on serum calcium, phosphorus, and alkaline phosphatase, and on growth and calcification were investigated, using chicks. Vitamin D at 550 ICU/kg diet resulted in increased calcium and strontium absorption and phosphorus retention when compared with suboptimal vitamin D levels. A tenfold increase in vitamin D to 5500 ICU/kg decreased Ca and Sr absorption and phosphorus retention. Cortisol decreased absorption of calcium and retention of strontium at all vitamin D levels. Estrogen reduced serum phosphorus and retention of calcium, strontium, and phosphorus. Lactose at 20% of the diet increased bone ash. Stearic acid at 10% decreased weight gains, bone ash, calcium absorption, and phosphorus retention.

Bergeim (1) observed in 1926 that the presence of 25 to 50% lactose in the diets of rats greatly increased calcium absorption. Migicovsky and Emslie (2) reported that vitamin D enhanced calcium absorption, but not retention. Wasserman (3) observed that vitamin D increased duodenal absorption of a variety of divalent ions including magnesium, calcium, strontium, barium, and cobalt.

Calcium and strontium absorption from the gut may be increased by various carbohydrates besides lactose, including cellobiose, sorbose, ribose, xylose, raffinose, melibiose, glucosamine, mannitol, and sorbitol (4). Arginine and lysine have similar effects (5). Increasing diet calcium decreases absorbability of the more saturated fats in chicks (6).

Calcium retention in sexually immature pullets was increased by estradiol and testosterone (7). The positive effect of vitamin D on calcium absorption was antagonized by cortisol in in vitro studies (8); recently (9) this phenomenon could be demonstrated in vitro, but not in vivo.

The study reported herein was conducted to determine the influence of many of the mentioned factors simultaneously on absorption and retention of calcium, strontium, and phosphorus under in vivo conditions.

EXPERIMENTAL

White Plymouth Rock (Arbor Acre) female chicks were obtained from dams which had been deprived of vitamin D and shielded from direct sunlight for 2 weeks prior to the saving of eggs. They were fed the following vitamin D depletion diet during a pre-experimental period of 7 days: (in %) ground yellow corn, 38.32; soybean meal (solvent), 38; glucose·H_2O,[3] 20; methionine hydroxy analogue calcium (90%), 0.05; calcium carbonate, 1; dicalcium phosphate (feed grade), 2; sodium chloride, 0.47; potassium iodide, 0.000291; manganese sulfate monohydrate, 0.0238; zinc carbonate, 0.00634; vitamin A acetate (10,000 IU/g), 0.044; vitamin E acetate (110 IU/g), 0.01; menadione, 0.00022; riboflavin, 0.00044; calcium pantothenate, 0.00132; niacin, 0.00176; vitamin B_{12} (0.1% trituration), 0.0022; pyridoxine·HCl, 0.00022; thiamine·HCl, 0.00022; biotin, 0.000022; folic acid, 0.00011; and choline chloride (25%), 0.07.

Received for publication April 3, 1964.

[1] This manuscript is published with the permission of the Directors of the University of Tennessee and University of Minnesota Agricultural Experiment Stations, Knoxville and St. Paul, respectively. Studies accomplished during leave of senior author from University of Minnesota.

[2] Operated by the Tennessee Agricultural Experiment Station for the U. S. Atomic Energy Commission under Contract no. AT-40-1-GEN-242.

[3] Cerelose, Corn Products Company, Argo, Illinois.

Analyses of variance and Duncan's multiple range tests were performed as described in Steel and Torrie (10). At 7 days of age the chicks were distributed into 21 lots of 12 chicks each. The chicks within each lot were assigned from 12 groups of varying body weight to provide equitable weight stratification within each lot. Variation due to starting weight was removed in the analysis of variance. These block effects were insignificant except for the weight gain measurement where there was a highly significant effect due to starting weight.

The experimental treatments were imposed during 7 to 16 days of age. Substitutions made in the above listed diet as indicated were in all cases for the glucose portion. Except when under study, the vitamin D level was 550 ICU/kg.

Cortisol was injected intramuscularly into the left breast muscle (saline carrier, 3 mg/ml), each chick receiving 0.12, 0.09, 0.09, and 0.12 mg/bird at 8, 10, 12 and 14 days of age. Estradiol benzoate and testosterone propionate (sesame oil carrier, 3 mg/ml) were injected as described for cortisol above, each chick receiving 0.12, 0.12, 0.15, and 0.15 mg/bird at 8, 10, 12, and 14 days of age, respectively.

Of each lot of 12 chicks, one-half were dosed orally and the other half dosed intramuscularly with isotopes at 14 days of age. The oral dose consisted of 20 μc Ca^{45}, 30 μc P^{32}, and 10 μc Sr^{89} in 0.50 ml of distilled H_2O. The intramuscular (right breast muscle) dose was 10 μc Ca^{45}, 15 μc P^{32}, and 5 μc Sr^{89} in 0.25 ml. The experiment was terminated at 16 days of age. Blood was combined from pairs of birds for later analyses of serum calcium, phosphorus, and alkaline phosphatase, using Auto-Analyzer methodologies.[4] The left tibia of each bird was cleaned, dried, and ashed. Bone ash was calculated as a percentage of dry bone. Calcium and strontium were separated from phosphorus by oxalate precipitation and all 3 isotopes counted using a Geiger-Müller instrument. Alkaline phosphatase values are in King-Armstrong units.

RESULTS

The effects of vitamin D, carbohydrate and cortisol on the experimental measurements are shown in table 1. The 550 ICU/kg level of vitamin D represents 2.5 times the National Research Council estimated requirement (11). Growth and bone ash were highly dependent upon vitamin D status. That chicks gained more weight with the highest vitamin D level appears to be the result of faster recovery from the depletion period. Cortisol injections markedly reduced growth rate. The presence of dietary lactose resulted in greater weight gains with inadequate vitamin D and consistently higher levels of bone ash regardless of vitamin D adequacy.

Vitamin D was shown to have marked effects upon the tibia deposition of orally administered calcium. Since the treatments shown in table 1 had no effect on intramuscularly administered calcium, it is concluded that the effects shown were on absorption. It was unexpected that the highest vitamin D level (25 times requirement) depressed calcium absorption as compared with the 550-unit level. Lactose did not result in improved calcium absorption. The administration of cortisol decreased calcium absorption regardless of vitamin D status.

Essentially the same effects were shown with orally administered strontium as with orally administered calcium except that the magnitude of absorption was considerably less. The highest level of vitamin D as well as the administration of cortisol decreased the deposition of orally administered strontium. Results with intramuscularly administered strontium with the glucose and lactose series suggest decreased tibia deposition with increasing vitamin D levels. Chicks receiving cortisol showed a highly significant reduction in deposition of intramuscularly administered strontium.

In most instances, orally and intramuscularly administered phosphorus were deposited with equal efficiency. The vitamin D and cortisol effects agreed closely with those of orally administered calcium.

The influence of the variables upon blood serum levels of calcium, phosphorus and alkaline phosphatase in the glucose and lactose series indicate that vitamin D increased the levels of calcium and phos-

[4] Technicon Instruments Corporation, Chauncey New York.

TABLE 1

Influence of vitamin D, carbohydrate, and cortisol on experimental measurements

Diet and vitamin D	Gain, 7–16 days	Bone ash	Deposition in tibia						Blood serum levels		
			Ca^{45}		Sr^{89}		P^{32}		Ca	P	Alkaline phosphatase
			Oral	im	Oral	im	Oral	im			
ICU/kg	*g*	*%*	*% of dose*		*% of dose*		*% of dose*		*mg/100 ml*		*KA units* [1]
A 20% Glucose·H_2O											
0	53.0	22.5	2.18	6.92	1.06	5.20	5.38	4.88	7.57	5.92	660
55	51.3	24.6	3.33	7.96	1.46	5.82	5.67	5.18	7.42	4.60	751
550	102.2	36.8	6.72	7.41	2.66	4.97	10.10	8.80	9.40	6.42	207
5500	113.8	36.8	4.94	7.32	1.20	4.96	8.42	7.84	8.00	6.33	233
B 20% Lactose											
0	66.3	25.0	2.37	7.73	0.97	6.20	5.59	5.03	7.12	5.78	548
55	66.5	27.3	2.17	6.85	0.88	5.90	5.37	5.35	5.62	4.67	418
550	97.1	40.1	6.46	7.44	2.20	5.19	8.68	8.30	9.53	6.08	240
5500	110.7	38.2	4.93	6.92	1.24	4.83	7.62	7.44	8.55	6.97	192
C 20% Glucose·H_2O, cortisol											
0	35.4	24.6	1.55	7.32	0.44	3.39	4.27	3.24	9.98	3.18	592
55	44.2	26.6	2.31	7.35	0.65	4.20	4.51	4.13	9.00	4.27	773
550	63.1	34.2	5.66	7.78	1.59	4.98	7.82	6.91	9.67	6.60	305
5500	67.9	35.7	3.81	7.12	0.70	3.99	6.80	5.99	9.40	6.48	384
Minimum required difference [2]	10.9	2.1	1.21	0.94	0.58	0.70	1.04	1.11	1.46	0.93	98
Analysis of variance [3]											
Treatments (11)	46.9**	87.5**	28.0**	1.36	14.6**	10.7**	27.6**	24.2**	6.82**	10.6**	14.1**
Vitamin D (3)	107**	801**	95.2**	0.98	38.0**	3.92*	73.7**	71.3**	9.80**	25.0**	40.4**
A, B, C (2)	80.4**	16.4**	8.10**	0.70	20.0**	35.1**	20.3**	25.0**	14.3**	5.37**	8.47**
D × ABC (6)	5.7**	4.28**	0.99	1.78	0.98	5.95**	6.95**	0.41	2.83*	5.05**	2.75*

[1] King-Armstrong units/100 ml.

[2] Minimum number of units required between adjacent groups for significance by Duncan's multiple range test ($P < 0.05$).

[3] Listed are degrees of freedom in parentheses and F values. Indication of significance: *, $P < 0.05$; **, $I < 0.01$. Degrees of freedom for error: gain and ash data, 121; all others, 55.

TABLE 2

Influence of carbohydrate, estradiol, and testosterone on experimental measurements

	Gain, 7–16 days	Bone ash	Deposition in tibia						Blood serum levels		
			Ca^{45}		Sr^{89}		P^{32}		Ca	P	Alkaline phosphatase
			Oral	im	Oral	im	Oral	im			
	g	*%*	*% of dose*		*% of dose*		*% of dose*		*mg/100 ml*		*KA units* [1]
20% Glucose·H_2O											
None	102.2	36.8	6.72	7.41	2.66	4.97	10.10	8.80	9.40	6.42	207
Estradiol (E)	108.8	37.0	5.78	5.75	2.33	4.04	8.37	7.61	7.68	5.37	233
Testosterone (T)	103.5	38.2	6.92	7.57	2.68	5.48	8.90	8.50	8.38	6.73	250
E + T	103.0	37.0	5.77	6.77	1.91	4.11	7.65	7.74	8.90	5.75	256
20% Lactose											
None	97.1	40.1	6.46	7.44	2.24	5.19	8.70	8.30	9.53	6.08	240
Estradiol	100.7	38.3	6.38	6.69	2.05	4.30	7.71	7.10	9.37	5.52	278
Testosterone	101.0	39.8	6.10	7.62	1.75	5.22	8.71	8.56	8.87	6.12	155
E + T	110.1	39.2	5.96	6.79	1.77	4.38	8.20	7.24	8.12	6.06	197
Minimum required difference [2]	10.9	2.1	1.21	0.94	0.58	0.70	1.04	1.11	1.46	0.93	98
Analysis of variance [3]											
Treatments (7)	1.32	22.3	0.89	3.25*	1.88	4.28**	3.80**	2.46*	1.53	3.62**	0.76
E + T (3)	1.39	7.25**	0.90	6.37**	1.74	9.42**	5.90**	5.02**	1.49	6.62**	0.48
E (1)	3.29	0.14	2.51	16.3**	2.78	27.2**	15.4**	14.9**	2.05	16.08**	0.78
T(1)	0.81	0.07	0.17	2.19	2.38	0.82	1.54	0.03	1.36	3.74	0.62
E × T (1)	0.04	5.16*	0.03	0.60	0.09	0.24	0.67	0.09	1.12	0.71	0.02
CHO (1)	1.08	34.4**	0.04	1.10	5.45*	0.39	2.37	1.48	1.06	0.53	0.35
E + T × CHO (3)	1.35	3.63*	0.70	0.84	0.83	0.05	2.17	2.23	1.73	1.62	1.19

[1] King-Armstrong units/100 ml.

[2] Minimum number of units required between adjacent groups for significance by Duncan's multiple range test ($P < 0.05$).

[3] Listed are degrees of freedom in parentheses and F values. Indication of significance: *, $P < 0.05$; **, $P < 0.01$. Degrees of freedom for error: gain and ash data, 75; all others, 36.

phorus and decreased alkaline phosphatase. With cortisol similar vitamin D effects were observed on phosphorus and alkaline phosphatase, but unexpectedly there was no comparable effect on calcium.

The influence of carbohydrate, estradiol, and testosterone on the experimental measurements is shown in table 2. The growth-stimulating effect due to estrogen was significant at $P < 0.10$. Dietary lactose again significantly increased the level of bone ash. With repect to the deposition of calcium, strontium, and phosphorus in the tibia, estrogen effects were generally highly significant, with the exception of orally administered calcium and strontium where the effects approached significance ($P < 0.10$). Apparently estrogen reduced the deposition of these isotopes metabolically. A highly significant effect of estrogen in reducing serum phosphorus was also evident.

The influence of carbohydrates and stearic acid on the experimental measurements is shown in table 3. Growth rate was depressed by 20% xylose or 10% stearic acid. The latter markedly reduced bone ash deposition. Lactose, xylose, and sorbitol did not result in greater deposition of either orally or intramuscularly administered isotopes of calcium and strontium. The presence of stearic acid markedly reduced calcium, but not strontium absorption. Both oral and parenteral phosphorus depositions were reduced by stearic acid. It was unexpected that sorbitol would result in reduced serum calcium. Stearic acid resulted in somewhat lower serum calcium and phosphorus and increased alkaline phosphatase.

TABLE 3

Influence of carbohydrates and stearic acid on experimental measurements

	Gain, 7–16 days	Bone ash	Deposition in tibia						Ca	P	Alkaline phosphatase
			Ca^{45}		Sr^{89}		P^{32}				
			Oral	im	Oral	im	Oral	im			
	g	*%*	*% of dose*		*% of dose*		*% of dose*		*mg/100 ml*		*KA units* [1]
20% Glucose·H_2O	102.2	36.8	6.72	7.41	2.66	4.97	10.12	8.80	9.40	6.42	207
20% Lactose	97.1	40.1	6.46	7.44	2.24	5.19	8.70	8.33	9.53	6.08	240
20% D(+)-Xylose	86.2	38.1	7.17	7.27	2.87	4.99	8.81	7.58	9.30	6.60	331
20% D-Sorbitol (hydrate)	99.7	36.6	6.38	7.20	2.42	4.96	9.08	7.95	7.37	6.03	213
10% Stearic acid + 10% glucose·H_2O	71.9	29.1	3.60	6.56	2.34	4.78	6.23	4.87	8.00	5.32	407
Minimum required difference [2]	10.9	2.11	1.21	0.94	0.58	0.70	1.04	1.11	1.46	0.93	98

[1] King-Armstrong units/100 ml.
[2] Minimum number of units required between adjacent groups for significance by Duncan's multiple range test ($P < 0.05$).

DISCUSSION

That lactose did not increase the absorption of Ca^{45} confirms the observation of Wasserman et al. (12) that lactose did not increase calcium absorption in the chick as it did in the rat. There are, however, effects in the present study due to lactose which should be considered. Lactose resulted in increased growth rate at suboptimal levels of vitamin D and in higher bone ash even with adequate vitamin D. These effects may be explained by the suggestion of Fox et al. (13) that

lactose, a relatively unabsorbable carbohydrate, may assist in removing nutritional deficiencies by decreasing the available energy of the diet and increasing its consumption by the chicks. In this study a relative increase in calcium, phosphorus, and vitamins might explain the observed effects of lactose.

The depressing effect of high vitamin D upon calcium and strontium absorption was unexpected. The data in table 1 indicate that 73% as much Ca^{45} was absorbed with 5500 ICU/kg vitamin D as with the 550 ICU/kg level. Similarly only 45% as much Sr^{89} was absorbed. These effects existed also in the lactose and cortisol modifications. In these comparisons, high vitamin D caused a greater percentage depression on strontium than on calcium absorption. Since the depression in calcium absorption and strontium retention due to cortisol occurred independently of the vitamin D effects, it should be noted that only 0.70% of the oral strontium dose was retained by the tibia of the chick with the cortisol-high vitamin D treatment.

The relative percentages of phosphorus deposited in the tibia were similar regardless of whether phosphorus was administered orally or intramuscularly, suggesting that phosphorus was absorbed equally well with the various imposed treatments. Since phosphorus deposition and retention in bone is dependent upon calcium and since the vitamin D and cortisol effects upon phosphorus were similar to those with orally administered calcium, it seems logical to conclude that phosphorus deposition was limited by calcium. If true, this demonstrates how nutritional effects between 2 nutrients may not be direct but rather dependent upon a third nutrient.

It seems probable that the effect of stearic acid in reducing growth and bone ash is related to its effect in making the calcium in the gut unavailable to the chick. This is further evident by a decreased calcium level in the serum. The increased level of alkaline phosphatase is also suggestive of insufficient calcium as observed previously by Hurwitz and Griminger (14). The results in this study show that alkaline phosphatase is a very sensitive indicator of vitamin D deficiency and extend previous observations of Martin and Patrick (15) and others.

The administration of estrogen and testosterone in this study did not increase calcium retention as reported previously by others (7, 16) using sexually immature pullets. Estrogen actually resulted in decreased retention of Ca^{45}, Sr^{89} and P^{32} by bone. This agrees to a limited extent with the stated effect of estrogen in assisting in the removal of calcium from the bone into circulation; however, there was no increase in serum calcium due to estrogen. Quite consistent with the administration of estrogen, however, was a decreased level of serum phosphorus, which factor might result in decreased calcification and therefore decreased deposition of all 3 isotopes.

LITERATURE CITED

1. Bergeim, O. 1926 Intestinal chemistry. V. Carbohydrates and calcium and phosphorus absorption. J. Biol. Chem., 70: 35.
2. Migicovsky, B. B., and A. R. G. Emslie 1950 Deposition of radiocalcium in rachitic and non-rachitic chick tibia from oral and intramuscular doses of Ca^{45}. Arch. Biochem., 28: 324.
3. Wasserman, R. H. 1962 Studies on vitamin D_3 and the intestinal absorption of calcium and other ions in the rachitic chick. J. Nutrition, 77: 69.
4. Wasserman, R. H., and C. L. Comar 1959 Carbohydrates and gastrointestinal absorption of radiostrontium and radiocalcium in the rat. Proc. Soc. Exp. Biol. Med., *101*: 314.
5. Wasserman, R. H., C. L. Comar and M. M. Nold 1956 The influence of amino acids and other organic compounds on the gastrointestinal absorption of calcium^{45} and strontium^{89} in the rat. J. Nutrition, 59: 371.
6. Fedde, M. R., P. E. Waibel and R. E. Burger 1960 Factors affecting the absorbability of certain dietary fats by the chick. J. Nutrition, 70: 447.
7. Common, R. H., W. A. Maw and J. R. Jowsey 1953 Observations on the mineral metabolism of pullets. IX. The effects of estrogen and androgen administered separately on the retention of calcium by the sexually immature pullet. Canad. J. Agr. Sci., 33: 172.
8. Harrison, H. E., and H. C. Harrison 1960 Transfer of Ca^{45} across intestinal wall *in vitro* in relation to action of vitamin D and cortisol. Am. J. Physiol., *199*: 265.
9. Williams, G. A., E. N. Bowser, W. J. Henderson and V. Uzgiris 1962 Calcium absorption in the rat in relation to excessive vitamin D and cortisone. Proc. Soc. Exp. Biol. Med., *110*: 889.
10. Steel, G. D., and J. H. Torrie 1960 Principles and Procedures of Statistics. The McGraw-Hill Book Company, Inc., New York.

11. National Research Council, Committee on Animal Nutrition 1960 Nutrient requirements for poultry, pub. 827. National Academy of Sciences — National Research Council, Washington, D. C.
12. Wasserman, R. H., C. L. Comar, J. C. Schooley and F. W. Lengemann 1957 Interrelated effects of L-lysine and other dietary factors on the gastrointestinal absorption of calcium^{45} in the rat and chick. J. Nutrition, *62:* 367.
13. Fox, M. R. S., and G. M. Briggs 1959 Effects of dietary lactose upon chicks fed a purified diet. Poultry Sci., *38:* 964.
14. Hurwitz, S., and P. Griminger 1961 The response of plasma alkaline phosphatase, parathyroids and blood and bone minerals to calcium intake in the fowl. J. Nutrition, *73:* 177.
15. Martin, W. G., and H. Patrick 1962 Radionuclide mineral studies. 5. The relationship of serum alkaline phosphatase of Ca^{45} metabolism in the chick as influenced by age, vitamin D_3 and treatment. Poultry Sci., *41:* 916.
16. Thompson, A., and R. C. Noble 1962 A study of the effect of gonadal hormones on the phosphorus balance of the domestic fowl. Poultry Sci., *41:* 347.

Effects of Chlortetracycline and Isoniazid on Body Weight Gain and Intestinal Urea Hydrolysis of Rats [1]

R. F. HENDRICKSON,[2] A. P. ALVARES AND W. J. VISEK
Department of Pharmacology, The University of Chicago, Chicago, Illinois

ABSTRACT Results are presented from 4 experiments using 144 weanling male Sprague-Dawley rats fed either a casein-sucrose basal diet or the USP XIV assay diet (fortified with vitamin A), each supplemented with chlortetracycline (100 ppm) and isoniazid (100 ppm), alone and in combination, for a 4-week experimental period. Chlortetracycline with or without isoniazid resulted in markedly increased weight gains, improved efficiency of feed utilization, and lowered intestinal urea hydrolysis when added to the lower quality casein-starch diet. Isoniazid, and antituberculosis agent, was generally ineffective in promoting growth or reducing ureolytic activity when added alone or in combination with chlortetracycline to either diet. Significantly increased growth was observed in one experiment with the casein-sucrose diet when isoniazid was the only additive fed. Results of a fifth experiment with 50 rats fed the USP XIV diet fortified with vitamin A and supplemented with graded amounts of isoniazid (zero to 400 ppm) indicated that 100 ppm was the most suitable concentration tested and that the failure of isoniazid to significantly stimulate growth with this diet was not due to the concentration of the additive used. In general, increase in weight gain due to additives appeared dependent on overall growth rate and nutritional adequacy of the diet.

It is generally recognized that dietary antibiotics exert their growth-promoting effects primarily as a result of changes in the intestinal flora (1). These effects are frequently associated with an increased efficiency of feed utilization and a sparing action on the requirements of essential nutrients in suboptimal diets (1–6). A possibility, to be considered in this paper, is that changes in the production of ammonia, a toxin to mammals (7), may be related to this growth response in rats.

Ammonia concentration in the intestinal tract has been reduced in animals fed antimicrobial agents (8, 9). Urea hydrolysis, one of the ammonia-producing systems in the intestinal lumen, has been inhibited by dietary antibiotics (9, 10), arsanilic acid (9), a cyclic urea derivative (11), and by active immunization to crystalline jackbean urease (12, 13). This depression has frequently occurred concurrently with increased rates of growth, although a quantitative relationship between weight gain and depression of ureolytic activity has not been established. This relationship has not been well demonstrated in rats fed antibiotics.

Isoniazid, an antituberculosis agent, has been associated with improved weight gains and efficiency of feed utilization when fed to 2- to 3-month-old pigs (14) and when fed in combination with chlortetracycline to young calves (15). Recent work in this laboratory has demonstrated enhanced growth and depressed ureolytic activity in chicks fed diets containing isoniazid and chlortetracycline, singly or in combination (16).

Some effects of dietary chlortetracycline and isoniazid, incorporated in 2 different diets, on rat growth, feed efficiency, and in vitro intestinal hydrolysis of urea are presented in the present paper.

EXPERIMENTAL

Five experiments were conducted with a total of 194 weanling male Sprague-Dawley rats having an average initial weight of 51.6 ± 3.9 g. All animals were individually housed in wire-bottom cages and supplied with feed and water ad

Received for publication February 26, 1964.
[1] Financial support for these studies was provided in part by the Herman Frasch Foundation, New York.
[2] Public Health Service Postdoctoral Fellow.

libitum. Body weight and feed consumption were recorded weekly during the 28-day experimental periods. The 2 × 2 factorial arrangement of treatments used in experiment 1 (6 rats/treatment) and experiments 2, 3, and 4 (10 rats/treatment) consisted of: 1) basal diet; 2) basal diet plus 100 ppm chlortetracycline; 3) basal diet plus 100 ppm isoniazid; and 4) basal diet plus 100 ppm chlortetracycline plus 100 ppm isoniazid. A purified casein-sucrose diet was fed in experiments 1 and 2, whereas in the other 3 experiments the USP XIV vitamin A assay casein-starch diet, supplemented with vitamin A, was fed (table 1). Five treatments were used in the last experiment (10 rats/treatment) where isoniazid was incorporated in the starch diet at concentrations of zero, 50, 100, 200, and 400 ppm.

Prior to the animals being killed, they were fasted 16 hours in experiment 1, but were not fasted at the termination of the other experiments. Large intestines including cecum plus contents were quickly removed, weighed, and homogenized for 3 minutes in 60 ml of 0.9% sodium chloride solution at 4°. Duplicate 3-ml aliquots were analyzed for ureolytic activity as previously described (17). Data from all experiments were treated by analysis of variance using orthogonal comparisons as described by Snedecor (18).

RESULTS

In the first experiment (table 2), combining chlortetracycline with isoniazid resulted in less growth than was observed with either additive alone. This negative interaction was statistically significant ($P < 0.03$). Because of this interaction, main effects were not tested and nonorthogonal comparisons were made among single treatments. During the 4-week period, rats receiving chlortetracycline alone and isoniazid alone gained 10 and 15% more ($P < 0.02$), respectively, than the basal group. Feed consumption paralleled weight gains and no differences in efficiencies of feed utilization were noted. In experiment 2 (table 2), the average gain of all animals was 27 g or 20% greater than in experiment 1, and no significant treatment differences in growth or feed efficiency were observed at 4 weeks.

Hydrolysis of urea by intestinal contents of rats fed the casein-sucrose diet is also presented in table 2. In the first experiment, analysis for main effects showed that incorporation of chlortetracycline reduced urea breakdown by 58% ($P < 0.01$). This reduction was only 16% in experiment 2 and no significant differences were found. The overall average ureolytic activity in experiment 2 was nearly 220% of the average activity in experiment 1 and was due, at least in part, to the non-

TABLE 1

Composition of diets

Casein-sucrose diet [1]		Casein-starch diet [2]	
	% of diet		*% of diet*
Casein	20.0	Casein	18.0
Sucrose	61.6	Starch	65.0
Gelatin	8.0	Brewer's yeast [4]	7.98
Bone ash	2.0	Irradiated yeast [5]	0.02
Ca gluconate	5.0	Corn oil [6]	5.0
Salt mixture	1.4	Salt mixture	4.0
Vitamin mixture [3]	1.0		
Choline mixture	1.0		
Total	100.0	Total	100.00

[1] Basal diet of Stokstad et al. (22).

[2] USP XIV Vitamin A assay diet, supplemented with vitamin A acetate in corn oil.

[3] Vitamin content of basal diet: (mg/100 g) thiamine·HCl, 1.0; riboflavin, 1.0; pyridoxine·HCl, 1.0; Ca pantothenate, 5.0; niacin, 5.0; biotin, 0.02; folic acid, 0.20; vitamin B_{12}, 0.05; menadione, 0.25; α-tocopherol, 34; vitamin A, 1500 IU; and vitamin D, 200 IU.

[4] Vitamin content of basal diet; (mg/100 g) thiamine·HCl, 1.2; riboflavin, 0.52; pyridoxine·HCl, 0.24; Ca pantothenate, 1.0; niacin, 3.8; inositol, 36.0; biotin, 0.018; folic acid, 0.18.

[5] Vitamin D, 100 IU/100 g diet.

[6] Vitamin A, 200 IU/100 g diet.

fasting state of the animals at the termination of the second experiment. Animals fed isoniazid were found to have slightly higher values for urea hydrolysis than their respective controls in both experiments.

In experiments 3 and 4, a casein-starch diet (USP XIV) was fed in an attempt to obtain a greater antibiotic response (19). These results are presented in table 3. Statistical analysis revealed highly significant increases in weight gains by all groups receiving chlortetracycline, with or without isoniazid. These increases ranged from 21 to 43% of control values, and were reflected in greater efficiency of feed

TABLE 2

Weight gain, efficiency of feed utilization, and intestinal urea hydrolysis of rats fed a casein-sucrose basal diet supplemented with chlortetracycline or isoniazid, or both

Diet [1]	Weight gain			Urea hydrolyzed [2]
	0–2 Weeks	0–4 Weeks	0–4 Weeks	
	g	*g*	*g/g feed*	*mg/g dry wt*
	Experiment 1 [3]			
Basal	75 ± 7 [4]	125 ± 11 [5]	0.36 ± 0.02	9.1 ± 3.3
Chlortetracycline	80 ± 5	137 ± 12	0.36 ± 0.03	4.5 ± 1.0 [6]
Isoniazid	81 ± 3	144 ± 9 [7]	0.37 ± 0.02	12.9 ± 2.0
Chlortetracycline + isoniazid	78 ± 9	132 ± 14 [5]	0.36 ± 0.02	4.8 ± 1.0 [6]
	Experiment 2 [8]			
Basal	86 ± 5	158 ± 15	0.46 ± 0.04	18.3 ± 2.8
Chlortetracycline	87 ± 6 [9]	160 ± 15	0.47 ± 0.03	17.3 ± 2.9
Isoniazid	82 ± 7	158 ± 15	0.46 ± 0.04	18.9 ± 3.5
Chlortetracycline + isoniazid	89 ± 6 [9]	171 ± 12	0.49 ± 0.03	14.0 ± 1.2

[1] Chlortetracycline (Lederle Laboratories, Pearl River, New York) and isoniazid (Hoffmann La Roche, Nutley, New Jersey) added at 100 ppm.
[2] Large intestine including cecum plus contents. Animals fasted 16 hours in experiment 1; not fasted in experiment 2.
[3] Six rats/treatment, initial weight 51.6 ± 1.5 g.
[4] Mean ± SE.
[5] Interaction between additives statistically significant at $P < 0.03$.
[6] Main effect: chlortetracycline vs. no chlortetracycline significant at $P < 0.01$.
[7] Non-orthogonal comparison between isoniazid and basal significant at $P < 0.02$.
[8] Ten rats/treatment, initial weight 54.0 ± 3.8 g.
[9] Main effect: chlortetracycline vs. no chlortetracycline significant at $P < 0.05$.

TABLE 3

Weight gain, efficiency of feed utilization, and intestinal urea hydrolysis of rats fed a casein-starch basal diet supplemented with chlortetracycline or isoniazid, or both [1]

Diet [2]	Weight gain			Urea hydrolyzed [3]
	0–2 Weeks	0–4 Weeks	0–4 Weeks	
	g	*g*	*g/g feed*	*mg/g dry wt*
	Experiment 3			
Basal	62 ± 5 [4]	106 ± 11	0.36 ± 0.03	17.0 ± 4.4
Chlortetracycline	79 ± 5 [5]	154 ± 11 [5]	0.51 ± 0.05 [5]	13.2 ± 2.6 [6]
Isoniazid	67 ± 5 [7]	112 ± 22	0.38 ± 0.07	24.7 ± 6.8
Chlortetracycline + isoniazid	83 ± 7 [5,7]	157 ± 18 [5]	0.52 ± 0.06 [5]	12.6 ± 2.3 [6]
	Experiment 4			
Basal	68 ± 4	116 ± 19	0.37 ± 0.06	27.3 ± 5.5
Chlortetracycline	78 ± 10 [5]	146 ± 13 [5]	0.47 ± 0.05 [5]	9.8 ± 2.0 [5]
Isoniazid	64 ± 9	113 ± 21	0.35 ± 0.06	31.3 ± 5.1
Chlortetracycline + isoniazid	82 ± 8 [5]	156 ± 23 [5]	0.49 ± 0.07 [5]	13.9 ± 3.9 [5]

[1] Ten rats/treatment; initial weights 50.5 ± 3.6 g (exp. 3), 49.7 ± 3.4 g (exp. 4).
[2] Chlortetracycline and isoniazid added at 100 ppm.
[3] Large intestine including cecum and contents. Animals not fasted.
[4] Mean ± SE.
[5] Main effect: chlortetracycline vs. no chlortetracycline significant at $P < 0.001$.
[6] Main effect: chlortetracycline vs. no chlortetracycline significant at $P < 0.09$.
[7] Main effect: isoniazid vs. no isoniazid significant at $P < 0.05$.

utilization by animals fed antibiotic. In contrast, isoniazid had no appreciable effect on either weight gain or feed efficiency.

The marked growth stimulation observed with chlortetracycline was accompanied by pronounced decreases in urea hydrolysis (table 3) of 38% ($P < 0.09$) and 60% ($P < 0.001$) for groups fed this additive in experiments 3 and 4, respectively. As in the first 2 experiments, ureolytic activity was slightly higher when isoniazid was added to the diet.

Results of a fifth study (table 4) suggest that the failure of isoniazid to significantly promote growth in rats fed the casein-starch diet was not due to the concentration of the additive used. Although none of the differences were statistically significant, the data lend support to the choice of 100 ppm as a favorable concentration and agree with the effects observed with isoniazid in the previous experiments. On gross observation, no signs of pyridoxine deficiency were noted in the animals.

DISCUSSION

The results of experiments 3 and 4 clearly demonstrate stimulation of growth concurrently with depression of intestinal urea hydrolysis when chlortetracycline is added to the USP XIV diet fed to rats for a 4-week experimental period; they also confirm the apparent relationship between growth and urea hydrolysis observed previously in this laboratory with chlortetracycline in chicks (11, 16, 17). Others have noted a depression of in vivo urea hydrolysis of intestinal ammonia with antibiotics in the diet (8, 10). Chlortetracycline did not significantly increase growth rate when rats were fed a casein-sucrose diet (exps. 1 and 2), and ureolytic activity was not markedly influenced except when the animals were fasted, in which case the lower level of urea hydrolysis (exp. 1) may reflect the more rapid passage of ingesta through the gastrointestinal tract sometimes observed in antibiotic-fed animals (20). However, retarded passage has also been reported (21), and others have observed conflicting effects which appear to be dependent on carbohydrate source (22) and on duration of antibiotic supplementation (23).

The data strongly suggest that diet was an important determinant in the growth response due to chlortetracycline. Antibiotics do not normally stimulate growth of animals fed nutritionally optimal diets. Probably neither of the semipurified diets used in these studies was optimal, although the casein-sucrose diet generally exceeds the current NRC requirements for nutrients for the growing rat (24). The type of carbohydrate fed was a primary variable, but there were many other differences in diet composition including a lesser amount of protein and a deficiency of sulfur amino acids and choline in the casein-starch diet which may have affected the response to the antibiotic. Previous work with chicks indicates that growth stimulation by chlortetracycline and barbituric acid is dependent on carbohydrate source (17, 22). Although the results obtained with chlortetracycline in these experiments with rats are in contrast to those obtained with chicks (where chlortetracycline increased growth when added to a casein-sucrose diet but not when added to a casein-starch

TABLE 4

Weight gain, efficiency of feed utilization, and intestinal urea hydrolysis of rats fed a casein-starch basal diet supplemented with isoniazid [1]

Isoniazid	Weight gain			Urea hydrolyzed [2]
	0–2 Weeks	0–4 Weeks	0–4 Weeks	
ppm	*g*	*g*	*g/g feed*	*mg/g dry wt*
0	64 ± 6 [3]	115 ± 12	0.35 ± 0.04	27.4 ± 8.1
50	65 ± 5	117 ± 12	0.34 ± 0.03	25.8 ± 4.6
100	68 ± 7	122 ± 14	0.35 ± 0.04	39.5 ± 7.8
200	65 ± 5	117 ± 9	0.33 ± 0.03	35.3 ± 6.4
400	60 ± 5	110 ± 9	0.31 ± 0.03	35.6 ± 11.2

[1] Ten rats/treatment, initial weight 51.1 ± 3.6 g.
[2] Large intestine including cecum and contents. Animals not fasted.
[3] Mean ± SE.

diet), the differences in diet composition previously mentioned may be partially responsible for this discrepancy.

Isoniazid did not consistently stimulate growth when fed as the only additive or in combination with chlortetracycline. This is in contrast with results obtained with swine (14), calves (15), and in this laboratory with chicks fed the same casein-sucrose diet (16). Although the intestinal ureolytic activity was substantially reduced by isoniazid in the chick studies, in these experiments rats fed isoniazid had slightly higher urea hydrolysis values — again suggesting a negative relationship between the degree of intestinal urease activity and growth rate. Whether this relationship is causative or coincidental to a mutal causative effect remains to be established. The USP XIV diet appears to be a useful test system for further investigation of this problem.

The evidence presented in this paper demonstrates that dietary chlortetracycline resulted in growth stimulation, increased efficiency of feed utilization, and depressed intestinal ureolytic activity when rats were fed the USP XIV casein-starch diet, but not when a higher quality casein-sucrose diet was fed. Isoniazid had no consistent effect on growth or urea hydrolysis when added to either diet fed to rats.

LITERATURE CITED

1. Jukes, T. H. 1957 Antibiotics in Nutrition. Medical Encyclopedia, Inc., New York.
2. Monson, W. F., A. E. Harper, M. E. Winje, C. A. Elvehjem, R. A. Rhodes and W. B. Sarles 1954 Mechanism of the vitamin-sparing effect of antibiotics. J. Nutrition, *52:* 627.
3. Barnes, R. H., E. Kwong, K. Delany and G. Fiala 1960 The mechanism of the thiamine-sparing effect of penicillin in rats. J. Nutrition, *71:* 149.
4. Peterson, G. E., E. C. Dick and K. R. Johansson 1953 Influence of dietary aureomycin and carbohydrate on growth, intestinal microflora and vitamin B_{12} synthesis of the rat. J. Nutrition, *51:* 171.
5. Scaletti, J. V., W. L. Kulp and R. C. Cleverdon 1952 Effect of terramycin on fecal microflora of rats. I. Interrelation of diet and terramycin. Proc. Soc. Exp. Biol. Med., *81:* 552.
6. Eyssen, H., and P. DeSomer 1963 Effect of antibiotics on growth and nutrient absorption of chicks. Poultry Sci., *42:* 1373.
7. Greenstein, J. P., M. Winitz, P. Gullino, S. M. Birnbaum and M. C. Otey 1956 Studies on the metabolism of amino acids and related compounds in vivo. III. Prevention of ammonia toxicity by arginine and related compounds. Arch. Biochem. Biophys., *64:* 342.
8. Francois, A. C. 1956 First International Conference on Antibiotics in Agriculture, National Academy Sciences — National Research Council, Washington, D. C., p. 84.
9. Visek, W. J., J. M. Baron and D. M. Switz 1959 Urea metabolism and intestinal ureolytic activity of rats fed antimicrobial agents. J. Pharm. Exp. Therap., *126:* 359.
10. Dintzis, R. Z., and A. B. Hastings 1953 The effect of antibiotics on urea breakdown in mice. Proc. Nat. Acad. Sci., *39:* 571.
11. Harbers, L. H., A. P. Alvares, A. I. Jacobson and W. J. Visek 1963 Effect of barbituric acid and chlortetracycline upon growth, ammonia concentration, and urease activity in the gastrointestinal tract of chicks. J. Nutrition, *80:* 75.
12. Dang, H. C., and W. J. Visek 1960 Effect of urease injection on body weights of growing rats and chicks. Proc. Soc. Exp. Biol. Med., *105:* 164.
13. Harbers, L. H., R. F. Hendrickson, H. N. Bass and W. J. Visek 1963 Effect of urease immunity upon rats with controlled vitamin A intakes. Proc. Soc. Exp. Biol. Med., *113:* 420.
14. Ševković, N., N. Stojanović, and M. S. Stojanović 1957 Uticaj isoniazida na prirast prasadi [Influence of isoniazid on weight gains of pigs.] Acta Vet. Belgrade, *7:* 69 (Cited in Nutr. Abstr. Rev., *29:* 341, 1959).
15. Wing, J. M., and P. T. D. Arnold 1960 Effects of isoniazid and chlortetracycline in the diet of young calves. J. Dairy Sci., *43:* 1656.
16. Alvares, A. P., R. F. Hendrickson and W. J. Visek 1964 Effects of isoniazid, chlortetracycline and copper upon growth and gastrointestinal urease activity of chicks. J. Nutrition, *83:* 79.
17. Alvares, A. P., L. H. Harbers and W. J. Visek 1964 Effect of barbituric acid, chlortetracycline and carbohydrates upon growth and gastrointestinal urease activity of chicks. J. Nutrition, *80:* 93.
18. Snedecor, G. W. 1956 Statistical Methods. Iowa State College Press, Ames, p. 237.
19. Guerrant, N. B. 1962 Efficiency of antibiotics in supplementing the growth effect of beta carotene in vitamin A-depleted rats. Proc. Soc. Exp. Biol. Med., *111:* 34.
20. Jukes, H. G., D. C. Hill and H. D. Branion 1956 Effect of antibiotic on the rate of passage of feed marker through the digestive tract of the chick. Poultry Sci., *35:* 232.
21. Tuckey, R., B. E. March and J. Biely 1958 Diet and the rate of food passage in the growing chick. Poultry Sci., *37:* 786.
22. Stokstad, E. L. R., T. H. Jukes and W. L. Williams 1953 The growth promoting effects of aureomycin on various types of diets. Poultry Sci., *32:* 1054.

23. Kimbel, K. H., W. Fischer, H. Bünte and W. Frik 1956 Untersuchungen über enterale Antibiose bei der Ratte. VIII. Mitteilung: Der Einfluss von Tetracyclingaben auf die Magen-Darm-Passage. Ztschr. Ges. Exp. Med., *126:* 587.

24. National Research Council, Committee on Animal Nutrition 1962 Nutrient requirements of laboratory animals, pub. 990. National Academy of Sciences — National Research Council, Washington, D. C.

Studies in Infantile Malnutrition

III. EFFECT OF PROTEIN AND CALORIE INTAKE ON NITROGEN RETENTION [1]

GEORGE G. GRAHAM, ANGEL CORDANO AND JUAN M. BAERTL
British American Hospital, Lima, Peru

ABSTRACT In severe marasmus and in partially recovered malnourished infants, the retention of nitrogen is shown to depend on the total intake of calories as well as on the quantity and quality of protein ingested. If the caloric requirements are not met, protein is "wasted." Early in the treatment of patients with hypoalbuminemia, with or without other signs of kwashiorkor, nitrogen retention is, to a great extent, independent of caloric intake but closely related to the quantity and quality of protein in the diet. After this initial period of repletion, nitrogen retention is also dependent on caloric intake.

The effect of caloric intake on the efficiency of protein utilization in animals is well documented: if it falls below the optimal range for a given protein intake, retention of nitrogen falls off; if caloric intake is increased beyond this range, additional retention of nitrogen can be demonstrated, accompanied by a change in body composition. The optimal level of caloric intake for maximal utilization of protein varies with the quality of the protein (1). Similar observations have been made in man, notably in premature infants (2) and in young adults (3).

In those areas of the world where severe infantile malnutrition is common, a very high mortality is consistently reported in cases of marasmus. At the same time great difficulty is encountered in obtaining a satisfactory, if any, gain in weight (4). Despite the much higher incidence of this gross, essentially uncomplicated form of undernutrition, it has not received the attention accorded in recent years to the more dramatic symptom complex of kwashiorkor, whether in marasmic or less severely undernourished infants. Most pediatric centers are reluctant to admit marasmic infants, in great part because of their prolonged hospital stays and poor response to treatment, which usually consists of an over-generous protein intake and an inadequate caloric intake. This is in direct contrast with the dramatic responses obtained in infants surviving an episode of kwashiorkor. The difference in responses can be shown to be due to the very high caloric requirements of the marasmic infants with severe deficits in height and weight, on the one hand, and to the initial high protein requirement of the acutely depleted kwashiorkor patient on the other.

Waterlow (5) related the weight gain of malnourished infants to caloric intake, and in a previous publication (6), we have made similar observations, suggesting that the energy requirements were directly related to the deficit in weight and height for age. It is generally agreed that nitrogen retention as determined by the balance method, is a crude but valuable index of growth. In only one study of malnourished infants (7) have we found an attempt to relate nitrogen retention and caloric intake. Just as we have noted that marasmic infants require exceptionally high caloric intakes to gain weight, we have also noted a direct relation between caloric intake and nitrogen retention in the same patients. On the other hand, in patients with hypoalbuminemia, with or without edema and other signs of kwashiorkor, we have noted high nitrogen retentions despite caloric intakes which are well below the minimum for growth, particularly when milk was the source of protein.

Received for publication January 21, 1964.
[1] This investigation was supported by Public Health Service Research Grants AM-4635 and AM-5935 from the National Institutes of Health.

METHODS

In 23 severely malnourished infants we have had the opportunity of studying two or more consecutive dietary periods in which the intakes of protein, water, sodium, potassium, and magnesium, on a per kilogram basis, recalculated daily, were kept constant, but the caloric intake was either increased or decreased progressively (100 ml of water, 2.0 mEq Na, 3.3 mEq K, and 0.55 mEq Mg/kg/day). The same dose (0.6 ml daily) of a multivitamin preparation [2] was given at all times. Collections of urine and stool were made, using metabolic beds, in an air conditioned environment, allowing a minimal period of adjustment of 3 days before determining the apparent nitrogen balance. Nitrogen in the diet, urine and feces was determined by the standard micro-Kjeldahl method. No attempt was made to estimate endogenous excretion or other possible nitrogen losses, or to interpret the results in absolute terms, as at all times we were comparing values in the same patient. The sources of nitrogen were one of the following: a modified cow's milk preparation,[3] a mixture of wheat and fish proteins (8), and a vegetable mixture based on cottonseed flour.[4] When desired, additional calories were added in the form of cottonseed oil and cane sugar, maintaining the proportion of fat and carbohydrate found in the modified milk. In all diets, 53% of non-protein calories came from fat and 47% from carbohydrates.

RESULTS

For purposes of comparison, the patients have been divided into 3 groups. Some patients are reported more than once, in the same or different groups, at different stages in the course of recovery.

In the first group (table 1) are included severely malnourished infants with normal serum proteins whose caloric intake was progressively increased, either to accelerate weight gain or for the explicit purpose of studying its effect on nitrogen retention. Nearly all of them had ratios of height age/chronologic age below 0.4, and in most of them, the weight age/height age ratio was also below 0.4, indicating both chronic and acute undernutrition. In practically every infant we see a clear tendency for nitrogen retention to increase with caloric intake, regardless of the source of protein. In a single case with a high nitrogen intake of 0.8 g/kg/day there was no apparent effect of caloric intake on nitrogen retention.

In the second group (table 2) are included infants with hypoalbuminemia (less than 2.5 g/100 ml of serum), with or without edema and other clinical signs of kwashiorkor, whose caloric intake was progressively increased. Caloric intake had no consistent effect on protein utilization. When milk was the source of protein, particularly at the higher levels of protein intake, initial nitrogen retention was high and tended to fall off as caloric intake was increased. In three of them, there was again an increase in retention when their caloric intake was further increased. The initial high retentions of nitrogen coincide with the rapid regeneration of serum albumin, particularly when milk was the source of protein.

The third group (table 3) includes those patients whose caloric intake was progressively reduced, either to control excessive weight gain or to study the effect on nitrogen retention. Although all of them had low height age/chronologic age ratios, the weight age/height age ratios were between 0.4 and 1.25, and they have thus been considered as "partially recovered." We see a consistent tendency for nitrogen retention to decrease as caloric intake was decreased, thus supporting the contention that the progressive improvement shown in table 1 is not the result of improvement in nutritional state and the ability to retain nitrogen but rather of more efficient protein utilization resulting from a higher caloric intake.

DISCUSSION

There is abundant evidence to the effect that the calorically deprived animal, whether fed a high or a low protein diet, achieves a very similar body nitrogen con-

[2] Abdecol (Parke, Davis and Company), 0.6 ml of which yield 50 mg ascorbic acid, 5000 units vitamin A, 1000 units vitamin D, 1.0 mg thiamine·HCl, 1.2 mg riboflavin, 1.0 mg pyridoxine·HCl, 5 mg pantothenic acid and 10 mg nicotinamide.

[3] Similac with Iron, supplied by Ross Laboratories, Columbus, Ohio.

[4] Bradfield, R. B. 1960 The development of a low-cost, high nutritive value food supplement for Peruvian children. Abstract no. 126, Fifth International Congress of Nutrition, Washington, D. C.

TABLE 1

Effect of protein and calorie intake on nitrogen retention in marasmic infants with normal serum albumin

Case and sex	Height	Wt	Age	Height age [1]	Wt age [2]	Source of protein	Daily N intake	Intake, kcal/kg/day				
								75	100	125–130	150	175
								Apparent nitrogen retention [3]				
	cm	*kg*	*months*	*months*	*months*		*mg/kg*	*mg/kg/day*	*mg/kg/day*	*mg/kg/day*	*mg/kg/day*	*mg/kg/day*
14M	64.2	5.30	13	4.7	2.2	WF [4]	320	−6(3)	−2(3)	90(12)	78(6)	—
19M	77.0	5.18	54	14.0	2.0	WF	320	118(10)	108(27)	160(6)	152(11)	—
17M	64.2	4.40	13	4.7	0.9	V [5]	320	57(16)	43(6)	86(6)	105(12)	—
16M	72.5	5.70	30	10.1	3.2	M [6]	320	107(10)	121(12)	111(12)	138(15)	—
36F	60.0	4.10	15	3.2	0.7	M	320	—	7(9)	65(3)	14(8)	75(37)
37F	61.5	4.20	15	3.9	0.8	M	320	—	41(9)	—	77(23)	102(12)
39F	57.0	3.95	6	2.2	0.5	M	320	−18(4)	32(6)	—	73(4)	102(12)
42M	56.0	3.02	15	1.6	0	M	320	—	91(6)	—	97(26)	145(9)
48M	75.2	8.37	23	12.2	7.5	M	320	—	55(6)	117(15)	—	—
36F	62.6	6.61	19	4.5	4.8	M	480	—	118(3)	142(6)	187(20)	—
37F	63.8	6.00	19	4.5	3.5	M	480	—	104(9)	102(6)	171(3)	—
49M	58.0	3.26	14	2.3	0	M	480	—	—	158(35)	177(6)	201(9)
07F	57.0	4.21	12	2.2	0.8	M	802	—	—	318(6)	—	332(9)

[1] Height age (Children's Medical Center, Boston).
[2] Weight age (Children's Medical Center, Boston).
[3] Figures in parentheses indicate days of metabolic collection.
[4] A mixture of wheat and fish proteins.
[5] A vegetable mixture based on cottonseed flour.
[6] Modified cow's milk.

TABLE 2

Effect of protein and calorie intake on nitrogen retention in malnourished infants with hypoalbuminemia

Case and sex	Height	Wt	Age	Height age [1]	Wt age [2]	Source of protein	Daily N intake	Intake, kcal/kg/day				
								75	100	125–130	150	175
								Apparent nitrogen retention [3]				
	cm	*kg*	*months*	*months*	*months*		*mg/kg*	*mg/kg/day*	*mg/kg/day*	*mg/kg/day*	*mg/kg/day*	*mg/kg/day*
13M	74.6	6.18	22	11.8	3.8	WF [4]	320	72(7)	9(3)	69(6)	131(9)	—
15M	82.0	10.35	25	18.0	13.0	V [5]	320	68(9)	68(15)	71(9)	88(18)	—
06M	69.0	6.07	13	7.5	3.6	M [6]	324	91(6)	100(8)	109(6)	—	—
21F	72.0	6.88	17	10.7	5.4	M	320	—	169(20)	159(6)	—	—
45F	61.0	4.10	12	3.7	0.7	M	320	98(3)	117(6)	107(6)	106(11)	135(15)
23F	75.6	6.80	30	13.4	5.2	M	480	228(6)	229(8)	167(9)	211(6)	—
26M	73.2	6.68	20	10.7	4.7	M	480	267(3)	207(9)	145(17)	174(9)	—
27M	85.7	8.66	30	22.1	8.1	M	480	222(6)	165(19)	111(19)	—	—

[1] Height age (Children's Medical Center, Boston).
[2] Weight age (Children's Medical Center, Boston).
[3] Figures in parentheses indicate days of metabolic collection.
[4] A mixture of wheat and fish proteins.
[5] A vegetable mixture based on cottonseed flour.
[6] Modified cow's milk.

TABLE 3

Effect of protein and calorie intake on nitrogen retention in partially recovered malnourished infants whose caloric intake was progressively decreased

Case and sex	Height	Wt	Age	Height age [1]	Wt age [2]	Source of protein	Daily N intake	Intake, kcal/kg/day				
								175	150	125–130	100	75
								Apparent nitrogen retention [3]				
	cm	*kg*	*months*	*months*	*months*		*mg/kg*	*mg/kg/day*	*mg/kg/day*	*mg/kg/day*	*mg/kg/day*	*mg/kg/day*
01M	69.0	9.23	10	7.6	9.5	F [4]	308	—	—	130(9)	122(9)	—
14M	66.0	7.45	16	6.0	5.8	V [5]	320	—	75(14)	—	81(14)	—
16M	75.5	8.55	33	12.3	7.8	V	320	—	138(9)	—	92(9)	84(27)
39F	59.4	4.87	8	2.9	1.8	WF [6]	320	127(14)	—	113(9)	—	—
11M	68.3	8.35	13	7.2	7.5	M [7]	320	—	207(9)	—	147(6)	75(21)
19M	85.0	8.42	66	21.4	7.6	M	320	—	153(6)	118(8)	—	66(12)
50F	73.5	8.34	21	11.8	8.5	M	320	111(9)	—	87(18)	—	—
52M	79.6	8.53	37	16.0	7.8	M	320	—	61(41)	—	35(15)	—
23F	78.6	7.68	33	16.8	7.0	M	480	—	159(27)	—	104(20)	—
42M	59.0	5.34	19	2.6	2.3	M	480	223(9)	213(3)	172(3)	—	—

[1] Height age (Children's Medical Center, Boston).
[2] Weight age (Children's Medical Center, Boston).
[3] Figures in parentheses indicate days of metabolic collection.
[4] Fish flour.
[5] A vegetable mixture based on cottonseed flour.
[6] Wheat and fish flours.
[7] Modified cow's milk.

tent and distribution, and that in severe caloric restriction protein in the diet becomes, in the main, another source of energy (9–11). In the malnourished infant, there is a compensatory reduction in basal metabolic rate; during treatment, as caloric intake is increased, BMR reaches higher than normal values (12), resulting in an increased requirement of energy and failure to gain weight if this is not satisfied. In chronic caloric deprivation it has also been shown that there is an increase in adrenal cortical function conducive to the conservation of liver and serum proteins at the expense of muscle protein (13). An adequate caloric intake favors the renewed utilization of amino acids by muscle proteins (14). In our marasmic patients, these mechanisms are apparently in operation. Our observations confirm the importance of meeting the markedly elevated caloric requirements of these infants and the futility of giving supplementary protein without doing so.

The initial situation of our patients with hypoalbuminemia is quite different and much akin to the acute protein depletion produced in experimental animals where there is a high retention of nitrogen, most particularly by the liver, well above that normally obtained with a given caloric intake (10, 15, 16). A similar effect has been reported in protein-depleted infants (17). The rapid regeneration of serum albumin and disappearance of the fatty liver of kwashiorkor which is observed when milk proteins are given is probably of a similar nature. After this initial period, nitrogen retention is more nearly dependent on caloric intake as in the normal or marasmic individual. In the protein-depleted animal and in kwashiorkor much if not most of the nitrogen retained initially is probably taken up by the liver and must depend to a great extent on the amount and sequence of amino acids available in the portal circulation. Since the action of insulin is essential for the uptake of amino acids by muscle cells, an adequate caloric intake is of greater importance for the subsequent retention of nitrogen, once the depleted liver proteins are regenerated.

LITERATURE CITED

1. Allison, J. B. 1958 Calories and protein nutrition. Ann. N. Y. Acad. Sci., 69: 1009.
2. Calcagno, P. L., and M. I. Rubin 1954 Effect of added carbohydrate on growth, nitrogen retention and renal water excretion in premature infants. Pediatrics, *13:* 193.
3. Clark, H. E., S. P. Yang, L. L. Reitz and E. T. Mertz 1960 The effect of certain factors on nitrogen retention and lysine requirements of adult human subjects. I. Total caloric intake. J. Nutrition, 72: 87.
4. Mönckeberg, F., G. Donoso, S. Oxman, N. Pak and J. Meneghello 1963 Human growth hormone in infant malnutrition. Pediatrics, *31:* 58.
5. Waterlow, J. C. 1961 The rate of recovery of malnourished infants in relation to the protein and calorie levels of the diet. J. Trop. Ped. Afr. Child Health, *7:* 16.
6. Graham, G. G., A. Cordano and J. M. Baertl 1963 Studies in infantile malnutrition. II. The effect of protein and calorie intake on weight gain. J. Nutrition, *81:* 249.
7. Holemans, K., and A. Lamhrechts 1955 Nitrogen metabolism and fat absorption in malnutrition and in kwashiorkor. J. Nutrition, 56: 477.
8. Graham, G. G., J. M. Baertl and A. Cordano 1962 Evaluation of fish flour in the treatment of infantile malnutrition. Fish in Nutrition, Fishing News (Books) Ltd., London, p. 271.
9. Dickerson, J. W. T., and V. Cabak 1962 The distribution of nitrogen in young rats which had lost equal amounts of weight after being fed diets (a) low in protein and (b) high in protein but limited in calories. Proc. Nutrition Soc., *21:* iii.
10. Cox, W. M., Jr., R. C. Elingson and A. J. Mueller 1953 Nitrogen utilization in caloric insufficiency. Pediatrics, *11:* 435.
11. Calloway, D. H., and H. Spector 1955 Nitrogen utilization during caloric restriction. I. The effect of dietary fat content. J. Nutrition, 56: 533.
12. Montgomery, R. D. 1962 Changes in basal metabolic rate of the malnourished infant and their relation to body composition. J. Clin. Invest., *41:* 1953.
13. Castellanos, H., and G. Arroyave 1961 Role of the adrenal cortical system in the response of children to severe protein malnutrition. Am. J. Clin. Nutrition, *9:* 186.
14. Munro, H. N., J. Chisholm and D. J. Naismith 1962 The influence of the calorie content of the diet on the uptake of labelled amino acids by tissue proteins. Brit. J. Nutrition., *16:* 245.
15. Summers, J. D., and H. Fisher 1962 Nutritional requirements of the protein-depleted chicken. III. Protein needs and utilization during repletion. J. Nutrition, 77: 465.
16. Summers, J. D., and H. Fisher 1960 Nutritional requirements of the protein-de-

pleted chicken. I. Effect of different protein depletion regimes on body composition during depletion, repletion and redepletion. J. Nutrition, 72: 153.

17. Rubin, M. I., P. K. Mukherji and P. L. Calcagno 1958 Nitrogen utilization in caloric and protein depleted infants. J. Dis. Child., 96: 491.

Metabolic Aspects of Urea Utilization by Ruminant Animals [1]

WILLIAM CHALUPA,[2] J. L. EVANS AND M. C. STILLIONS [3]
Department of Animal Sciences, Rutgers — The State University, New Brunswick, New Jersey

ABSTRACT Growing 267-kg steers were fed a low-nitrogen (N), semipurified diet with varying combinations of urea and corn gluten meal to supply either zero, 46 or 92% of the animals' N intake as urea. N balances were 20, 14 and 1 g/day for the respective diets. Fecal and urinary N excretions increased with increasing amounts of urea N in the diet. Other indices of N utilization followed similar trends. Although microbial activity (estimated by redox potential) was higher with urea-containing diets, measurement of ruminal ammonia and partition of the urinary N fraction indicated a loss of N by the animal as a result of ruminal ammonia concentration. Ruminal ammonia peak and biological value were inversely related. Evidently microbial protein synthesis was not comparable in rate to urea hydrolysis. The small change in ruminal ammonia concentration produced from corn gluten meal suggested that this protein is relatively insoluble or has a slow rate of release in the rumen. The inferiority of urea diets can therefore be explained as a combination of urea hydrolysis occurring at a faster rate than synthesis of microbial protein and a portion of less soluble N sources escaping rapid proteolysis in the rumen.

One of the major developments in ruminant feeding practices during recent years has been the use of urea as a protein substitute. The function of urea in ruminant diets has been covered in several reports (1–8). In most of the experiments reviewed in these reports, urea did not supply more than 30 to 40% of the total nitrogen (N). More recently, however, higher levels of urea feeding have been attempted (9, 10). Although much research effort has been directed towards finding optimal conditions for the efficient utilization of urea, in terms of productive capacity, diets using urea as a protein substitute are usually inferior to diets using protein. It seems important, therefore, that basic studies on urea utilization by ruminant animals be continued.

The experiments reported herein were designed to supply almost all of the animals' N requirement through the test sources considered by feeding a low N, semipurified diet (0.23% N on a dry matter basis). In this way it was possible to supply either zero, 46 or 92% of the N requirement from urea and thereby to study the effect of urea N per se on both the rumen microorganism and the intact animal metabolism.

EXPERIMENTAL

Growing 267-kg Jersey steers, fitted with permanent rumen cannula, were fed the low N basal diet (table 1) with combinations of urea and corn gluten meal to supply either zero, 46 or 92% of the animals' N requirement as urea. These diets were fed once daily according to the NRC (11) protein and energy recommendations.

TABLE 1
Composition of basal diet

	%
Oat straw	19.6
Cornstarch	19.6
Ground corn cobs	19.6
Glucose monohydrate [1]	39.2
Mineral mix [2]	1.5
Vitamin mix [3]	0.4

[1] Cerelose, Corn Products Company, Argo, Illinois.
[2] In mg/g of diet: $CaHPO_4$, 6.85; NaCl, 2.00; and Na_2SO_4, 6.36. Offered separately ad libitum: $CaCO_3$; $CaSO_4$; $KHCO_3$; $MgCO_3$; NaCl; and NaH_2PO_4.
[3] In IU/g of diet: vitamin A, 3.88; vitamin D, 0.39; vitamin E, 0.08; vitamin B_{12}, 0.005.

Received for publication March 13, 1964.
[1] Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers — The State University, New Brunswick. Data presented are from a thesis submitted by the senior author in partial fulfillment for the Ph.D. degree.
[2] Present address: Department of Dairy Science, Clemson University, Clemson, South Carolina.
[3] Present address: Morris Research Laboratories, Topeka, Kansas.

Urea-containing diets were supplemented with starch to compensate for their lower energy content. Adjustments in amounts of N and energy for animals of differing size were made as a function of body weight$^{0.73}$.

The experimental design consisted of (a) a 50-day adjustment period in which equal portions of the animals' N requirement were supplied by corn gluten meal and urea to determine the efficacy of the semipurified diet; (b) a metabolism trial (21-day preliminary and 14-day experimental periods) in which the various combinations of urea and corn gluten meal were fed to 4 animals in an incomplete (less 1 column) 4×4 Latin square design (12); and (c) a N-free period to estimate urinary endogenous and metabolic fecal N excretions.

During the experimental period of the metabolism trial (b), samples of rumen liquor were collected via the fistula at zero, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0 and 5.0 hours after feeding and analyzed for ammonia concentration, pH, and redox potential, to ascertain the influence of urea versus plant protein N on the metabolism of rumen microorganisms. Total collection of urine and feces and N partition provided information on the overall metabolism of the animal.

Redox potential measurements were made with a Beckman Model H-2 ph meter fitted with a calomel and gold electrode. Redox potential measurements were corrected for pH effect as suggested by Chalupa[4] and by Baldwin and Emery (13). Redox values presented are expressed in terms of the gold versus the calomel electrodes. Ammonia concentration in rumen liquor and urine and urinary creatine and creatinine were determined according to procedures outlined by Hawk et al. (14). Proximate analyses were carried out according to AOAC (15) recommendations; cellulose, by a modification of the Crampton and Maynard (16) technique; and energy, by calorimetry in a Parr oxygen bomb.[5]

RESULTS

Adjustment period. To evaluate the efficacy of the semipurified diet for growing ruminant animals, a 50-day test trial was conducted. Approximately one-half of the animals' N requirement was in the form of urea, with remainder supplied by corn gluten meal. Average daily weight gain for the 50-day period was 0.29 kg, which indicated that growth of ruminant animals could be maintained with this diet.

N-Free period. Total collections of urine and feces were made on days 3 to 5, 7 to 9, and 10 to 12 of N-free feeding. No differences in N excretions existed between collection days 7 to 9 and 10 to 12, which indicated that metabolic and endogenous excretion levels had been reached by the seventh day. The mean metabolic fecal N excretion was 0.281 ± 0.048 g/100 g of dry matter consumed. Mean urinary endogenous N excretion was 0.0315 ± 0.0007 g/kg body weight. These values fall within the ranges observed by other workers and cited by Chalupa.[6]

Metabolism trial. It is well known that the amount of available energy as well as the level of protein feeding will markedly influence the magnitude of N utilization. Thus a constant calorie-to-N ratio is an important factor when comparing utilization of dietary N sources. The values presented in table 2 show that diets fed during this study were both equicaloric and isonitrogenous. Thus, the metabolic differences in ruminal ammonia content, ruminal redox potential, N balance and excreted N noted between dietary N sources can be attributed to the N source per se.

Nitrogen excretion data are presented in table 3. Since fecal N values were significantly affected by level of urea N, there

TABLE 2

Nitrogen intake and calorie-to-nitrogen ratio

Urea N	Corn gluten meal N	N intake	Calorie-to-N ratio
%	%	*g/day*	*kcal digestible energy/g N*
0	92	81.0	175 ± 4 [1]
46	46	78.6	171 ± 9
92	0	78.2	170 ± 2

[1] SE of mean.

[4] Chalupa, W. V. 1962 Nitrogen utilization and microbial activity. Ph.D. Thesis. Rutgers University, New Brunswick, New Jersey.

[5] Parr Manual no. 120 1948 Oxygen bomb calorimetry and oxygen combustion methods. Parr Instrument Company, Moline, Illinois.

[6] See footnote 4.

TABLE 3

Influence of dietary nitrogen source upon nitrogen utilization

Measurement	Nitrogen source, % [1]		
	Zero U 92 CGM	46 U 46 CGM	92 U Zero CGM
Fecal N, g/day[b]	20.5 ± 2.58 [2]	22.6 ± 1.70	25.9 ± 2.74
Urinary N, g/day [b]	40.8 ± 3.29	42.4 ± 2.56	51.1 ± 2.87
N retained:			
g/day [a]	19.6 ± 1.96	13.6 ± 4.57	1.2 ± 1.37
as % intake [b]	24.2 ± 1.71	16.8 ± 5.46	1.6 ± 1.75
Biological value, % [a]	56.5 ± 3.69	50.6 ± 5.28	36.8 ± 3.79
Net protein utilization, % [a]	51.2 ± 2.66	44.4 ± 5.16	30.7 ± 2.67
Urinary creatine, g/day [b]	4.3 ± 1.12	4.5 ± 0.70	7.2 ± 0.82
Wt change, kg/day [a]	0.5 ± 0.08	0.3 ± 0.05	−0.1 ± 0.11

[1] U indicates N% supplied by urea and CGM indicates N% supplied by corn gluten meal.
[2] SE of mean.
[a] Significant linear regression of criterion of measurement and percentage of N source ($P < 0.05$) (12).
[b] Significant linear regression of criterion of measurement and percentage of N source ($P < 0.10$) (12).

were differences in both apparent and true digestibility of N. Similarly, urinary N excretion was increased when urea supplied increasing amounts of the animals' N requirement. These increased fecal and urinary excretions were reflected in significant decreases in N retention, biological value and net protein utilization. The weight changes (table 3) followed the same pattern as the N utilization data.

Since a major change, in terms of N utilization, occurred in the excretion of urinary N, this fraction was partitioned as indicated in table 4. Significant increases in urinary urea plus ammonia N and in creatine N were observed with the diets which supplied increasing amounts of the total N as urea. Since urinary creatine arises as a result of tissue catabolism or the reduction of muscle mass, this increase may be due, in part, to muscular catabolism resulting from an inferior dietary N source in the form of urea. The large undetermined urinary N fraction observed with the diets supplying either zero or 46% of the N as urea probably represents amino N and small amounts of intact protein.

In an attempt to determine the origin of the increased urinary excretion of urea plus ammonia, rumen liquor samples obtained at various time intervals after feeding were analyzed for ammonia concentration. The data in figure 1 indicate that urea was rapidly hydrolyzed to ammonia in the rumen. The small change in rumen

TABLE 4

Influence of dietary nitrogen source upon urinary nitrogen distribution

Component	Nitrogen source, % [1]		
	Zero U 92 CGM	46 U 46 CGM	92 U Zero CGM
Total urinary N, g/day	40.8 ± 3.29 [2]	42.4 ± 2.56	51.1 ± 2.87
Nitrogen partition: (% of total urinary N)			
Urea + NH_3 N	72.1 ± 1.23	73.3 ± 2.38	86.0 ± 1.64
Creatinine N	6.3 ± 0.79	7.0 ± 0.88	6.3 ± 0.49
Creatine N	3.6 ± 0.79	3.7 ± 0.48	5.1 ± 0.85
Undetermined N	18.0 ± 0.95	16.0 ± 1.80	2.6 ± 1.50

[1] U indicates N% supplied by urea and CGM indicates N% supplied by corn gluten meal.
[2] SE of mean.

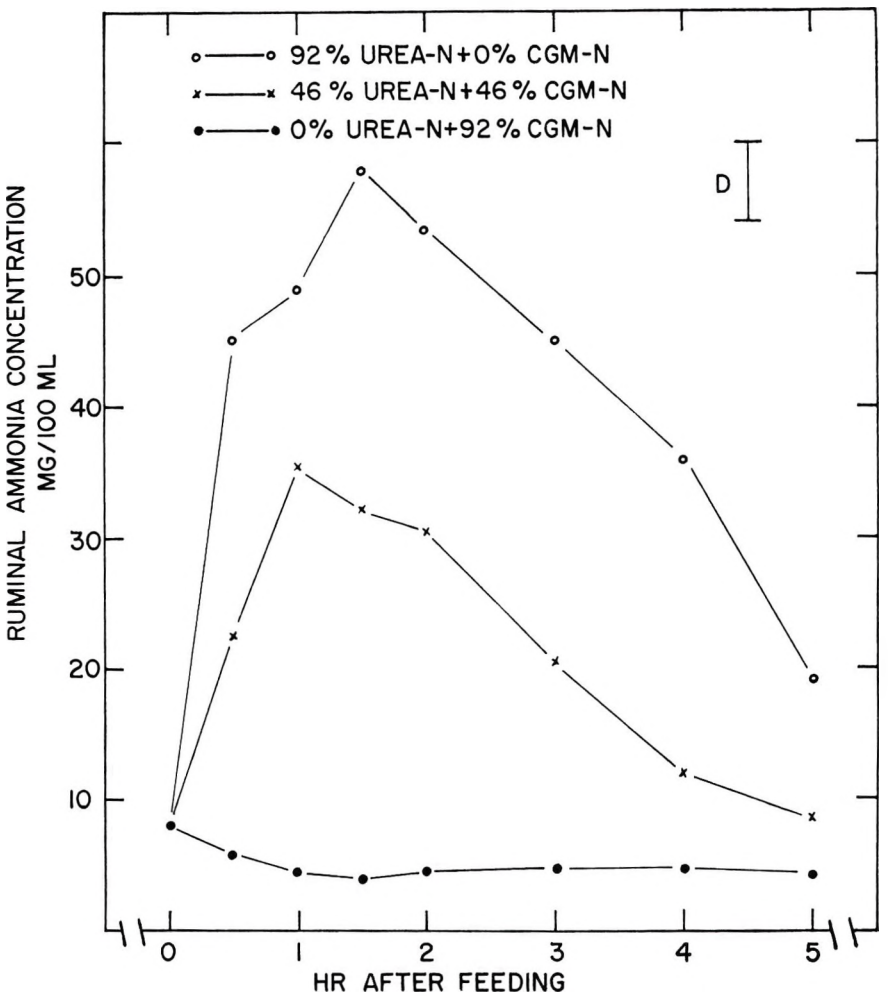


Fig. 1 Influence of urea upon ruminal ammonia concentrations at various times after feeding (Tukey's *D*, differences with $P < 0.05$) (12). CGM indicates corn gluten meal.

ammonia concentration produced from the corn gluten meal diet suggests that this N source is relatively insoluble or has a slow rate of release in the rumen.

Rumen liquor samples, obtained at various time intervals after feeding, were subjected to redox potential measurments in an attempt to ascertain whether differences in rumen metabolic activity existed as a result of varying the dietary N source. Values presented in figure 2 have been corrected for differences existing at the zero-hour sampling period and are to be interpreted as reflecting quantitative changes in microbial activity as estimated by redox potential. Animals fed diets with 92% of the N supplied by urea exhibited an immediate and marked increase in ruminal redox potential, whereas at first those fed the corn gluten meal had a lower redox potential although subsequently it increased. We interpreted these data to mean that microbial activity was greatest with diets containing the most soluble N, that is, urea N.

DISCUSSION

Since microbial activity, as estimated by changes in redox potential, was higher with urea-containing diets, the factor limiting efficient N utilization of urea diets by the animal was assumed to be the rate of

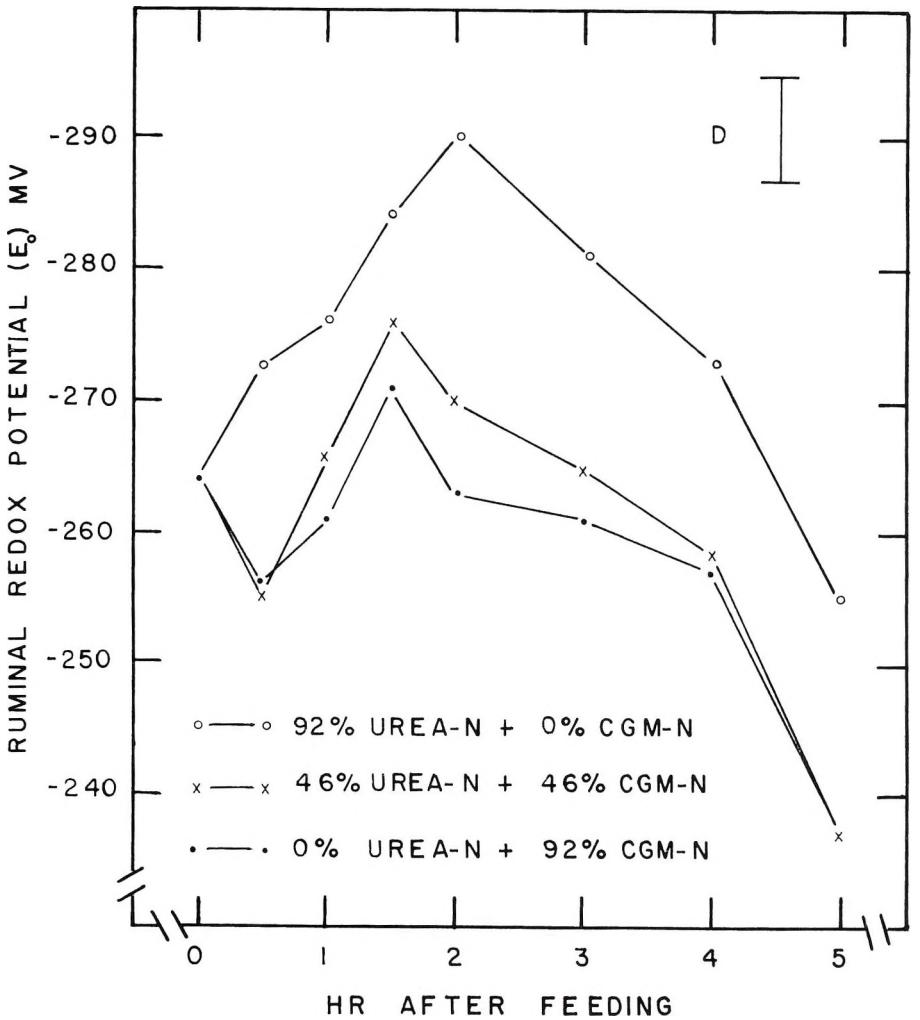


Fig. 2 Influence of urea upon ruminal redox potential at various times after feeding (Tukey's *D*, difference with $P < 0.05$) (12). CGM indicates corn gluten meal.

formation of microbial protein in the rumen. Since urea is rapidly hydrolyzed to ammonia in the rumen, the urea fed must by synthesized into protein by the rumen microorganisms for the best utilization of dietary N by the animal; if otherwise, urea hydrolysis occurs at a faster rate than protein synthesis, and a loss of N to the animal could result. These data are in agreement with those previously reported (20, 21), namely, that N sources of lower solubility have higher utilization values for ruminant animals.

N utilization data indicated that with increasing urea N supplementation N utilization was decreasing. The decreased N utilization with the diets supplying an increasing amount of N as urea resulted in an increased urinary N excretion. Partition of urinary N revealed that the increase was in the form of urea plus ammonia. Ruminal ammonia concentrations indicated that urea was rapidly hydrolyzed.

The relationship of increasing ruminal ammonia concentration and decreasing N utilization existed, supporting the concept of ammonia absorption into the portal blood system with subsequent conversion back to urea in the liver and excretion in the urine. The greater urinary N excre-

tion and percentage of urea N in the urine supported this hypothesis. Similar results have been reported by Drori and Loosli (9). Not so obvious, however, was the amount of ammonia that was not converted to urea in the liver and which eventually re-entered the rumen via the peripheral circulation as suggested by Houpt (17). Similar to the report of Oberleas,[7] this latter fraction was reflected, in part, by the increased fecal N excretion reported in table 3 with increasing dietary N from urea. The greater return of urea to the rumen reported by Lewis (18), Houpt (17), and Hirose et al. (19) when rumen ammonia concentration were elevated could result in this anomaly.

Since the limiting factor for optimal utilization of urea appears to be the rate of urea hydrolysis and microbial protein synthesis from its N, future studies should be directed towards decreasing the former and increasing the latter, or both. And as microbial activity is related to the amount of soluble N in the rumen, the caloric density or the amount of fiber in a diet may determine the optimal ratio of soluble to insoluble N in the ruminant diet.

LITERATURE CITED

1. Armsby, H. P. 1911 The nutritive value of the non-protein feeding stuffs. U. S. Bureau of Animal Industries, bull. 139, Washington, D. C.
2. Krebs, K. 1937 Value of the amides in the feeding of dairy cattle. Historical consideration of the development of the amide question, critical evaluation of the state of our present knowledge (translated title). Biedermanns Zentr. B. Tierernahr., 9: 394.
3. Goss, H. 1943 Some peculiarities of ruminant nutrition. Nutrition Abstr. Rev., *12:* 531.
4. McNaught, M. L., and J. A. B. Smith 1947 Nitrogen metabolism in the rumen. Nutrition Abstr. Rev., *17:* 18.
5. Huffman, C. F. 1953 Ruminant nutrition. Ann. Rev. Biochem., 22: 399.
6. Reid, J. T. 1953 Urea as a protein replacement: a review. J. Dairy Sci., 36: 955.
7. Loosli, J. K. 1958 Chemical sources of nitrogen as supplements to protein feeds. In Processed Plant Protein Foodstuffs, ed., A. M. Altshul. Academic Press, Inc., New York.
8. O'Rourke, W. F. 1960 Urea for ruminants. Proc. Am. Feed Manufacturers Assoc. Nutrition Council (November), p. 8.
9. Drori, D., and J. K. Loosli 1961 Urea and carbohydrates versus plant protein for sheep. J. Animal Sci., 20: 233.
10. Lassiter, C. A., R. M. Grimes, C. W. Duncan and C. F. Huffman 1958 High level urea feeding to dairy cattle. I. Effect of high-level urea feeding on the growth and metabolism of growing dairy heifers without sulfur supplementation. J. Dairy Sci., *41:* 281.
11. National Research Council, Committee on Animal Nutrition 1958 Nutrient requirements of dairy cattle, pub. 579. National Academy of Sciences — National Research Council, Washington, D. C.
12. Snedecor, G. W. 1956 Statistical Methods, ed. 5. Iowa State College Press, Ames.
13. Baldwin, R. L., and R. S. Emery 1960 The oxidation-reduction potential of rumen contents. J. Dairy Sci., *43:* 506.
14. Hawk, P. B., B. L. Oser and W. H. Summerson 1954 Practical Physiological Chemistry, ed. 13. McGraw-Hill Book Company, Inc., New York.
15. AOAC 1960 Official Methods of Analysis, ed. 9. Association of Official Agricultural Chemists. Washington, D. C.
16. Crampton, E. W., and L. A. Maynard 1938 The relation of cellulose and lignin content to the nutritive value of animal feeds. J. Nutrition, *15:* 383.
17. Houpt, T. R. 1959 Utilization of blood urea in ruminants. Am. J. Physiol., *197:* 115.
18. Lewis, D., K. J. Hill and E. F. Annison 1957 Studies on the portal blood of sheep. I. Absorption of ammonia from the rumen of the sheep. Biochem. J., *66:* 587.
19. Hirose, Y., R. S. Emery, C. F. Huffman and G. H. Conner 1960 Effect of protein status on salivary urea secretion. J. Dairy Sci., *43:* 874.
20. Little, C. O., W. Burroughs and W. Woods 1963 Nutritional significance of soluble nitrogen in dietary proteins for ruminants. J. Animal Sci., 22: 358.
21. Tagari, H., I. Ascarelli and A. Bondi 1962 The influence of heating on the nutritive value of soya-bean meal for ruminants. Brit. J. Nutrition, *16:* 237.

[7] Oberleas, D. 1959 A biological evaluation of urea with mature wethers. M. S. Thesis. University of Kentucky, Lexington, Kentucky.

Estimation of Net Phosphorus Utilization by the "Slope" Method [1]

S. HURWITZ
The National and University Institute of Agriculture, Rehovot, Israel

ABSTRACT A mathematical model for the estimation of phosphorus (P) availability was derived and tested in 3 separate trials. This method is based on the determination of the regression slope of tibia P as a function of total P intake, under conditions of controlled feeding. Carcass P-to-tibia P was found to be 19.6 ± 1.0. The value of the regression slope multiplied by this factor and 100 yields the percentage of net phosphorus utilization (% NPU). The latter allows for the endogenous P excretion, in contrast with percentage of apparent retention. The % NPU for reagent grade dicalcium phosphate was 94.1, 98.0 and 78.4, respectively, in 3 trials. Defluorinated rock phosphate, soft phosphate and reagent grade tricalcium phosphate were 95, 76 and 92%, respectively, as available as reagent grade dicalcium phosphate. Feed grade dicalcium phosphate was as available as reagent grade dicalcium phosphate. Plasma inorganic phosphorus was determined to be a useful measure for estimation of the relative P availability. Under ad libitum feeding conditions, total P requirement level was found to be 0.65, 0.65 and 0.66% for maximal growth, feed efficiency and tibia P, respectively. The Ca-to-P ratio of bone decreased with P supplementation. The relationship between feed efficiency and dietary P is discussed.

The comparative utilization of phosphorus from various supplements has been studied by several investigators (1–4). Gillis et al. (5) were the first to quantitate the availability of phosphorus from various phosphorus compounds, for chicks. Their method was based on relating percentage of tibia ash from chicks fed a certain test supplement, to that of chicks fed a reference standard, thus obtaining relative "biological availability" values. Such relative values were also determined by Gardiner (6) who used plasma inorganic phosphorus as an index of phosphorus availability. Ammerman et al. (7) suggested the slope of the regression line of percentage tibia ash on percentage of dietary phosphorus as a means of comparing supplements. This method partly overcomes the necessity of selecting an arbitrary level of supplementation for comparisons.

To obtain values of phosphorus utilization in terms of percentage retention, balance techniques have been used (8). However, these techniques as employed, yielded values of apparent retention with no allowance for the endogenous phosphorus excretion.

In the present paper a new method is described for obtaining the percentage of net phosphorus utilization. This value differs from percentage of apparent retention by allowing for the endogenous excretion, and is, therefore, a true measure of phosphorus utilization.

EXPERIMENTAL AND RESULTS

Theoretical considerations. Considering phosphorus retention in terms of balance, the equation for net phosphorus utilization (NPU) will be, by definition:

$$\text{NPU} = \text{P intake} - (\text{P feces} + \text{P urine}) + \text{P endogenous (fecal and urinary)} \quad [1]$$

or, also by definition:

$$\text{NPU} = \text{apparent P retention} + \text{P endogenous.} \quad [2]$$

When chicks are fed 2 levels of phosphorus, the difference between the corresponding NPU's will be given by:

$$\Delta\,\text{NPU} = \Delta\,\text{apparent P retention} + \Delta\,\text{P endogenous.} \quad [3]$$

If body weight and feed intake of the 2 groups are equal, the endogenous phos-

Received for publication April 20, 1964.

[1] Contribution from The National and University Institute of Agriculture, Rehovot, Israel, 1964 Series no. 691-E. Supported by a grant-in-aid from the Board for the Production and Marketing of Eggs and Poultry.

phorus excretion can be assumed to be equal, an assumption also inherent in the intercept method for the estimation of endogenous phosphorus (9). In this case, equation 3 can be simplified to:

$$\Delta \text{ NPU} = \Delta \text{ apparent P retention.} \quad [4]$$

In terms of body composition, after such a feeding trial,

$$\text{Final body P} = \text{initial body P} + \text{apparent P retention.} \quad [5]$$

When the initial body phosphorus is equal for the groups, a comparison as in equation 3 will yield:

$$\Delta \text{ Final body P} = \Delta \text{ apparent P retention.} \quad [6]$$

Rewriting, using equation 4,

$$\Delta \text{ Final body P} = \Delta \text{ NPU.} \quad [7]$$

Percentage of net phosphorus retention will be, by definition:

$$\% \text{ NPU} = \frac{\Delta \text{ NPU}}{\Delta \text{ P intake}} \times 100 \quad [8]$$

or,

$$\% \text{ NPU} = \frac{\Delta \text{ P body}}{\Delta \text{ P intake}} \times 100 \quad [9]$$

When Δ P intake approaches zero, equation 9 will become:

$$\% \text{ NPU} = \frac{\text{d P body}}{\text{d P intake}} \times 100 \quad [10]$$

The % NPU would therefore be the slope $\times$ 100 of the body phosphorus as a function of total phosphorus intake. When such a function is linear, as is the case within a certain range below the minimal requirement, equation 10 equals a constant and the function, Y (body P) = a + b X (P intake) may be written:

$$\text{P body} = a + \frac{\% \text{ NPU}}{100} \text{ P intake.} \quad [11]$$

Since carcass analysis is rather cumbersome, the possibility that tibia phosphorus may be a reliable measure of body phosphorus was investigated. Sixteen chicks from 3 trials fed different phosphorus levels were analyzed for both carcass and tibia phosphorus. Results of these analyses indicated a fairly constant ratio between carcass and tibia phosphorus. This ratio was found to be 19.6 with a standard deviation of 1.0. The ratio was unaffected by the nutritional treatments used in these trials. It appears, therefore, that tibia phosphorus may serve as a good estimate of carcass phosphorus.

Using this availability method it is essential to start with groups of chicks of equal average body weight, minimizing any differences in the initial body phosphorus, and to keep the level of feed intake equal in all groups to obtain an increment of phosphorus intake derived entirely from supplementary phosphorus and not from consumption of greater amounts of phosphorus contained in the basal diet. It can be shown easily that the phosphorus level of the basal diet is of no consequence in this type of assay, as long as it is not high enough to minimize the range in which a reliable measure of the regression slope can be made. The % NPU is calculated by multiplying the regression slope by a factor of 1960.

Before actual testing of the method, it was necessary to determine the phosphorus requirement, under our experimental conditions, in order to define the range of dietary phosphorus to be used.

Materials. The following phosphorus supplements were tested: Dicalcium phosphate, $CaHPO_4 \cdot 2H_2O$, reagent grade;[2] tricalcium phosphate, reagent grade;[3] a 1:1 mixture of monosodium and monopotassium phosphate, analytical grade (NaKP);[4] defluorinated rock phosphate;[5] dicalcium phosphate, feed grade;[6] soft phosphate.[7]

General procedure. In all trials White Rock male chicks were used. They were raised to one week of age with a commercial starter and weighed individually at this age. About 60% of the chicks, representing the middle weight range, were distributed among the experimental lots according to their body weight. Average body weight was equal for all lots at the start of the experiment. Each lot contained 20 chicks housed in electrically heated batteries with raised wire floors.

Body weight was recorded individually and feed intake on a group basis, after one week and at the end of the 16-day trial. In the trials in which paired-feeding was practiced, feed consumption records were

[2] Produced by Riedel de Hoen Ag., Seelze-Hannover, Germany.
[3] See footnote 2.
[4] Produced by The British Drug Houses Ltd., Poole, England.
[5] Produced by Chemicals and Phosphates, Haifa, Israel.
[6] See footnote 5.
[7] From U. S. commercial stock, origin unknown.

kept daily. At the end of the experiment, 9 birds of each lot, representing the middle weight range of the lot, were killed and their tibias removed for analysis. These bones were analyzed for total ash, calcium and phosphorus as previously described (10).

In trials 1 and 4, blood samples were obtained by heart puncture prior to death, using heparin as an anticoagulant. Plasma inorganic phosphorus was estimated in the trichloroacetic acid extract of the plasma using the spectrophotometric method of Gomori (11).

Trial 1. The purpose of this trial was to determine the suitable range of dietary phosphorus for the availability test, to determine the minimal phosphorus requirement under the present experimental conditions, and to compare various phosphorus supplements under ad libitum feeding conditions.

The composition of the experimental rations is shown in table 1. Dicalcium phosphate, reagent grade, dicalcium phosphate, feed grade, NaKP, and defluorinated rock phosphate were used in this trial as phosphorus supplements, each fed at 4 levels. Thus, the total phosphorus of the diets varied from 0.44 to 0.90%. One lot of chicks received the basal ration with no phosphorus supplement. Feed and water were given ad libitum.

Body weight, feed intake and feed efficiency as functions of dietary phosphorus level, are shown in figure 1. Although the differences among the various supplements were small, growth and feed efficiency tended to be highest with NaKP and lowest with defluorinated rock phos-

TABLE 1

Composition of experimental diets (trial 1)

		%
a Constant		
	Soybean meal (50% protein)	29.00
	Soybean oil, refined	1.00
	Vitamin mixture [1]	0.30
	Mineral mixture [2]	0.40
	Coccidiostat	0.10
	DL-Methionine	0.15

b Variable

Lot	1	2	3	4	5	6	7	8	9
NaKP [3]	—	0.44	0.88	1.34	2.22	—	—	—	—
Dicalcium phosphate, reag. grade	—	—	—	—	—	0.56	1.12	1.67	2.79
Limestone	2.70	2.70	2.70	2.70	2.70	2.36	2.01	1.68	1.00
Milo	66.35	65.91	65.47	65.01	64.13	66.13	65.92	65.70	65.26
P content [4]	0.36	0.44	0.55	0.60	0.78	0.49	0.56	0.67	0.88
Ca content [4]	1.2	1.3	1.2	1.3	1.2	1.3	1.3	1.2	1.2

Lot	10	11	12	13	14	15	16	17
Dicalcium phosphate, feed grade	0.58	1.16	1.74	2.90	—	—	—	—
Defluorinated rock phosphate	—	—	—	—	0.77	1.54	2.31	3.87
Limestone	2.32	1.95	1.57	0.81	2.27	1.85	1.42	0.57
Milo	66.15	65.94	65.71	65.34	66.01	65.66	65.32	64.61
P content [4]	0.47	0.59	0.68	0.88	0.48	0.58	0.68	0.85
Ca content [4]	1.3	1.3	1.3	1.3	1.3	1.3	1.4	1.4

[1] Supplying/kg of diet: vitamin A, 10,000 IU; vitamin D_3, 1000 ICU; riboflavin, 5 mg; Ca pantothenate, 11 mg; niacin, 25 mg; choline chloride, 300 mg; vitamin B_{12}, 10 μg; vitamin E, 2 IU; menadione sodium bisulfite, 2 mg; procaine penicillin and bacitracin, 10 mg; butylated hydroxytoluene, 125 mg.

[2] Sodium chloride supplemented to supply/kg of diet: (in milligrams) manganese, 78; zinc, 60; iron, 4.2; copper, 3; iodine, 1.4; molybdenum, 0.132; cobalt, 0.240.

[3] A 1:1 mixture of monosodium and monopotassium phosphates, analytical grade.

[4] Estimated by chemical analysis.

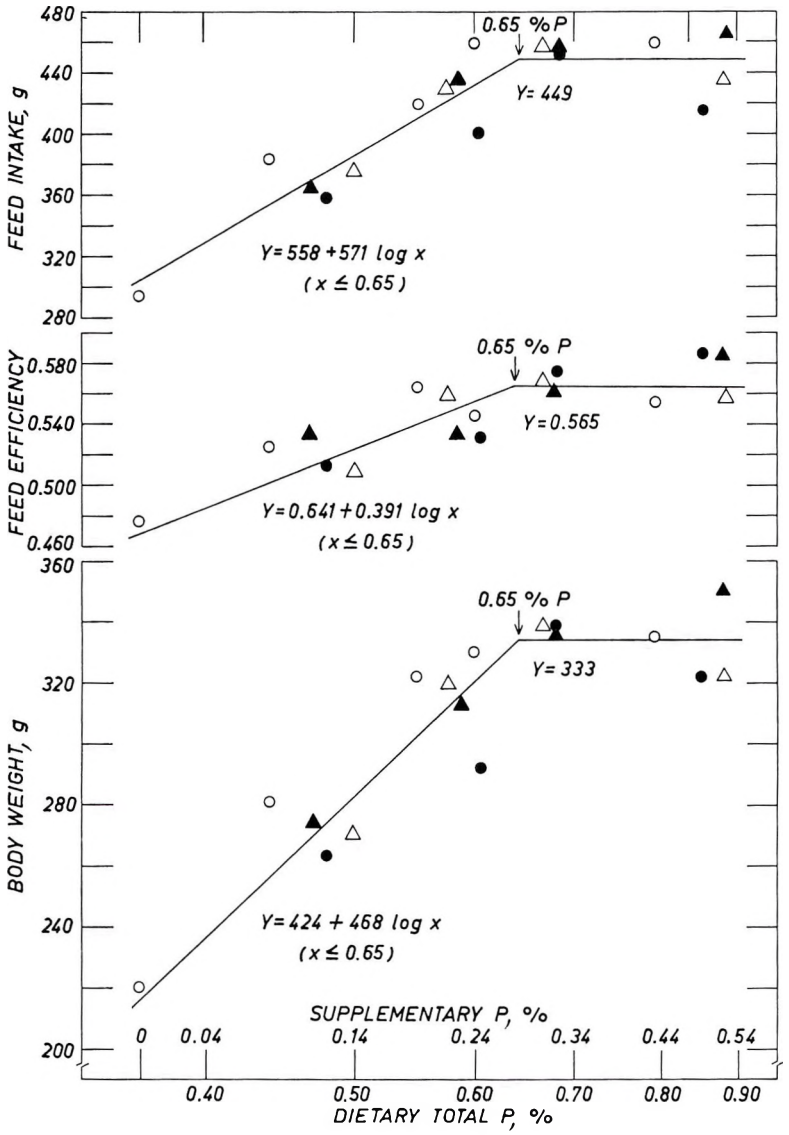


Fig. 1 The response of body weight, feed consumption and feed efficiency to a 16-day ad libitum feeding of different levels of phosphorus. Averages of groups of chicks fed sodium and potassium monophosphates (○); dicalcium phosphate, reagent grade (△); dicalcium phosphate, feed grade (▲); and defluorinated rock phosphate (●), trial 1.

phate. Averages of each supplement and phosphorus level were used to calculate the regressions of body weight, feed consumption and feed efficiency on the logarithm of the percentage of dietary phosphorus. The regression equations indicated that growth and feed efficiency responded to increasing levels of dietary phosphorus up to 0.65%. Feed consumption responded likewise.

Total tibia phosphorus, Ca-to-P ratio and plasma inorganic phosphorus are shown in figure 2. The calculated regression equation indicated that tibia phosphorus responded to dietary phosphorus up to 0.66%. The differences among the

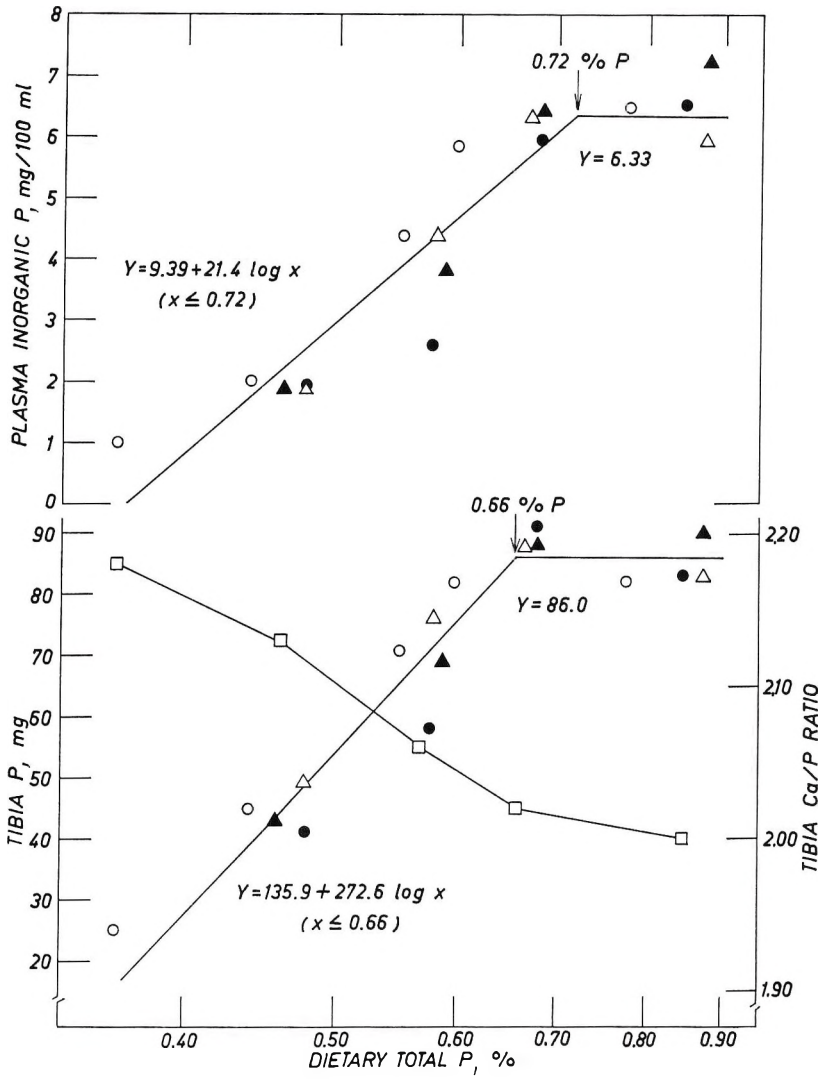


Fig. 2 The response of tibia phosphorus, plasma inorganic phosphorus and tibia Ca-to-P ratio (□) to a 16-day ad libitum feeding of different levels of phosphorus. Averages of groups of chicks fed sodium and potassium monophosphates (○); dicalcium phosphate, reagent grade (△); dicalcium phosphate, feed grade (▲); and defluorinated rock phosphate (●), trial 1.

supplements were small but consistent with those observed for body weight and feed efficiency.

The Ca-to-P ratio of the tibia decreased with increasing phosphorus supplementation. The decrease was rapid up to the requirement level and slower above it.

Plasma inorganic phosphorus markedly responded to dietary phosphorus level. It reached a maximum at 0.72% of dietary phosphorus, a dietary level much higher than that observed for maximal growth, feed efficiency and tibia phosphorus. Again, the differences among the supplements were small but consistent with those observed for growth, feed efficiency, and tibia phosphorus.

Trials 2–4. In these trials the mathematical model for evaluating the percentage of net phosphorus utilization (% NPU)

was tested, by comparing defluorinated rock phosphate, soft phosphate, and tricalcium phosphate, reagent grade, with dicalcium phosphate, reagent grade. In each experiment, all lots were daily pair-fed with the lot consuming least feed. The phosphorus content of the diets (table 2) varied in a range below the minimal requirement level estimated in trial 1. Each phosphorus supplement was fed at 3 different levels.

Body weight, feed intake, feed efficiency and percentage of tibia ash (of dry non-defatted bone) are shown in table 3. With all supplements, body weight and feed efficiency were somewhat increased with phosphorus supplementation; significant differences ($P < 0.05$) were noted only between dicalcium phosphate, reagent grade, and soft phosphate in trial 3. Percentage tibia ash significantly ($P < 0.05$) responded to phosphorus supplementation with all supplements. However, among the supplements, the only significant difference was noted between dicalcium phosphate, reagent grade, and soft phosphate in trial 3.

Total tibia phosphorus as a function of total phosphorus intake is shown in figure 3. In all cases the regression slopes for dicalcium phosphate, reagent grade, were greater than those of the test supplements. The % NPU was calculated for each supplement by multiplying the value of the corresponding regression slope by 1960. This calculation was followed by calculation of the relative availability, also presented in table 4. In trial 2 the % NPU for dicalcium phosphate, reagent grade, was similar to that determined in trial 3 but it was considerably reduced in trial 4. The % NPU for defluorinated rock phosphate and tricalcium phosphate, reagent grade, was only slightly different than that of dicalcium phosphate, reagent grade, but soft phosphate had a markedly lower value.

In trial 4, the usefulness of plasma inorganic phosphorus as a criterion for the relative phosphorus availability was also

TABLE 2

Composition of experimental diets (trials 2, 3 and 4)

Lot no.	1	2	3	4	5	6
	%	%	%	%	%	%
Trial 2						
Constant ingredients [1]	30.95	30.95	30.95	30.95	30.95	30.95
Dicalcium phosphate, reag. grade	0.44	0.89	1.33	—	—	—
Defluorinated rock phosphate	—	—	—	0.62	1.23	1.85
Limestone	2.43	2.16	1.89	2.36	2.02	1.68
Milo	66.18	66.00	65.83	66.07	65.80	65.52
P content [2]	0.44	0.52	0.59	0.44	0.51	0.58
Ca content [2]	1.2	1.3	1.1	1.1	1.2	1.3
Trial 3						
Constant ingredients [1]	30.95	30.95	30.95	30.95	30.95	30.95
Dicalcium phosphate, reag. grade	0.44	0.89	1.33	—	—	—
Soft phosphate	—	—	—	0.89	1.78	2.67
Limestone	2.43	2.16	1.89	2.28	1.86	1.44
Milo	66.18	66.00	65.83	65.88	65.41	64.94
P content [2]	0.48	0.56	0.64	0.47	0.54	0.61
Ca content [2]	1.2	1.2	1.2	1.2	1.2	1.2
Trial 4						
Constant ingredients [1]	30.95	30.95	30.95	30.95	30.95	30.95
Dicalcium phosphate, reag. grade	0.44	0.89	1.33	—	—	—
Tricalcium phosphate, reag. grade	—	—	—	0.40	0.80	1.20
Limestone	2.43	2.16	1.89	2.22	1.82	1.41
Milo	66.18	66.00	65.83	66.43	66.43	66.44
P content [2]	0.45	0.52	0.60	0.44	0.51	0.58
Ca content [2]	1.2	1.2	1.2	1.2	1.2	1.2

[1] For composition of constant ingredients, see table 1.
[2] Estimated by chemical analysis.

TABLE 3

Body weight, feed intake, feed efficiency and percentage tibia ash of chicks fed diets containing graded levels of phosphorus, derived from various supplements, under controlled feeding conditions [1]

Trial	Supplement	Dietary P [2]	Body wt	Feed intake [3]	Feed efficiency [4]	Tibia ash [5]
		%	g	g		%
2.	Dicalcium phosphate, reag. grade	0.44	302 ± 5 [6]	390	0.526	29.6 ± 0.3
		0.52	309 ± 6	399	0.532	32.5 ± 0.2
		0.59	318 ± 7	408	0.539	36.4 ± 0.3
	Defluorinated rock phosphate	0.44	303 ± 2	404	0.512	29.9 ± 0.6
		0.51	306 ± 6	398	0.523	32.7 ± 0.5
		0.58	312 ± 4	402	0.537	35.7 ± 0.4
3	Dicalcium phosphate, reag. grade	0.48	312 ± 5	398	0.540	27.6 ± 0.5
		0.56	313 ± 5	402	0.537	33.0 ± 0.5
		0.64	317 ± 7	404	0.545	35.1 ± 0.6
	Soft phosphate	0.47	299 ± 7	399	0.504	24.5 ± 0.3
		0.54	306 ± 6	398	0.523	27.4 ± 0.9
		0.61	305 ± 7	399	0.521	31.6 ± 1.3
4	Dicalcium phosphate, reag. grade	0.45	258 ± 6	345	0.501	29.3 ± 0.6
		0.52	265 ± 7	352	0.514	32.0 ± 0.5
		0.60	272 ± 5	353	0.532	37.1 ± 0.9
	Tricalcium phosphate, reag. grade	0.44	265 ± 7	353	0.512	28.4 ± 0.8
		0.51	261 ± 5	347	0.507	32.2 ± 0.4
		0.58	269 ± 6	351	0.530	34.3 ± 0.6

[1] Trials conducted with 7-day-old chicks, and lasted 16 days.
[2] Percentage of total phosphorus.
[3] Average feed intake per bird, for the entire experimental period.
[4] Gain in body weight/food intake.
[5] Percentage ash of oven-dried bones at 105°.
[6] Average ± SE of the mean.

investigated. Plasma inorganic phosphorus as a function of dietary phosphorus level is presented in figure 3. The ratio of the regression slopes of dicalcium phosphate, reagent grade, and tricalcium phosphate, reagent grade, indicated that tricalcium phosphate, reagent grade, was about 90% as available as dicalcium phosphate, reagent grade, in good agreement with the relative availability determined from the % NPU values (table 4).

DISCUSSION

The minimal requirement of total dietary phosphorus for chicks for maximal growth and feed efficiency was 0.65%, and for maximal phosphorus accumulation in tibia, 0.66%. Taking the accepted value of available phosphorus in plant materials to be one-third of the total phosphorus, the requirement for available phosphorus would then be 0.43 to 0.44%, in close agreement with the accepted requirement level of 0.45% available phosphorus (12).

The response of feed efficiency to dietary phosphorus deserves special comment. It has been shown previously that feed efficiency improves with phosphorus supplementation (13). Such an improvement may result from better feed utilization per se, or from increased growth rate as the result of a larger feed intake. The results of trial 1 show a marked increase in feed consumption due to phosphorus supplementation and an appreciable improvement in feed efficiency (fig. 1). When feed intake is restricted to that of the birds receiving the lowest phosphorus level, there still remains a slight but consistent improvement of feed efficiency with phosphorus supplementation (table 3). Possibly, phosphorus deficiency in chicks reduces the partial efficiency of energy utilization as observed by Kleiber et al. (14) in heifers.

As in observations of maturing pullets (15), the Ca-to-P ratio in bones decreased with increased levels of phosphorus supplementation. The reverse effect was dem-

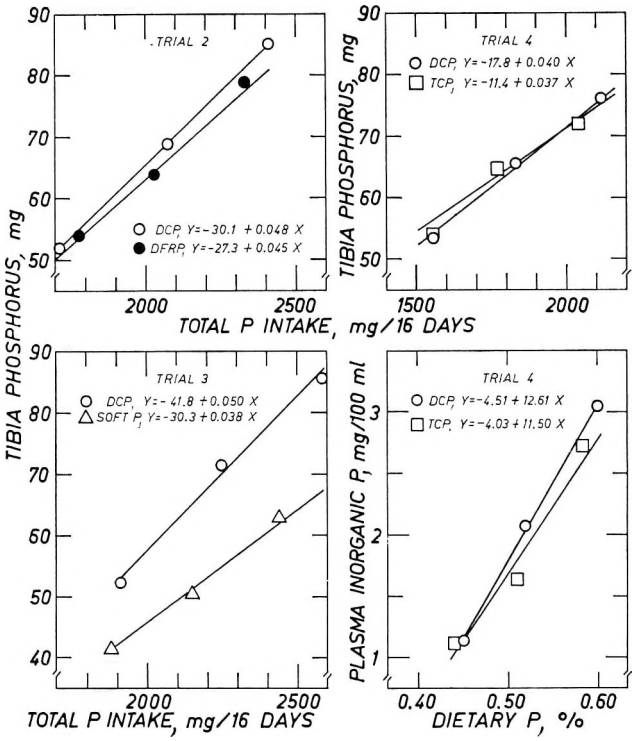


Fig. 3 Tibia phosphorus as a function of total phosphate intake of chicks, fed various phosphorus supplements during a 16-day period of controlled feeding (trials 2–4), and plasma inorganic phosphorus as a function of the percentage of dietary phosphorus (trial 4).

TABLE 4

Percentage of net phosphorus utilization (% NPU) and relative availability of phosphorus from various phosphate supplements for chicks

Trial no.	Supplement	% NPU [1]	Relative availability
2	Dicalcium phosphate, reag. grade	94.1	100.0
	Defluorinated rock phosphate	88.2	95.0
3	Dicalcium phosphate, reag. grade	98.0	100.0
	Soft phosphate	74.5	76.5
4	Dicalcium phosphate, reag. grade	78.4	100.0
	Tricalcium phosphate, reag. grade	72.5	91.8

[1] Per cent NPU is calculated by multiplying the corresponding regression slopes (fig. 3) by a factor of 1960.

onstrated in chicks (10) with increasing levels of dietary calcium. This observation emphasizes the importance of phosphorus analysis in bone, as compared with percentage ash, in studies of phosphorus availability.

In addition to the importance of controlled feed intake in the availability test described here, results indicate a more uniform response to dietary phosphorus by parameters such as tibia phosphorus and plasma inorganic phosphorus, as compared with ad libitum feeding (fig. 3 vs. fig. 2). The importance of controlled feed intake was demonstrated 2 decades ago for studies of phosphorus requirement, availability and deficiency (16–18), but was somehow neglected in later studies.

The results of trials 2–4 indicate that the model described above can be used for the estimation of % NPU. The linearity of the response of tibia phosphorus to total dietary intake of phosphorus, appears to indicate equal phosphorus utilization along the range of dietary phosphorus used in these trials. The % NPU values obtained for dicalcium phosphate (table 4) are similar to the percentage of apparent phosphorus retention determined by Edwards and Gillis (8) who used balance techniques. The "slope" method for phosphorus was also applied for the study of calcium availability,[8] and xanthophyll utilization.[9]

Calculation of the biological availability of phosphorus, on the basis of the percentage of bone ash, according to the method of Gillis et al. (5), showed that defluorinated rock phosphate and soft phosphate were 92 and 63% as available as dicalcium phosphate, reagent grade. These values are somewhat lower than the relative availability calculated from % NPU. This discrepancy, especially in the case of soft phosphate may be due to either a different effect of soft phosphate on percentage bone ash and phosphorus accumulation, or to an inherent difference between both methods.

Plasma inorganic phosphorus was found to be a sensitive indicator of phosphorus nutriture. In trial 1, plasma inorganic phosphorus correlated well with tibia phosphorus, but reached its maximum well above the dietary phosphorus level which gave maximal tibia phosphorus. In trial 4, plasma inorganic phorphorus, when expressed as a function of percentage dietary phosphorus, indicated the relative availability of tricalcium phosphate, reagent grade as compared with dicalcium phosphate, reagent grade, to be 90%, in close agreement with the relative availability determined by % NPU. This observations confirms the results reported by Gardiner (6).

Although the purpose of this study was mainly to test the applicability of the proposed method for measurement of phosphorus utilization, some comment is appropriate concerning the actual phosphorus utilization values determined. As reviewed by Gueguen (19) disagreement exists among the investigators with respect to phosphorus availability from various supplements. This appears to be true for both defluorinated rock phosphate and soft phosphate. Gillis et al. (5) observed high chick mortality with soft phosphate feeding, and that the availability of soft phosphate was only 25% of that of β-tricalcium phosphate. On the other hand, Almquist (20) quotes a value of 80% for the availability of soft phosphate, somewhat higher than the relative value obtained in this study (table 4). This discrepancy may be explained either by a difference in the experimental methods employed, or by the non-uniformity of the samples tested.

To further clarify this variance, it would seem advisable to test a single homogeneous sample in several laboratories simultaneously.

ACKNOWLEDGMENTS

The technical assistance of Mrs. M. Cotter is gratefully acknowledged. The author wishes to thank Dr. J. D. Summers, Ontario Agricultural Experiment Station, for supplying the soft phosphate used in this study.

LITERATURE CITED

1. Krieger, C. H., R. Bunkfeldt and H. Steenbock 1940 Cereals and rickets X. The availability of phytic acid phosphorus. J. Nutrition, *20:* 7.
2. Gerry, R. W., C. W. Carrick, R. E. Roberts and S. M. Hauge 1947 Phosphate supplements of different fluorine content as sources of phosphorus for chickens. Poultry Sci., *26:* 323.
3. Miller, M. W., and V. V. Joukovsky 1953 Availability of phosphorus from various phosphate materials for chicks. Poultry Sci., *32:* 78.
4. Matterson, L. D., E. P. Singsen and H. M. Scott 1945 Rock phosphates as phosphorus supplements for growing chicks. Poultry Sci., *24:* 188.
5. Gillis, M. B., L. C. Norris and G. F. Heuser 1954 Studies on the biological value of inorganic phosphates. J. Nutrition, *52:* 115.
6. Gardiner, E. E. 1962 The relationship between dietary phosphorus level and the level of plasma inorganic phosphorus of chicks. Poultry Sci., *41:* 1156.
7. Ammerman, C. B., H. W. Norton, H. M. Scott and A. H. Nesbit 1960 Rapid assay of in-

[8] Unpublished data, S. Hurwitz and N. T. Rand, 1964.
[9] Unpublished data, I. Bartov and S. Bornstein, 1964.

organic phosphates for chicks. Poultry Sci., 39: 245.
8. Edwards, H. M., Jr., and M. B. Gillis 1959 A chromic oxide balance method for determining phosphate availability. Poultry Sci., 38: 569.
9. Mitchell, H. H. 1962 Comparative Nutrition of Man and Domestic Animals, vol. 1. Academic Press, Inc., New York, p. 242.
10. Hurwitz, S., and P. Griminger 1961 The response of plasma alkaline phosphatase, parathyroids and blood and bone minerals to calcium intake in the fowl. J. Nutrition, 73: 177.
11. Gomori, G. 1942 A modification of the colorimetric phosphorus determination for the use with photometric colorimeter. J. Lab. Clin. Med., 27: 955.
12. Almquist, H. J. 1954 The phosphorus requirement of chicks and poults. A review. Poultry Sci., 33: 937.
13. Motzok, I., D. Arthur and H. D. Branion 1956 Utilization of phosphorus from various phosphate supplements by chicks. Poultry Sci., 35: 627.
14. Kleiber, M., H. Goss and H. R. Guilbert 1936 Phosphorus deficiency metabolism and food utilization in beef heifers. J. Nutrition, 12: 121.
15. Taylor, T. G., and J. H. Moore 1958 The effect of high and low levels of dietary inorganic phosphate on the pre-laying storage of calcium and phosphorus and on the composition of medullary and cortical bone in pullets. Brit. J. Nutrition, 12: 35.
16. Williams, D. E., F. L. MacLeod and E. Morrell 1940 Availability to white rats of phosphorus in Lespedeza sericea and alfalfa hays. J. Nutrition, 19: 251.
17. Watkins, W. E., and H. H. Mitchell 1936 The phosphorus requirements of growing chickens, with a demonstration of the value of controlled experimental feeding. Poultry Sci., 15: 32.
18. Follis, R. H., Jr., H. G. Day and E. V. McCollum 1940 Histological studies of the tissues of rats fed a diet extremely low in phosphorus. J. Nutrition, 20: 181.
19. Gueguen, J. 1961 Valeur comparée des phosphates numéraux comme sources de phosphore pour les animaux. Ann. Zootech., 10: 177.
20. Almquist, H. J. 1962 Soft phosphate in poultry nutrition. Feedstuffs, 34 (11): 62.

LETTERS

IRON DEFICIENCY STUDIES IN CHICKS [1]

In a recent paper (1) we reported washing isolated soybean protein at pH 4.3 with a 0.06% solution of the disodium salt of ethylenediaminetetraacetic acid dihydrate (EDTA) in order to remove or reduce the quantity of trace elements in the protein. After the fourth treatment, the protein was washed 5 times with distilled water. In order to determine if any EDTA remained in the final wash water, 10 ml were added to 5 ml of a saturated solution of ammonium oxalate, the pH adjusted to 11.0 with sodium hydroxide and one drop of a saturated solution of calcium chloride added. A precipitate of calcium oxalate formed which was thought to indicate that the final wash water was free of EDTA.

Following publication of the paper, doubt concerning the sensitivity of the calcium oxalate procedure, used in the investigation, was expressed by a colleague of the authors. He also suggested that some EDTA, because of possible adsorption on the soybean protein, might not have been removed by repeated washing with distilled water.

The calcium oxalate procedure was, therefore, re-examined. By calculation 10 ml of the original EDTA solution contained 6.0 mg of EDTA equivalent on a molecular basis to 0.646 mg calcium. Each drop of the calcium chloride solution at the rate of 20 drops/ml, on the other hand, contained approximately 12.1 mg calcium/drop. As a consequence the calcium content of the calcium chloride solution was much too concentrated to show that the isolated soybean protein was free of EDTA.

In order, therefore, to have a solution of calcium chloride sufficiently dilute to test for traces of EDTA, one containing 30 mg calcium chloride/liter was made up. Each drop of this solution contained 0.5415 μg calcium/drop. This was molecularly equivalent to 5.03 μg EDTA in 10 ml of the wash solution. Since the addition of one drop of the dilute calcium chloride solution to this quantity of wash solution resulted in the formation of a readily observable precipitate of calcium oxalate, it was adjudged that the quantity of EDTA in the solution remaining in the sedimented isolated soybean protein was too small to be of any experimental significance.

In order to ascertain whether some EDTA had been adsorbed on the isolated soybean during treatment and thus not removed by washing with distilled water, three 200 mg samples of protein were washed at pH 4.3 in a solution containing one milligram EDTA/milliliter and sufficient EDTA-2-C^{14} to give 37,600 counts/min/ml. The protein was washed 4 times with 10 ml of the radioactive solution, and 5 times with demineralized water, according to the original procedure. Two of the samples of protein were heated to 50° and washed with shaking. One of these samples was centrifuged and decanted after each washing with the chelate solution, whereas the second sample was allowed to settle and then decanted. The third sample was washed with the EDTA solution without shaking and allowed to settle before decantation. After the washing process, the protein samples were dried at 50°. The C^{14} activity was determined after wet combustion of the sample by the method of Van Slyke et al. (2) by counting the C^{14} as barium carbonate, using a windowless gas-flow counter. Self absorption fac-

[1] Supported in part by grant no. A-5334 from the National Institutes of Health, U. S. Public Health Service.

Letters may be considered for publication when the writer comments constructively concerning a paper that has been published in the *Journal of Nutrition*. Such letters will be subject to the usual editorial review and at the same time, the author or authors of the paper in question will be given the privilege of submitting a rebuttal. Final acceptance of Letters shall be the prerogative of the Editorial Board.

tors were applied to compensate for self absorption errors.

The average activity of the 3 samples was 228 counts/min/200 mg of protein. The average apparent adsorption of EDTA by the protein was accordingly 30.3 µg/g. The range in values was 0.0 to 63.0 µg/g. This variability is believed to be due for the most part to experimental errors inherent in the procedure used and the counting equipment. It seemed probable, consequently, that little EDTA was adsorbed on the protein. The evidence indicates, therefore, that washing isolated soybean protein repeatedly with distilled water to get rid of EDTA after treatment to remove or reduce the quantity of trace elements in the protein is effective.

P. N. Davis
L. C. Norris
F. H. Kratzer
Department of Poultry Husbandry
University of California
Davis, California

LITERATURE CITED

1. Davis, P. N., L. C. Norris and F. H. Kratzer 1962 Iron deficiency studies in chicks using treated isolated soybean protein diets. J. Nutrition, *78:* 445.
2. Van Slyke, D. D., J. Folch and J. Plazin 1940 Manometric carbon determination. J. Biol. Chem., *136:* 509.

The Journal of Nutrition announces the following new editorial policies

LETTERS

Letters may be considered for publication when the writer comments constructively concerning a paper that has been published in the *Journal of Nutrition*. Such letters will be subject to the usual editorial review and at the same time, the author or authors of the paper in question will be given the privilege of submitting a rebuttal. Final acceptance of Letters shall be the prerogative of the Editorial Board.

PAPERS BASED ON NUTRITION LITERATURE

In addition to reports derived from original experimental observations, the Editorial Board will give consideration to papers in which new nutritional concepts are developed from information available in the existing nutrition literature.

ERRATUM

King, D. W. 1964 Comparative effects of certain antioxidants on gestational performance and teratogeny in vitamin E-deficient rats. J. Nutrition, 83: 123. Table 1, column 2, the percentage of dextrin should have read: 49%. Also, in the abstract, text and tables, wherever the term N-propyl gallate appears, this should have read: *n*-propyl gallate.