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Walter Bradford Cannon — A Biographical Sketch

(October 19, 1871 — October 1, 1945)

In his preface to "The Way of an Investigator — a Scientist's Experiences in Medical Research," Walter Cannon quotes William James' view that everybody has as many social selves, or social me's, as there are distinct persons or groups of persons who see him in a particular role. He goes on to describe his book as being about one of the me's in his social complex, his life as a scientific worker — essentially the forty years he spent as a physiologist at the Harvard Medical School. Today, twenty years after his death, we can look with some perspective at the many me's which combined to produce the unified and balanced personality of Walter Cannon — the investigator, the philosopher of biology, the teacher, the servant of his country in war and peace, the statesman of science, the foe of tyranny, the friend of mankind, who was also a devoted husband, a tender father, and a friend of many both in this country and abroad.

Walter Bradford Cannon was born at Prairie du Chien, Wisconsin, October 19, 1871; his ancestry was Scotch-Irish (the name Cannon was a corruption of Carnahan), English and French. Prairie du Chien, incidentally, contains the site of old Fort Crawford where in the 1820's the U. S. Army surgeon, Beaumont, had made his memorable observations on gastric digestion in a subject with a fistula resulting from a gunshot wound. (It is a curious coincidence that Cannon's initial work was the first *in vivo* study of digestion which made use of the newly discovered Roentgen rays.) His father was an inventive railroad engineer; his mother died when he was ten. Just before she died, she called him to her bedside and said to him: "Walter, be good to the world," a wish which he amply fulfilled. Cannon was educated in public schools in Milwaukee and St. Paul, being much interested during his high school years in the controversy carried out by Huxley with the Bishop of Peterborough and with Gladstone. In spite of his family's reduced circumstances, he decided not to attend the University of Minnesota but to go to Harvard, a venture made possible by his securing a series of scholarships. Walter Cannon was a brilliant student; in 1896, he graduated *summa cum laude*, having completed enough graduate courses during his undergraduate years that he could be awarded the degree of Master of Arts at the end of his first year at Harvard Medical School. At the beginning of his fourth year at the Harvard Medical School, he was invited to conduct at Harvard College and at Radcliffe College the course in comparative anatomy of vertebrates (in which he had been student assistant while an undergraduate). At the end of the fourth year when he received the M.D. degree, he was offered an instructorship in that course as well as one in physiology at the Medical School. He accepted the latter; from 1900 to 1902 he had the title of Instructor. From 1902 to 1906 he was Assistant Professor. In 1906, he succeeded Henry P. Bowditch as George Higginson Professor of Physiology, an appointment which continued until he became Professor Emeritus in August, 1942. A year after graduating from Medical School, he married Cornelia James, whom he had known at St. Paul High School and later when she came to Radcliffe as a student. Mrs. Cannon was and is a gifted writer who has written a number of excellent children's books. (If I may be permitted an autobiographical note, I will say that when I was a child and the Cannon's came to visit my parents in Paris, I was much more impressed to meet the author of "Lazaro in the Pueblos" than that of "Bodily Changes in Pain, Hunger, Fear and Rage.")

Doctor Cannon's career as an independent contributor to physiology started on

December 9, 1906, when he was a first-year medical student; with the aid of a primitive x-ray apparatus, he watched pearl buttons pass down the esophagus of a dog and thus was able for the first time in history to study the motor activity of the alimentary tract under conditions uncomplicated by anesthesia or operating procedure. Twenty days later, at the ninth annual meeting of the American Physiological Society, he demonstrated by the same technique the nature of deglutition in the goose.

Cannon and a second-year student, Albert Moser, went on to show that both buccal pressure and esophageal peristalsis were involved in deglutition with the degree to which either factor was predominant dependent on the consistency of the food and on the species. Cannon introduced the use of bismuth salts in radiology and on April 23, 1897, recorded the first observation by means of x-rays of peristaltic waves passing over the stomach content. He went on to describe the adjustment of the stomach to its contained volume, the behavior of the cardia, the origin and course of gastric peristalsis, the influence of the nature of foodstuffs on passage out of the stomach into the duodenum, the rythmic segmentation of the small bowel, and the characteristics of intestinal peristalsis. He extended his studies to those of the mechanisms by which these phenomena were made possible, and was interested in turn in the role of the smooth muscle, the intramural nervous elements and the extrinsic innervation. He extended the significance of Pavlov's discovery of the psychic secretion of gastric juice by showing that there was also a physiologically important psychic tonus of the gastric musculature. His studies of the digestive tract ended with his fundamental demonstration, in 1912, that in man, the pangs of hunger are associated with cramp-like contractions of the gastric wall, an observation which was to give a direction and impetus to the work of Anton Carlson on the role of the stomach in hunger in health and disease.

Doctor Cannon's consideration of the disturbance of digestive processes led him to study the functional significance of the sympathetic nervous system. A number of papers, spread over a period of twenty years, demonstrated that stresses such as pain, rage, fear, asphyxia, hypoglycemia, and exposure to cold led to increased secretion of adrenalin. (The action of this hormone increases the effect of the widespread discharge of sympathetic nerve impulses to produce changes which enable the animal to fight, flee or adapt to the emergency.)

In the process of developing a better assay for adrenal secretion, Cannon developed a new method, based on the denervated heart. He showed that in the lasting preparations, there could be demonstrated a substance which produced cardiac acceleration, but only after the heart had been sensitized by denervation. The eventual result of this observation was the discovery of sympathin. Dr. Cannon and his collaborators showed that this substance is produced in the normal animal, is carried by the blood and may thus act on sympathetically innervated effectors some distance from its origin. The initial work on chemical mediation was extended, in particular in collaboration with Arturo Rosenblueth, and culminated in their writing together a highly useful book entitled, "Anatomic Neuro-Effector Systems."

The factual discoveries of Doctor Cannon, significant as they are, should not obscure his equally important contributions to the philosophy of physiology. His several books and essays on the organization of the body (in particular his book, "The Wisdom of the Body") have had a lasting influence on the thinking of physiologists. His ideas on homeostasis have been and continue to be of such importance that a brief analysis of his concepts is not out of place even in a short essay such as this.

The roots of the theory of homeostasis go back to Claude Bernard and his "Leçons sur les Phénomènes de la Vie" (1878): "All the vital mechanisms, however varied they may be, have only one object, that of preserving constant the conditions of the internal environment which make a free and independent life possible." Charles Richet, a French physiologist who was one of the early winners of the Nobel Prize, further elaborated on his intellectual ancestor's philosophy when he wrote in 1900

in his "Dictionnaire de Physiologie": "The living being is stable. It must be so in order not to be destroyed, dissolved or disintegrated by the colossal forces, often adverse, which surround it . . . In a sense, it is stable because it is modifiable — the slight instability is the necessary condition for the true stability of the organism." Walter Cannon tried to go beyond these general expressions and furnish more specific postulates for what he called at first "biological homeostatics" as well as "homeostasis," and to give specific illustrations of the application of these postulates.

1. "In a biological system, constancy (e.g., homeothermia) is in itself evidence that agencies are acting or are ready to act to maintain this constancy." As examples of such agents, Dr. Cannon cited antagonists, e.g., cardiac nerves; overflow, e.g., by the kidneys; distribution of stimuli, e.g., thirst; magnification of a constant process, e.g., effects of CO_2; structural adjustments, e.g., more erythrocytes at high altitude, etc.

2. "If a homeostatic condition continues, it does so because any tendency towards a change is automatically met by increased effectiveness of a factor or factors which lessen the change" (e.g., increased hepatic release from the liver in hypoglycemia, immediately arrested by intravenous glucose injection).

3. "A homeostatic agent does not act in opposite directions at the same point." Dr. Cannon points out the fact that while insulin may lessen sugar storage in normal animals and increase it in diabetic animals, this type of apparent contradiction is resolved if one considers the very different conditions in which these phenomena take place.

4. "Homeostatic agents, antagonistic in one region of the body, may be cooperative in another region" (e.g., opposition in action of the "sympathico-adrenal factor" and insulin in their action on glycogen deposition in the liver but synergism in the muscles in that "each causes acceleration of metabolism and increased utilization of sugar).

5. "The regulating system which determines a homeostatic state may comprise a number of cooperating factors brought into action at the same time or successively." As an illustration of this postulate, Dr. Cannon cites the successive steps by which the body resists cold and heat both in terms of increasing or decreasing heat production and in terms of decreasing or increasing heat transfer.

6. "When a factor is known which can shift a homeostatic state in one direction, it is reasonable to look for automatic control of that factor or for a factor or factors having an opposite effect." This last "postulate" is essentially a basis for the construction of satisfactory working hypotheses, and Dr. Cannon offered no specific examples of its application; but his own research — particularly that on the action of the sympathetic and the parasympathetic nervous system — offers many illustrations of its fruitfulness.

Dr. Cannon's influence as a teacher was considerable, both because of his considerable gifts and because it was during his lifetime — and largely under his influence that the major development of physiology in America took place. Dr. Henry Pickering Bowditch, whom Dr. Cannon succeeded, had been the first full-time professor of physiology in America. Dr. Cannon conceived his responsibility as a professor to be a mandate to develop a course which would serve as a model for all medical schools in the United States and to train a number of his best students to be the professors of physiology for these schools. He succeeded spectacularly well in both respects. His laboratory manual, which first appeared in 1911, went through many revisions and served as the basis of physiological laboratory teaching throughout the country. As the years went on, the experiments on cats — and on students — used more sophisticated equipment, but all remained demonstrations of the basic facts that physicians should know; at no time was Professor Cannon seduced by facility or the attraction of the easily spectacular nor was he diverted from the most important subject matters. Similarly, Cannon's lectures were erudite yet clear summaries of rapidly expanded knowledge and these too were under constant revision. Dr. Cannon's outstanding success as a teacher was not, however, so much due to his laboratory course or his lectures as to his

gift for attracting young men to his laboratory, to make them feel the joy of research, the excitement of discussion between dedicated investigators. In short, he made them partake of the enthusiasm and the scholarship which he himself brought to his life's work. His simplicity, his kindness, his frankness, the fact that, just as he had done some of his most important work when he was very young, he expected very young men to do important work and thus treated them as important workers — all these traits were important components in the creation of a fertile climate in which young physiologists grew up to become masters in their craft. And in their turn, they became professors in other departments of the Medical School, in other medical schools in the United States, and in countries around the world.

When the United States entered the first World War, Walter Cannon, then forty-six, enlisted and joined the Harvard Hospital Unit, being commissioned a first lieutenant on April 21, 1917. At that time, wound shock was a little-understood problem. As soon as the Unit arrived at the front (at No. 33 Casualty Clearing Station at Béthune, France), Cannon started his studies of this condition; he eventually ascribed it to a combination of acidosis and a reduction of blood volume, and recommended treating it by the intravenous injection of warm alkaline solution. He was commissioned a Captain on August 11, 1917, and became chairman of the National Research Council's Subcommittee on the Physiology of Shock on October 19, 1917 (his committee being essentially on the other side of the Atlantic). A series of papers on shock, published in United States, British and French journals on wound shock during and immediately following the war, and a series of experimental studies on traumatic shock in the three years following the end of the war eventually culminated in the publication of Cannon's important book, "Traumatic Shock." From October 23, 1917, to February 15, 1918, Cannon continued his studies on shock in the Physiological Laboratory of the University of London, collaborating with Professor Bayliss in the examination of the effect of cold on blood pressure in traumatic shock. On February 10, 1918, he was promoted to the rank of Major, and sent to the inter-allied conference on gas warfare (February 15 to April 18) in Paris, where he worked with the two physiologists who had been in charge of the biological aspects of chemical warfare for the Allies, André Mayer and Joseph Barcroft. From April 1 to December 25, 1918, Walter Cannon was placed in charge of a research unit at the Central Medical Laboratory of the U. S. Army in Dijon, France. He was promoted to the rank of Lieutenant-Colonel on October 23, 1918; in 1919, he was made a Commander of the Order of the Bath for "meritorious services to the Allied Cause;" in 1922, he received the Distinguished Service Medal.

During the second World War, Dr. Cannon again became immersed in problems related to shock. As early as the spring of 1940, he became chairman of the committee on blood transfusions and blood substitutes and their use in the treatment of shock of the National Research Council. The work of this committee laid the basis for the gigantic blood procurement program of the American Red Cross, for studies on blood substitutes, procedures for drying blood plasma so as to make it possible to transport it safely all over the world, and studies on the fractionation of blood plasma conducted, in particular, under the direction of his Harvard Medical School colleague, E. J. Cohn.

Doctor Cannon was always interested in international affairs. This interest was kindled by the fact that in his thirty-six years as professor, he had more than fifty foreign students from seventeen different countries doing research in his laboratory. The close bonds which he formed with French, Belgian and British scientists during World War I, his links with European universities (he was exchange professor at the University of Paris during the second half of the 1929–1930 academic year, and was made a *doctor honoris causa* of Liège, Strasbourg, Paris, Madrid and Barcelona), his clear view of the threat to free men everywhere posed by Fascism led him to take the lead in organizing United States help to the Spanish

Republicans. The fact that the head of the hard-pressed Loyalist government, Dr. Juan Negrin, was a physiologist probably gave Dr. Cannon a feeling of kinship with those men who were fighting what should have been clearly recognized as the first round of the defense of Western democracies against the greatest threat they had ever faced. Walter Cannon braved the criticism of isolationists and fascist sympathizers in this country to organize medical help for the Spanish Republican Army and later for the Spanish Republican refugees in France.

Dr. Cannon's visit to the Peiping Union Medical College, as well as the great scholarly interest of his daughter, Wilma, and her husband, John K. Fairbank (now Francis Lee Higginson Professor of History and Director of the East Asian Research Center at Harvard) led him to similarly raise his voice to protest the rape of China by the Japanese and the indifference of this country and of the world to the outrages perpetrated daily by the invaders.

Dr. Cannon, an old friend of Pavlov, visited the Soviet Union on the occasion of the International Physiological Congress and took an extended trip through Russia as a guest of the government. While he was quite conscious of the severe restrictions of freedom imposed on Soviet citizens, he was struck by the considerable progress achieved in fifteen years to change Russia from a backward, almost medieval country to a technologically advanced country; he was impressed by the enthusiasm of Russian scientists and physicians and on his return, accepted the presidency of the newly formed American-Soviet Medical Society. (As the only person then member of both the National Academy of Sciences of the United States and the Academy of Science of the U.S.S.R., he was uniquely qualified to try to establish some contact between the scientists of the two countries.) This effort was going to prove particularly useful when, a few years later, the United States and the Soviet Union became allies during World War II.

In 1942, on the death of his Harvard colleague, L. J. Henderson, Dr. Cannon succeeded him as Foreign Secretary of the National Academy of Sciences. In this position, he devoted particular attention to efforts destined to improve scientific relations with Latin America and to preserve the international scientific unions. Perhaps his last contribution to society was his compilation, with R. M. Field of Princeton, of a report entitled, "International Relations in Science, a Review of Their Aims and Methods in the Past and the Future," which laid the basis for the renewal of activities by the International Scientific Union, and for the work of UNESCO in the field of international scientific collaboration.

Dr. Cannon was a happy man in his family, his friends and his neighbors. His wife was — and still is, at 88 — a most charming, vivacious and liberal person. She could at the same time not only bring up and help educate five busy and lively children, but write books, engage in significant social activities and provide for Dr. Cannon both a peaceful environment and the encouragement that, in spite of his achievements, he sometimes required. She is still at work on a book for children — a travel book on Asia, I am told. Their children all grew up to be interesting, creative persons: Wilma (Mrs. John K. Fairbank), a distinguished Sinologist; Linda Burgess, the director of an important social agency in Washington, D.C.; Marian (Mrs. Arthur C. Schlesinger, Jr.), a gifted painter; Helen (Mrs. Douglas Bond), a prime mover in social reform movements in Cleveland; Dr. Bradford Cannon, a noted plastic surgeon. His sister, Miss Ida Cannon, a trained nurse who also moved from the Middle West to Cambridge, was one of a small band who with Dr. Richard Cabot, started medical social work in this country.

His friends both in the Boston area and in the world at large were numbered in the hundreds if not in the thousands. Dr. Cannon himself, in his autobiography, "The Way of an Investigator," marvels at the number of cities and countries in which he had at least one intimate friend. Certainly, the whole city of Cambridge and the Commonwealth of Massachusetts regarded him as "one of our own," and his simplicity, his good humor and the stories — some apocryphal — of his

absent-mindedness endeared him to his fellow citizens of all walks of life.

Ralph Barton Perry, speaking at memorial exercises for Walter Cannon, pointed out rightly that "his contributions to his age and to posterity were deeply personal" and that the achievements of his life and character were "an answer to much of the cynicism and misanthropy of this age." The example of Walter Cannon, twenty years after his death, continues to be to all of us who work as research men and as teachers, a reminder of the many qualities it takes to make a great man who is also a good man.

JEAN MAYER
Department of Nutrition
Harvard University School of Public Health
Boston, Massachusetts

Unrecognized Amino Acid Deficiencies of Cottonseed Protein for the Chick [1]

HANS FISHER

Nutrition Laboratories, Department of Animal Sciences, Rutgers, The State University, New Brunswick, New Jersey

ABSTRACT A series of studies was carried out with growing chicks fed diets in which 13 or 20% cottonseed protein provided the only protein source. In all cases, the cottonseed protein was supplemented with lysine and methionine, the 2 amino acids which have been known to be limiting in cottonseed protein. It was found that in addition to these two amino acids, cottonseed protein was also limiting in threonine, leucine, and isoleucine. A factorially designed experiment in which all combinations of these 3 amino acids were added, indicated that no single amino acid was first-limiting, that at least 2 amino acids, one of which had to be threonine, must be added to give a growth response. The addition of leucine plus isoleucine depressed growth below that of the control group. All 3 amino acids were necessary for optimal growth expression. Both pressed and solvent-extracted meals were similarly deficient in the 3 amino acids.

We have previously called attention (1) to unsatisfactory results obtained with cottonseed meal in protein evaluation studies with growing chickens by the carcass nitrogen retention method. A marked improvement in growth rate was observed when cottonseed meal was supplemented with lysine and methionine, but net protein utilization (NPU) was improved only slightly. Studies with rats suggested that digestibility was probably not a factor in interpreting these results (1).

According to the amino acid analysis for cottonseed protein reported by Block and Weiss (2), this protein, when supplied at the 20% level, is deficient in leucine, and just meets the National Research Council (3) requirements for threonine and isoleucine.

Grau (4) showed almost 20 years ago that in cottonseed protein lysine was the first and methionine the second limiting amino acid, but that neither threonine nor tryptophan provided additional benefit for the growing chick. Very recently Howe et al. (5) have shown that for growing rats cottonseed protein was equally limiting in threonine and lysine.

The following experiments were carried out with leucine, threonine and isoleucine supplementation of cottonseed meal. The results indicated all three of these amino acids to be limiting in cottonseed protein for the growing chick.

EXPERIMENTAL

Day-old male chicks, from a cross between Columbian females and New Hampshire males, in duplicate lots of eight were fed the experimental diets for 3-week feeding periods. The chicks were kept in electrically heated battery cages and had continuous access to feed and water. The composition of the basic diet was the same as that used in our previous protein evaluation studies (1, 6). Cottonseed protein was the only protein source in these diets and it was usually used at the 13% protein level, except for one experiment where it was also used at the 20% level ($N \times 6.25$). Since the lysine and methionine limitation of cottonseed protein has been well established (4), the basic diet was always supplemented with these 2 amino acids. In experiment 2, in which NPU was determined, the chicks were raised to one week of age with a corn-soybean 22% protein starter ration, and then placed into triplicate groups of 5 chicks for a 2-week period during which they were fed the experimental diets. At the end of this time they were weighed and killed and N was determined on the dried carcasses. NPU values were calculated as follows (6):

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NPU = $(B_f - B_k + I_k)/I_f$ where B_f and I_f denote, respectively, carcass N and N intake of animals fed the cottonseed diet, and B_k and I_k carcass N and N intake of those fed the N-free control diet.

RESULTS

Table 1 shows the results of 2 experiments with one source of cottonseed meal (41.3% protein) which show that cottonseed protein supplemented with lysine and methionine is further improved through addition of 0.2% each of L-leucine, L-isoleucine and L-threonine. Experiment 2 also shows an improvement in the NPU value due to the triple amino acid addition. Table 2 gives results obtained with 2 samples of cottonseed meal different than that used in the studies described in table 1. Both the pressed (43.2% protein) and solvent-extracted (43.7% protein) meals responded to threonine, leucine, and isoleucine supplementation beyond that obtained from lysine and methionine addition (table 2). This table further shows that the deficiency also existed when cottonseed protein was provided at the 20% level.

Table 3 gives the results of a factorially designed experiment with the 3 amino acids added to a 13% cottonseed protein diet (supplemented with methionine and

TABLE 1

Body weight, feed utilization and net protein utilization of chicks fed amino acid-supplemented or unsupplemented cottonseed meal

Amino acid supplement [1]	3-Week period		Net protein utilization
	Body wt gain	Feed utilization	
	g	*g gain/g feed consumed*	%
Experiment 1			
None	162 ± 8 [2]	0.36	
L-Leucine, 0.2% + L-threonine, 0.2% + L-isoleucine, 0.2%	188 ± 8	0.40	
Experiment 2			
None	169 ± 8 [3]	0.34	46.7
L-Leucine, 0.2% + L-threonine, 0.2% + L-isoleucine, 0.2%	205 ± 6	0.41	50.3

[1] Added to a diet providing 13% (N × 6.25) cottonseed protein supplemented with 0.4% L-lysine·HCl and 0.2% DL-methionine.
[2] Mean value ± SE for duplicate groups of 8 male chicks.
[3] Mean value ± SE for triplicate groups of 5 male chicks.

TABLE 2

Body weights and feed utilization of chicks fed differently processed cottonseed meal, with or without amino acids supplementation

Protein source and level [1]	Amino acid supplement [2]	3-Week period	
		Body wt gain	Feed utilization
		g	*g gain/g feed consumed*
Pressed cottonseed			
13%	−	122 ± 7 [3]	0.30
13%	+	160 ± 7	0.32
Solvent-extracted cottonseed			
13%	−	148 ± 8	0.35
13%	+	169 ± 4	0.42
20%	−	196 ± 9	0.46
20%	+	220 ± 10	0.48

[1] Supplemented with 0.5% L-lysine·HCl and 0.3% DL-methionine.
[2] Provided: 0.2% L-leucine, 0.2% L-isoleucine, and 0.2% L-threonine.
[3] Mean value ± SE for duplicate groups of 8 male chicks.

TABLE 3

Body weights and feed utilization of chicks fed a diet containing 13% cottonseed protein plus addition of lysine and methionine, factorially supplemented with leucine, isoleucine and threonine

Amino acid supplement [1]	3-Week period	
	Body wt gain	Feed utilization
%	g	*g gain/g feed consumed*
None	140 ± 8 [2]	0.32
L-Leucine, 0.2	126 ± 7	0.37
L-Threonine, 0.2	135 ± 6	0.36
L-Isoleucine, 0.2	122 ± 7	0.32
L-Leucine, 0.2 + L-threonine, 0.2	155 ± 6	0.39
L-Leucine, 0.2 + L-isoleucine, 0.2	127 ± 8	0.32
L-Threonine, 0.2 + L-isoleucine, 0.2	166 ± 8	0.38
L-Leucine, 0.2 + L-isoleucine, 0.2 + L-threonine, 0.2	175 ± 5	0.40

[1] Added to a diet providing 13% (N × 6.25) cottonseed protein supplemented with 0.4% L-lysine·HCl and 0.2% DL-methionine.
[2] Mean value ± SE for duplicate groups of 8 male chicks.

lysine) in all possible combinations. Of the single additions, leucine and isoleucine both caused a growth depression, in comparison with the unsupplemented diet, whereas threonine exerted no effect on growth rate. The combination of leucine plus threonine improved growth: threonine plus isoleucine was still more effective than leucine plus threonine, but the addition of all 3 amino acids appeared necessary for optimal utilization of cottonseed protein supplemented with lysine and methionine.

DISCUSSION

That cottonseed protein was found to be deficient for the chick in leucine, threonine, and isoleucine suggests that other plant proteins that have not been fully explored might also be studied for possible undetected amino acid deficiencies. Although a leucine deficiency of cottonseed protein appeared plausible, based on the known amino acid composition (2), that of the other 2 amino acids, threonine and isoleucine, was not obvious. Results obtained recently with peanut meal (7) indicated a major deficiency for this protein in threonine that was not obvious when related to amino acid composition.

The results presented in table 2 indicate that the newly observed deficiencies are not influenced by the 2 types of processing used currently in preparing commercial cottonseed meal. The results of the last experiment (table 3) point up the difficulties in tracing amino acid deficiencies, particularly if they are multiple in character. This is well illustrated by the fact that Grau (4), who studied threonine supplementation of cottonseed meal, missed its importance because he was unaware of the leucine and isoleucine deficiencies.

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Interrelationship of Plasma Amino Acid Levels and Weight Gain in the Chick as Influenced by Suboptimal and Superoptimal Dietary Concentrations of Single Amino Acids

R. A. ZIMMERMAN AND H. M. SCOTT
Department of Animal Science, University of Illinois, Urbana, Illinois

ABSTRACT The concentration of either lysine, arginine or valine was varied over a wide range, in a basal diet containing crystalline amino acids in order to study the relationship between weight gain and the concentration of free amino acids in the blood plasma of chicks. It was shown that the first-limiting amino acid in the diet remains at a very low and constant level in the blood irrespective of the severity of the deficiency. Increments in excess of the amount needed to maximize weight gain resulted in a rapid and linear accumulation of that amino acid in the blood. Since the point at which the amino acid starts to accumulate in the plasma coincides with the break in the growth curve, it is concluded that the plasma technique can be used to determine the chick's requirement for amino acids.

Although assay procedures used in plasma amino acid studies have varied considerably, the results obtained have been quite consistent in demonstrating that dietary amino acid deficiencies result in reduced plasma concentration of that amino acid (1–3),[1,2] whereas dietary amino acid excesses have resulted in accumulation of that amino acid in the plasma (4–7).[3]

However, it has not been possible to characterize the plasma amino acid response curve since in most instances only 2 dietary levels of an amino acid have been fed. The data of Morrison et al. (8) suggest that lysine does not accumulate in the plasma of growing rats when lysine is the growth-limiting amino acid in the diet. However, it has been demonstrated that plasma lysine accumulates in a linear manner when the concentration of dietary lysine is moderately in excess of that required to maximize growth in the turkey poult (6) and the growing rat (8), respectively.

It appears that when the growth of a young animal is restricted by an amino acid deficiency, the need of the tissues for that amino acid tends to maintain that nutrient at a minimal level in the blood. The present investigation was undertaken, therefore, to examine the effect of varying the dietary levels of single amino acids (lysine, arginine and valine) on the concentration of these amino acids in the blood plasma of growing chicks and to relate these observations to the growth-promoting ability of the experimental diets. Data were also obtained on the plasma pattern of amino acids other than the one varied in the diet.

EXPERIMENTAL

Male chicks, carefully selected from a large population for uniformity in initial weight after having been fed a pretest diet for seven or more days, were used in all experiments. Pertinent information concerning pretest diets, care and selection of experimental chicks has been described elsewhere (9).

The composition of the basal diet and the amino acid mixtures used are shown in tables 1 and 2, respectively. Except for methionine (DL-) only the L-isomer was used. Amino acid mixture A [4] was used in

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[3] See footnote 1.

[4] See footnote 1.

TABLE 1

Composition of basal diet

	% of diet
Cornstarch	variable
Amino acid mixtures	variable [1]
Corn oil, refined	15.00
Salt mixture [2]	5.37
Cellulose [3]	3.00
$NaHCO_3$	1.00
Choline chloride	0.20
Vitamins [4]	+
Total	100.00

[1] See experimental section and table 2.
[2] Mineral mixture, as per cent of the total diet: $CaCO_3$, 0.3; $Ca_3(PO_4)_2$, 2.8; K_2HPO_4, 0.9; $MgSO_4 \cdot 7H_2O$, 0.35; ferric citrate, 0.05; $ZnCO_3$, 0.01; KI, 0.004; $CuSO_4 \cdot 5H_2O$, 0.002; H_3BO_3, 0.0009; $CoSO_4 \cdot 7H_2O$, 0.0001; $MnSO_4 \cdot H_2O$, 0.065; NaCl, 0.88; $Na_2MoO_4 \cdot 2H_2O$, 0.0009; total 5.37.
[3] Solka Floc, Brown Company, Chicago 3, Illinois.
[4] G. J. Klain, H. M. Scott and B. C. Johnson, Poultry Sci., 39: 39, 1960.

TABLE 2

Composition of crystalline amino acid mixtures

Amino acid	Mixture A	Mixture B
	% contributed to the diet	
Arginine [1]	1.10	1.00
Histidine [1]	0.30	0.30
Lysine [1]	1.12	0.95
Tyrosine	0.63	0.45
Tryptophan	0.225	0.15
Phenylalanine	0.68	0.50
Methionine	0.55	0.35
Cystine	0.35	0.35
Threonine	0.65	0.65
Leucine	1.20	1.20
Isoleucine	0.80	0.80
Valine	0.82	0.82
Glycine	1.60	1.20
Proline	1.00	0.20
Glutamic acid	12.00	10.00
Total	23.025	18.92

[1] Supplied as arginine·HCl, histidine·HCl·H_2O and lysine·HCl.

experiment 5, and mixture B in experiments 2, 3, and 4. In experiments 1 and 6, mixture B, slightly modified to provide arginine, glycine and glutamic acid at the concentration indicated in mixture A was used. Access to feed and water was permitted at all times.

Blood samples were taken by heart puncture at the termination of the experiments immediately after obtaining final chick weights, using a syringe previously washed with a heparinized (0.6 mg/ml) 0.93% NaCl solution. The blood was then transferred to a heparinized (0.2 mg/ml blood) centrifuge tube and stored in an ice bath until it was centrifuged. In experiments 1, 5, and 6, plasma from 8 chicks was pooled, and plasma from 3 chicks was pooled in experiments 2, 3, and 4. After centrifuging the pooled plasma sample, a 10-ml aliquot was taken for analysis. The protein was precipitated with picric acid and further prepared for analysis as described by Stein and Moore (10). Two milliliters of the resultant 10-ml protein-free plasma were then added to the columns of a Beckman-Spinco Automatic Amino Acid Analyzer for amino acid analysis.

Where possible, the data for weight gain (gain/chick/day) and plasma amino acid (μg/ml) were analyzed statistically by the method of least squares.

RESULTS

The relationship of weight gain and plasma lysine to dietary lysine for chicks at different stages of growth [5] (exps. 1–4) are presented in tables 3 and 4. In the first experiment (table 3) the plasma lysine profile indicates that this amino acid did not accumulate in the plasma so long as the concentration of dietary lysine was less than that needed (0.83%) to maximize weight gain. Not until lysine levels in excess of 0.94% were fed did this amino acid start to accumulate rapidly in the plasma. This accumulation appears to proceed in a linear manner once the dietary requirement has been met.

In the older and larger chicks also, there was no suggestion that lysine would accumulate in the plasma when the concentration of dietary lysine was less than that needed to maximize weight gain (table 4). In general, lysine started to accumulate in the plasma at a rapid rate whenever the level of dietary lysine was approximately 10% in excess of that required to maximize weight gain.

Tables 5 and 6 record the results noted when the dietary concentration of valine and arginine were varied in a manner comparable to that described for lysine. In these instances also, the plasma pattern

[5] The experimental diets were fed over the period of 8 to 15 days (70), 15 to 21 days (157), 22 to 28 days (278) and 29 to 33 days (424) in experiments 1, 2, 3 and 4, respectively. The numbers in parentheses represent the average chick weight (g) of all experimental groups at the start of each assay. In both experiments 5 (valine assay) and 6 (arginine assay) the experimental diets were fed for 7 days extending from the 8th to the 15th day. The initial starting weights were 73 and 77 g for experiments 5 and 6, respectively.

TABLE 3

Effect of feeding graded levels of lysine on plasma amino acids and weight gain (exp. 1)

Amino acid	% Dietary lysine							
	0.60	0.70	0.80	0.90	1.00	1.20	1.40	1.80
	μg of amino acid/ml plasma							
Threonine	141.4	192.1	108.8	82.8	47.6	37.0	44.8	126.1
Proline	25.2	24.4	22.0	22.4	25.8	22.2	22.0	19.5
Glutamic acid	44.4	58.4	44.3	66.2	73.5	32.3	42.8	27.7
Glycine	108.0	97.1	73.8	84.1	125.1	60.4	78.1	64.8
Valine	31.9	31.0	29.3	20.4	16.9	15.2	15.1	22.7
Cystine	21.2	—	32.7	25.6	—	30.4	26.4	33.2
Methionine	17.2	17.4	15.5	14.2	13.5	10.6	12.9	12.4
Isoleucine	17.1	14.6	13.5	9.2	8.8	8.2	9.8	12.0
Leucine	21.3	21.3	17.2	14.6	12.6	10.7	13.1	14.6
Tyrosine	23.9	17.3	13.9	9.7	5.4	—	4.7	11.2
Phenylalanine	15.5	14.5	12.4	9.1	4.9	—	4.7	8.4
Histidine	21.2	15.9	10.4	5.0	4.1	1.7	3.4	10.2
Arginine	66.0	69.7	59.5	61.8	45.2	25.0	25.3	6.1
Lysine	6.8	9.7	7.2	15.5	23.1	65.7	106.7	233.7
Gain/chick/ day, g [1]	8.2	11.1	12.2	13.6	12.8	12.9	13.2	10.7
Gain/feed	0.57	0.64	0.68	0.74	0.75	0.77	0.73	0.64

[1] Average of duplicate groups of 5 chicks/treatment. See footnote 5 in text for data concerning initial starting weight and duration of assay.

TABLE 4

Effect of feeding graded levels of lysine on plasma lysine and weight gain

Dietary lysine	Experiment 2		Experiment 3		Experiment 4	
	Plasma lysine	Gain/ chick/ day [1]	Plasma lysine	Gain/ chick/ day [1]	Plasma lysine	Gain/ chick/ day [1]
%	*μg/ml*	*g*	*μg/ml*	*g*	*μg/ml*	*g*
0.4	7.5	6.3	5.4	10.8	5.7	15.5
0.5	9.8	10.5	4.8	15.8	4.7	20.5
0.6	7.8	14.5	4.4	23.0	6.2	29.3
0.7	6.2	19.8	9.9	26.8	20.4	29.0
0.8	9.4	19.8	18.2	27.2	15.9	29.5
0.9	27.8	19.2	44.4	27.0	26.5	28.0
1.0	—	—	—	—	62.4	32.5

[1] Average of triplicate groups of 3 chicks/treatment. See footnote 5 in text for data concerning initial starting weight and duration of assay.

for valine and arginine reflect the same basic characteristics as observed for lysine.

DISCUSSION

From this series of experiments, it has been shown that there is a definite relationship between plasma amino acid levels and the amino acid adequacy of the diet. The first-limiting amino acid remains at a very low level in the blood irrespective of the severity of the amino acid deficiency ("imbalance"). This observation helps to explain why in some instances, supplementing diets with the first-limiting amino acid has failed to increase the concentration of this amino acid in the plasma (4, 11). According to observations noted herein, no increase would be anticipated until the dietary level exceeded that needed to maximize growth.

The presence of an amino acid in the diet, in excess of that required to maximize weight gain, consistently resulted in a rapid accumulation of that amino acid in the plasma. This accumulation appears to proceed in a linear manner, even though, as was the case for lysine (table 3) and valine (table 5), the greatest dietary concentration used was more than twice that required to maximize weight gain. More-

TABLE 5

Effect of feeding graded levels of valine on plasma amino acids and weight gain (exp. 5)

Amino acid	% dietary valine							
	0.57	0.67	0.72	0.77	0.82	0.87	1.07	1.82
	μg of amino acid/ml plasma							
Threonine	78.2	65.0	46.0	35.2	36.0	40.6	27.3	49.5
Proline	40.5	46.2	36.5	38.4	34.1	44.7	30.3	48.7
Glutamic acid	35.6	36.4	36.2	43.4	39.7	44.7	22.0	42.6
Glycine	88.0	93.9	75.8	73.8	56.7	94.8	44.4	76.2
Cystine	25.3	27.0	33.0	32.2	34.5	29.7	44.6	29.6
Methionine	15.3	18.0	16.6	13.9	13.4	18.9	14.7	18.7
Isoleucine	14.2	13.9	10.8	10.3	7.4	10.7	8.0	9.5
Leucine	21.7	17.3	13.5	13.7	10.6	12.9	9.8	10.7
Tyrosine	30.6	25.8	24.4	25.9	21.1	27.1	18.7	21.0
Phenylalanine	16.6	12.6	10.3	11.0	9.1	10.6	8.9	8.2
Lysine	39.9	60.7	46.4	40.5	34.3	24.5	19.0	65.0
Histidine	3.8	5.6	2.2	2.4	0.4	0.6	nd [1]	4.3
Arginine	—	38.9	23.8	21.2	16.5	—	19.1	26.8
Valine	6.3	9.1	6.3	10.7	10.6	19.2	35.7	127.7
Gain/chick/day, g [2]	6.6	11.1	12.4	13.7	14.1	13.3	13.9	13.6
Gain/feed	0.57	0.72	0.76	0.78	0.79	0.76	0.78	0.76

[1] None detectable.
[2] Average of duplicate groups of 5 chicks/treatment. See footnote 5 in text for data concerning initial starting weight and duration of assay.

TABLE 6

Effect of feeding graded levels of arginine on plasma amino acids and weight gain (exp. 6)

Amino acid	% dietary arginine					
	0.60	0.70	0.80	0.90	1.00	1.10
	μg of amino acid/ml plasma					
Threonine	217.7	164.7	123.1	79.5	81.4	65.6
Proline	30.8	32.0	24.0	26.5	23.7	25.6
Glutamic acid	55.1	102.4	103.1	117.5	87.6	117.4
Glycine	98.1	86.3	134.3	128.3	125.3	86.9
Valine	39.8	35.7	28.6	24.1	28.7	22.6
Cystine	38.5	22.1	21.5	19.9	15.4	20.5
Methionine	16.6	13.4	17.6	14.2	17.0	13.3
Isoleucine	23.3	22.9	19.1	14.7	18.0	13.9
Leucine	31.9	28.7	21.8	19.3	23.0	18.5
Tyrosine	—	20.8	14.2	9.7	10.3	8.0
Phenylalanine	—	15.5	12.0	10.7	12.1	9.2
Lysine	109.2	97.0	65.4	39.3	37.5	30.8
Histidine	21.7	20.0	14.1	5.7	6.3	5.8
Arginine	4.8	7.9	23.1	11.4	39.5	46.0
Gain/chick/day, g [1]	9.9	11.3	12.4	13.6	13.8	13.8
Gain/feed	0.54	0.59	0.65	0.69	0.72	0.72

[1] Average of triplicate groups of 5 chicks/treatment. See footnote 5 in text for data concerning initial starting weight and duration of assay.

over, in the case of lysine, this amino acid continued to accumulate linearly in the plasma, even though growth was depressed 20% by the highest (1.8%) level fed. Exceedingly high plasma amino acid levels have been reported by others (5) [6] under similar conditions.

That the shape of the plasma amino acid curve can be used to determine the amino acid requirement of the chick has been demonstrated in these studies. More recently, Mitchell [7] has reported good agreement between data acquired by the plasma technique and nitrogen balance in deter-

[6] See footnote 1.
[7] Mitchell, J. R., Jr. 1965 Effect of level of amino acid intake on the nitrogen metabolism and plasma free amino acids of the young pig. Doctoral Thesis, University of Illinois, Urbana.

mining the young pig's requirement for several amino acids.

From the data obtained in this series of experiments, it was possible to calculate the chick's requirement for lysine at successive stages of development. Analysis of the data by the method of least squares indicates that the lysine requirement, expressed as a percentage of the diet, was 0.83, 0.70, 0.67 and 0.59% for the second, third, fourth and fifth week of life, respectively.

In experiments 1, 5 and 6, plasma samples were analyzed for both essential and non-essential amino acids (tryptophan excepted) but only data concerning the amino acids provided in the experimental diets are presented in the tabulations. In experiments 2, 3 and 4 the plasma was analyzed for lysine only. Severe deficiencies of either lysine or arginine markedly increased plasma threonine. Plasma threonine varied from a high of approximately 190 to a low of 40 μg/ml in the lysine assay (table 3) and from 217 to 65 μg/ml in the arginine experiment (table 6). Each increment of the first-limiting amino acid (lysine or arginine) resulted in a progressive decline in plasma threonine. However, the point where plasma threonine reaches its lowest concentration does not coincide with the dietary concentration of the first-limiting amino acid that maximizes weight gain, but rather at a level somewhat in excess of that needed for most rapid weight gain. However, this does not appear to be unique for threonine since many of the other amino acids also behave in this manner although obviously to a lesser degree. It follows also that the rapid decline in plasma threonine concentration should not be interpreted as indicating that it is the next limiting amino acid. Large excesses of dietary lysine or valine also increased the concentration of plasma threonine. Others (2, 3, 7) [8] have shown that amino acid imbalances will influence the concentration of threonine in the plasma. Lysine also appears to accumulate in the plasma to a marked degree when the diet is deficient in arginine (table 6). There is a suggestion that plasma arginine tends to accumulate in the plasma when the diet contains suboptimal amounts of either lysine or valine. As for the other amino acids, it appears that histidine, tyrosine, phenylalanine, leucine and isoleucine tend to accumulate in the plasma to a slight degree irrespective of whether lysine, arginine or valine were deficient whereas proline, glutamic acid, glycine, methionine and cystine were, for the most part, either unaffected by the treatments imposed, or were so variable as to preclude the establishment of a definite plasma pattern. Dean,[9] however, has reported that all amino acids (except lysine) tend to accumulate in the plasma when chicks are fed diets grossly deficient in lysine. In general, the observations noted herein support the view that diets containing well balanced protein would tend to minimize the accumulation of free amino acid nitrogen in blood plasma.

In experiment 1 where the greatest amount of lysine fed (1.8%) exceeded the requirement level approximately twofold, several amino acids tended to accumulate in the plasma. A notable exception was arginine, whose concentration continued to decrease (table 3), and the possibility should be considered whether this observation is in some way related to the demonstrated ability of arginine to reverse the growth-depressing effect of excess dietary lysine (12). Furthermore, the data presented herein appear to indicate that variations in the concentration of dietary lysine or arginine exert a more pronounced effect on the plasma concentration of other amino acids than does valine which may reflect the difference in "relative toxicity" among amino acids for the chick (13).

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[8] See footnote 1.
[9] See footnote 1.

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Protein Utilization in Growing Rats at Different Levels of Intake [1,2]

D. M. HEGSTED AND YET-OY CHANG
Department of Nutrition, Harvard School of Public Health, Boston, Massachusetts and the Division of Biochemistry, University of Wyoming, Laramie, Wyoming

ABSTRACT Four different proteins, lactalbumin, casein, soy protein and wheat gluten, were fed to groups of weanling rats by diets supplying 7 different levels of each protein for a 3-week test period. It is concluded that the net nitrogen utilization is essentially constant for each of these proteins at all levels of intake up to those which will support approximately maximal growth. The relative nutritive value of casein, soy protein, and wheat gluten appear to be 0.7, 0.34, and 0.14 relative to lactalbumin. When the intake of each of these proteins was multiplied by the appropriate factor to yield "lactalbumin equivalents," the performance of all proteins per lactalbumin equivalent appears to be the same.

It is well known that the utilization of dietary proteins is maximal at low levels of intake. Biological values by the Thomas-Mitchell method (1) are measured at limited intakes to assure maximal values (2). On the other hand, when the intake of protein exceeds the requirement, the efficiency of utilization must decrease rapidly since protein cannot be stored in the body to any appreciable extent. The utilization of protein at intermediate levels of intake, between mere maintenance and optimal growth, has not been studied adequately and has led to divergent conclusions. Perhaps the best data available, from Barnes et al. (3), appear to lead to an indeterminate conclusion, although as one of us has discussed elsewhere (4, 5) the data suggest that utilization may be maximal and essentially constant up to levels which approach optimal growth. Miller and Payne (6, 7), however, suggest that the net utilization of dietary protein falls linearly at levels of intake above the amount required for maintenance. They conclude that the regression line relating utilization and the amount of protein in the diet is such that it reaches zero for all proteins when approximately 54% of the calories are supplied as protein. Morrison et al. (8) interpret their data to mean that the decrease in utilization is proportional to the logarithm of the level of protein in the diet.

It is extremely important to know what happens to protein utilization in the growing animal at levels of intake above those required for maintenance. All recent estimates of the protein needs of children (9–11) assume that the biological value of the dietary protein is a constant, applicable to children and adults alike. On the other hand, if the thesis of Miller and Payne is correct and utilization is much less efficient as the intake approaches that needed for maximal growth, these estimates of protein needs would be grossly inadequate and need revision.

We have investigated this problem utilizing 4 proteins which vary greatly in nutritive value. We are unable to confirm the conclusions of Miller and Payne.

EXPERIMENTAL

The experimental procedures and the design of the experiments were described previously (12). Each of 4 proteins, lactalbumin, casein, soy protein, and wheat gluten, was fed at 7 different levels of dietary protein. There were 5 or 6 weanling male rats per group and the test period was 3 weeks. Food consumption

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[2] The second paper in a series: Protein Utilization in Growing Rats.

was measured and total body nitrogen was determined at the end of the test period. At the start of each experiment a group of 4 or 5 animals was killed to determine the body nitrogen at zero time. An additional group of animals received the nitrogen-free diet for 3 weeks and their body nitrogen was then determined. The 4 proteins were tested at different times during the year.

RESULTS

Many authors have demonstrated that the body nitrogen content is approximately proportional to body weight in young rats over the first few weeks of life. In figure 1 the relationship between body weight and total body nitrogen is shown for the animals that received each protein. The lowest values on each line represent those obtained for the animals that received the nitrogen-free diet. These values serve to stabilize the regression lines at the lower end. Two values for the casein-fed animals and one value for the gluten-fed animals appeared grossly abnormal and were arbitrarily omitted. When the regression lines were calculated, the regression line for the animals that received soy protein fell much below the zero intercept. We concluded that for reasons unknown the group which received the lowest level of soy protein was not analyzed correctly and these values were also omitted. The regression lines then obtained are shown in figure 1. These lines appear to be strictly linear and do not depart significantly from zero at the intercept. The regression coefficients are measures of the average nitrogen content, i.e., in the animals fed lactalbumin 0.029 (2.9%) of the body weight was nitrogen.

Covariance analysis showed that the slopes of the regression lines were signifi-

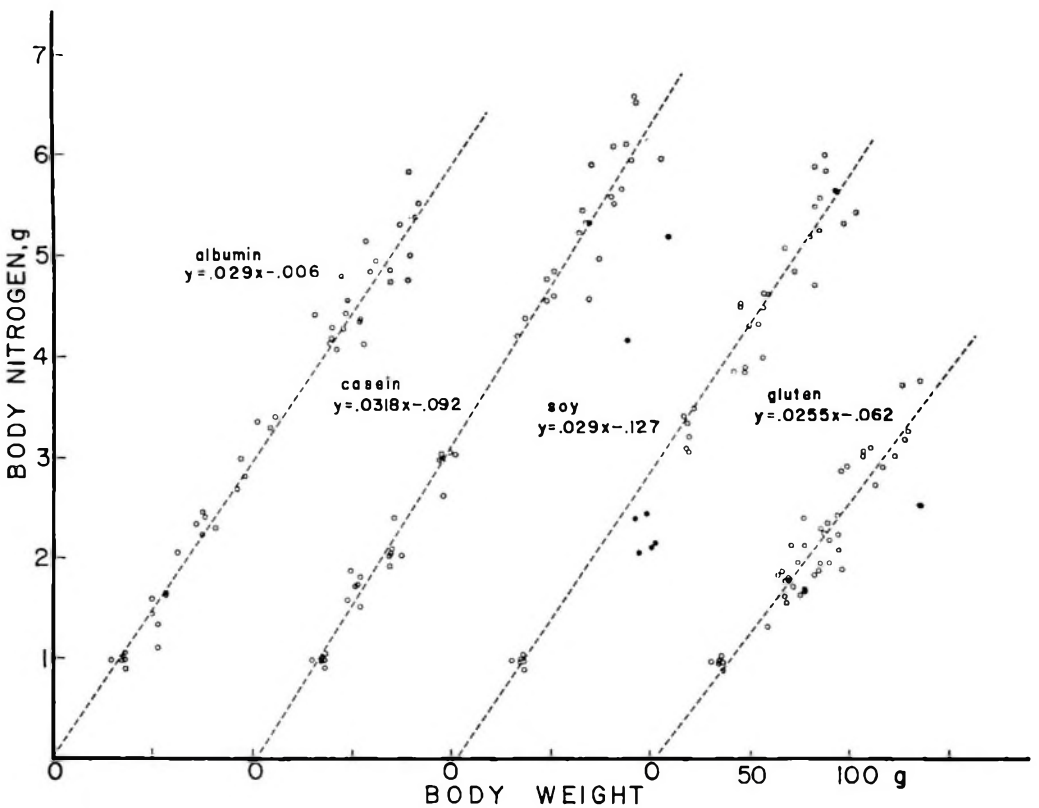

Fig. 1 The relationship between body weight and total body nitrogen in animals fed at varying levels of 4 proteins. As explained in the text, the values indicated by the solid circles were omitted in calculating the regression lines.

cantly different ($P < 0.005$). The mean slope obtained for the combined data was 0.0300 indicating an average body nitrogen content of 3.00 %. The gluten-fed animals had approximately 85% of this value, 2.55% body nitrogen, whereas the casein-fed animals had slightly higher values, 3.18% nitrogen. The low value for the gluten-fed animals appears to confirm the observations of Allison (13) that less nitrogen is stored per unit body weight than in animals fed higher quality proteins. Whether the slightly higher value for casein is of any practical significance is unknown.

Our previous paper (12) showed that the relative nutritive value of these 4 proteins based upon body weight gains was 100, 70, 34, and 22 for lactalbumin, casein, soy protein, and wheat gluten, respectively. Had body nitrogen rather than body weight been used as the parameter of growth, these values would have been changed in proportion to the regression coefficient in figure 1. The nutritive value of casein would have been increased slightly, relative to albumin, and that of gluten decreased. The values thus corrected would be 100, 76, 34, and 18.7, respectively.

If such indexes are valid estimates of the nutritive value of the proteins as compared with lactalbumin, the intake of each protein should be convertible into "lactalbumin equivalents" by multiplication of the actual intake by the appropriate factor. Thus, the intake of soy protein nitrogen would be multiplied by 0.34 to yield the equivalent amount of lactalbumin nitro-

TABLE 1

Mean nitrogen intake, lactalbumin equivalents and net nitrogen utilization at varying levels of dietary protein

Protein and relative growth index [1]	Dietary protein	Total N intake	N intake (lactalbumin N equivalents)	Body N stored [2]	Net N utilization [3]
	%	g	g	g	g
Lactalbumin (100)	2.21	0.40		0.085	0.421
	3.69	0.99		0.924	1.260
	5.16	1.81		1.722	2.058
	7.38	3.43		3.185	3.521
	9.59	4.07		3.025	3.361
	11.80	4.84		3.265	3.601
	14.75	6.48		3.692	4.028
Casein (70)	3.47	0.57	0.40	0.013	0.349
	6.08	1.19	0.84	0.393	0.729
	8.69	2.38	1.67	1.264	1.600
	12.16	4.72	3.31	2.884	3.220
	15.64	6.25	4.37	3.664	4.000
	19.11	7.42	5.20	3.958	4.294
	26.06	11.47	8.00	4.041	4.377
Soy protein (34)	8.51	3.28	1.12	0.326	0.662
	11.92	5.17	1.76	1.373	1.709
	15.32	7.87	2.67	1.916	2.252
	18.73	9.78	2.94	2.551	2.887
	25.54	12.90	3.32	2.926	3.262
	34.05	17.33	5.90	3.670	4.006
	42.56	20.90	7.10	3.654	3.990
Wheat gluten (18.7)	11.38	2.36	0.44 (0.33) [4]	0.296	0.632
	14.63	3.56	0.66 (0.50)	0.147	0.483
	17.88	4.63	0.86 (0.65)	0.481	0.817
	21.13	6.14	1.15 (0.86)	0.779	1.115
	24.38	7.45	1.39 (1.04)	0.525	0.861
	32.50	12.74	2.38 (1.78)	1.628	1.994
	40.63	15.05	2.81 (2.11)	1.445	1.781

[1] See Hegsted and Chang (12).
[2] Average body nitrogen minus body nitrogen of animals killed at the start of the experiment.
[3] Body nitrogen stored plus nitrogen lost by animals fed a nitrogen-free diet (0.336 g).
[4] Lactalbumin equivalents if gluten is considered to have a relative value of 14 rather than 18.7 (see text).

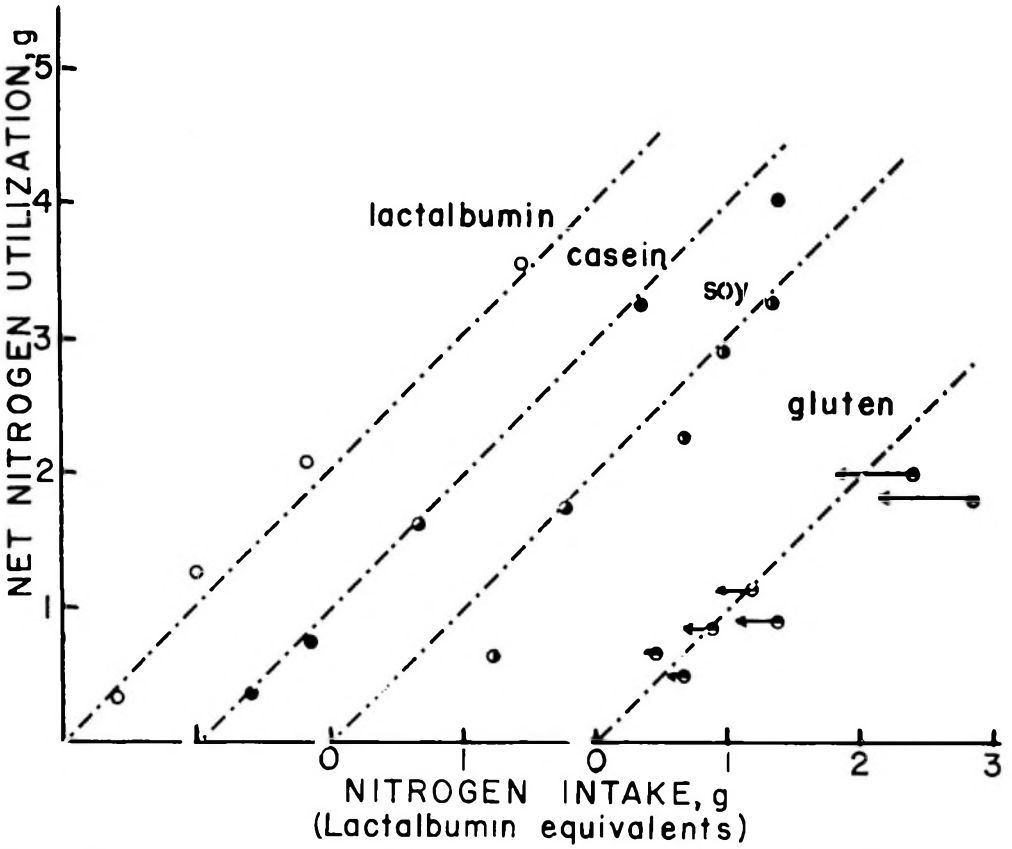

Fig. 2 The net nitrogen utilization in animals fed at the lower levels of each protein expressed as "lactalbumin nitrogen equivalents." The lines represent 100% utilization. The arrows on the gluten values show their position when gluten is considered to have 14% of the value of lactalbumin rather than 18.7%.

gen. The mean nitrogen intake of each group of animals and the "lactalbumin equivalent" is shown in table 1. The net nitrogen utilization is also shown. This represents the difference between the body nitrogen at the termination of the experiment and the start plus the amount of nitrogen lost by the animals that received the nitrogen-free diet.

In figure 2 the net nitrogen utilization of those groups of animals that received the lower levels of protein are shown plotted against the "lactalbumin nitrogen equivalents." The lines have been drawn to represent maximal utilization, i.e., where the nitrogen utilization is equal to the "lactalbumin equivalent." The data show that the net nitrogen utilization of lactalbumin was essentially complete and constant for the 4 levels shown. It appears therefore to be a satisfactory protein standard.

The data for casein (relative nutritive value, 70) and soy protein (relative nutritive value, 34) appear to approximate the response of lactalbumin reasonably well. However, the values for gluten tend to fall away from the line at the higher intakes. The arrows in figure 2 show the fit that would be obtained if the relative nutritive value of wheat gluten was 14 rather than 18.7. A correction of this order of magnitude would have little significance when good quality proteins are considered, but when the relative nutritive value is low it becomes substantial.[3] With

[3] In the previous paper (12) in which the various methods of evaluating the biologic assays were compared, other estimates of the relative nutritive value of wheat gluten were 17.7, 16.9, and 16.5 compared with that for lactalbumin.

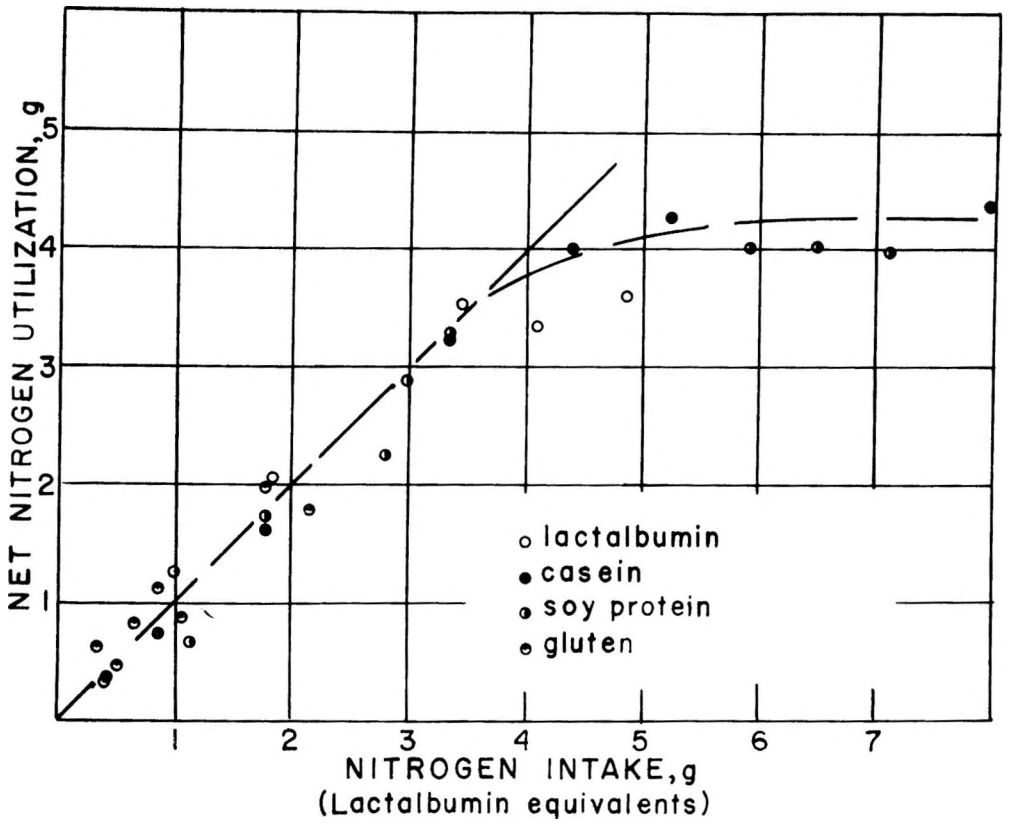

Fig. 3 Combined net nitrogen utilization of all proteins at all levels studied expressed as "lactalbumin nitrogen equivalents." Utilization is essentially complete up to levels closely approaching maximal growth.

wheat gluten it amounts to a reduction in lactalbumin equivalents of about 25%.

The combined data are shown in figure 3 where net nitrogen utilization for all groups has been plotted against "lactalbumin nitrogen equivalent." The relative nutritive values of 100, 70, 34, and 14 for the 4 proteins have been used. Net nitrogen utilization appears to be essentially complete and constant and similar for each protein up to levels which closely approach those required by maximal growth.

DISCUSSION

As pointed out in our previous paper (12), the data under discussion have certain limitations. In well designed studies to compare the effects of various proteins, animals from the same lot should be distributed randomly into the various assay groups. This was not possible in these studies because of the large number of animals used. Also, data are available only from one group of animals fed the nitrogen-free diet. Thus, the absolute values of nitrogen utilization may be somewhat in error and this may account for somewhat over 100% utilization of lactalbumin. However, the correction for the nitrogen-free group would be a constant for each protein and this would not affect the general thesis presented here. Also, percentage errors in nitrogen utilization are likely to be large when high quality proteins are fed at low levels of intake, such as in the first few lactalbumin groups. In the group fed 3.69% lactalbumin, the total nitrogen retention was estimated to be 1.26 g compared with an intake of 0.99 g. Errors of this magnitude might be the result of differences in the starting weights or the body nitrogen content of

different groups, errors in analysis, and others. The method assumes that the animals fed the nitrogen-free diet are exactly comparable to the test groups and this is never possible to ascertain. We are, however, primarily concerned with the general principles rather than the absolute values obtained for the several proteins under test.

Contrary to the conclusions of Miller and Payne (7) or of Morrison et al. (8), we are not able to show any consistent decrease in the utilization of nitrogen as the intake is increased until approximately maximal growth was achieved. In fact, the utilization of wheat gluten appears to be essentially constant up to the highest level fed, 40% of the diet. The reason for this discrepancy is unknown but it appears possible, at least, that it may relate to the time allowed for the test period. We pointed out earlier (12) that biological values and net protein utilization (NPU) values for low quality proteins appear to be substantially higher than the values we have obtained. Furthermore, Bender (14) reported an NPU value of approximately 40% for amino acid mixtures containing no lysine at all. Such a value does not appear meaningful since it is known that animals will not grow with lysine-free diets. We conclude that the rather elaborate scheme of Miller and Payne (7) for the evaluation of the nutritive value of dietary proteins rests upon an inadequate basis and upon generalizations that have not been proved. Njaa (15) has also justly criticized several of the assumptions made by these authors.

The work reported here supports the procedure we have used in estimating protein requirements for growth (11, 16), i.e., that the relative nutritive value of proteins is essentially constant at varying levels of intake up to those levels which closely approach maximal growth. However, as discussed in our previous paper (12), the textbook values for biological values or the usual amino acid scoring systems may not be appropriate for growing rats. Direct studies on infants and children are urgently needed to determine the significance of these observations upon the estimates of the protein requirements at these ages.

According to figure 3, the lactalbumin requirement for maximal growth in these rats was about 4 g of nitrogen during the 3-week period. This was achieved with a diet which provided about 12% of protein in the diet. Assuming that wheat gluten has approximately 14% of the activity of lactalbumin, maximal growth would require that 86% of the diet be supplied as gluten. This is in substantial agreement with the observations of Munaver and Harper (17) who obtained good growth with a diet containing 70 to 80% of gluten. It has been reported (10) that adequate protein intakes cannot be achieved with proteins which have a biological value of less than 60. This conclusion may not be correct if the nutritive value of such proteins was overestimated and they were not studied at high enough levels.

The approach which has been used here has some similarity to that proposed by Howard et al. (18, 19) who attempted to convert the protein content of diets containing wheat flour and protein supplements into comparable amounts of "complete protein" based upon the limiting amino acid content. It appears more useful, however, to have the bioassay procedures for protein self-contained. By following classical assay procedures in which an unknown is compared with a standard, no assumption need be made as to the cause of variations in potency. In an ideal assay the standard should have a maximal potency and the potency of the unknown expressed as "activity" relative to the standard. The method would then be similar to the familiar vitamin assays in which several forms with differing degrees of biological activity occur. This approach requires, of course, an adequate assay of measure "activity." We believe we have demonstrated that more satisfactory assays than those in use can be developed along the lines proposed in this and our preceding paper (12) which deals with the assay in detail.

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Levels of Liver Retinol and Retinol Ester in Pregnant and Pseudopregnant Rats [1,2]

WARREN N. DANNENBURG [3] AND RICHARD L. BURT
Department of Obstetrics and Gynecology, Section of Reproductive Biology, Bowman Gray School of Medicine of Wake Forest College and North Carolina Baptist Hospital, Winston-Salem, North Carolina

ABSTRACT Retinol and retinol ester concentrations were determined in the livers of pregnant rats throughout the 21-day period of gestation and compared with those observed in pseudopregnant controls. Data were analyzed by analysis of variance and the results showed that levels of retinol and its ester in the livers of pregnant animals were significantly higher than those for the pseudopregnant controls; differences were attributed to group × time interaction. The presence of decidual cells had no apparent effect on the concentration of liver retinol, but did appear to increase the concentrations of retinol ester in the liver. The relationship between liver retinol and its ester during pregnancy was discussed, and the evidence suggested that physiological changes in the fetal placenta or fetus were responsible for changes in the maternal levels of retinol and its ester.

Recent evidence suggested that retinol and retinol ester levels in the liver of pregnant rats showed significant increases over nonpregnant controls (1). This observation did not agree with reports that showed vitamin A concentrations were decreased in the liver during pregnancy (2–3), or with the concept of a proportional relationship between liver and plasma retinol which decreased in the latter part of pregnancy (1). Although changes in plasma retinol are generally assumed to reflect similar changes in the liver (4), this concept is equivocal as various investigators have demonstrated (5–7). Conflicting results concerning concentrations of retinol and its ester in the liver during pregnancy may be due to the comparison of nonpregnant and pregnant animals in different stages of estrus and gestation. This is not without basis as estrogen was shown to increase the levels of vitamin A in the liver of the rat (8). The present study was undertaken to determine whether levels of liver retinol and retinol ester in pregnant animals differed from those in pseudopregnant controls that were reported to have comparable endocrine changes (9), and possibly account for the discrepancies reported in the literature.

EXPERIMENTAL METHODS

Nonpregnant, female rats of the Wistar strain were obtained from the Dublin Farms in Dublin, Virginia, and placed in quarantine until it was ascertained they were free of disease. They were then randomized, placed in cages with raised wire floors, and subjected to a 10-hour day and 14-hour night by artificial lighting in rooms controlled at 22°. Two animals were placed in each cage, and food [4] and water supplied ad libitum. The animals remained under these conditions for at least one week prior to experimentation to adjust to their environment. Pseudopregnant animals with and without a deciduomata were produced according to the procedure of DeFeo (10) as modified by Bo et al. (11) by stimulating the cervix and traumatizing a uterine horn; pseudopregnancy was verified daily by vaginal smear. Pregnant animals were produced by placing a male animal of the same strain with the females, and pregnancy was confirmed by the presence of sperm in the vaginal smear which was recorded as day zero of gestation. Liver retinol and retinol ester concentra-

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[2] Presented in part at the 49th Annual Meeting of the Federation of American Societies for Experimental Biology, April 13, 1965, Federation Proc., 24: 656, 1965 (abstract).
[3] Supported by Public Health Service research career program award no. 1-K3-HD 22,873-01 from the National Institute of Child Health and Human Development.
[4] Purina Rat Chow, Ralston Purina Company, St. Louis.

TABLE 1

Retinol and retinol ester concentrations of liver for pseudopregnant, pseudopregnant(traumatized), and pregnant rats

	No. of rats	Body wt	Liver wt	Retinol and retinol ester concentrations			
				Retinol ester	Retinol	Retinol ester	Retinol
		g	*g*	*μg/g liver*		*μg/total liver/ 100 g body wt*	
Pseudopregnant	24	242 [1]	9.6	153 ± 16 [2]	29 ± 2	605	114
Pseudopregnant (traumatized)	37	256	9.6	211 ± 13	31 ± 2	792	115
Pregnant	53	231	8.3	248 ± 17	75 ± 4	892	270

[1] Fifteen animals.
[2] Mean ± SE.

tions were determined from the fourth to fifth day of pregnancy and throughout gestation at daily intervals as were the pseudopregnant controls. The animals were fasted 4 to 6 hours and killed by anesthetizing with diethyl ether. The livers were immediately excised, weighed, frozen on dry ice, wrapped in Parafilm, and placed in the deep freeze at −20° until analyses were made. Retinol and retinol ester were extracted according to Gade and Kadlec (12) and resolved on alumina by a modification (1) of the method of Eden (13).

Computations of variance and regression analysis as well as other statistical procedures used were described in Walker and Lev (14) and differences were considered significant at the 5% level of confidence. Results were expressed as micrograms per gram of liver or micrograms per total liver per 100 g body weight.

RESULTS

The mean concentrations of retinol and retinol ester for the pseudopregnant, pseudopregnant-traumatized, and pregnant rats (table 1) showed that both retinol and its ester were larger in the pregnant animals than those values recorded in the pseudopregnant animals. Comparison of the 2 pseudopregnant groups showed that retinol concentrations were the same, but differences were apparent with respect to the retinol ester. Although retinol ester levels were slightly higher in the pseudopregnant-traumatized animals, they were not considered highly significant based on the numerical value of *t*. Consequently, the values found for retinol and retinol ester in the 2 pseudopregnant groups were combined to represent the pseudopregnant control.

Analysis of variance (table 2) showed that significant differences ($P < 0.05$) existed between the groups and between the times of gestation in relation to retinol and retinol ester levels when the ratio of variance was determined from the error term. However, significant differences between these main sources of variation were accounted for by the group × time interaction as the variance ratio was not significant when determined on this basis. When group comparisons were made, however, the proportion of retinol to its ester in pseudopregnant and pregnant animals was 15 and 23%, respectively. These differences persisted when they were determined as micrograms per total liver per 100 g body weight to allow for differences attributable to body weight.

The differential responses of retinol and retinol ester in the 2 groups studied with time of gestation are shown in figures 1 and 2, respectively. In these figures, the observed mean values for retinol and retinol ester were plotted against the gesta-

TABLE 2

Variance analysis for liver retinol and retinol ester concentrations in pseudopregnant and pregnant rats with time of gestation

Source of variation	df	Mean squares	
		Retinol	Retinol ester
Groups	1	8784	2650435
Time, days	7	2308	64798
Group × time	7	47973 [1]	1872971 [1]
Error	98	414	7152

[1] Significant at $P < 0.05$.

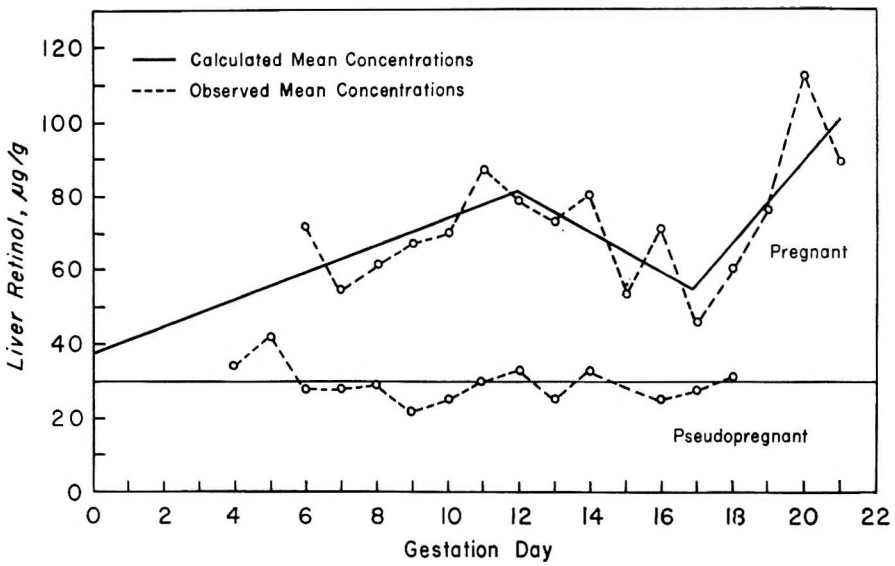

Fig. 1 Relationship between liver retinol concentrations and day of gestation in the pregnant and pseudopregnant rat.

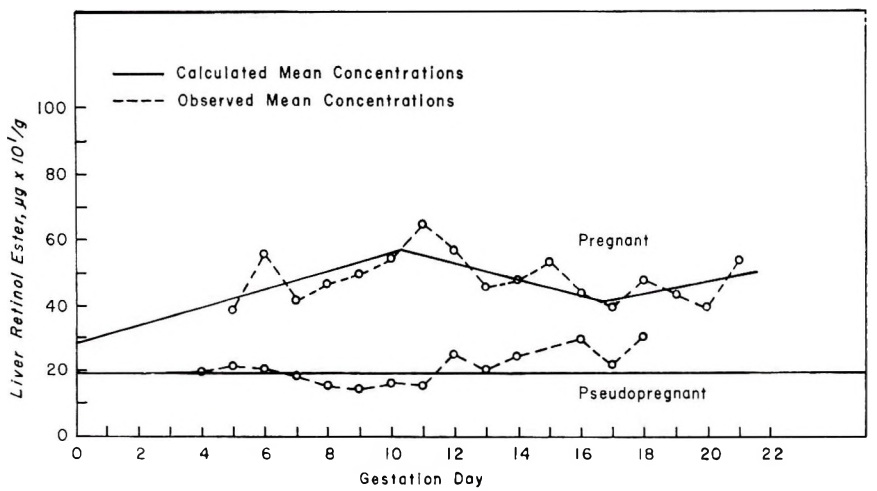

Fig. 2 Relationship between liver retinol ester concentrations and day of gestation in the pregnant and pseudopregnant rat.

tion day and a curve was calculated by regression analysis for each segment of the curve in the pregnant group and showed the average relationship between these variables. The mean levels for retinol and retinol ester in the pseudopregnant control were plotted in a similar way except the mean of the total values was determined as changes observed with time were were not significant and was explained on the basis of chance or random variation. The equations that described the regression of y (retinol) on x (time) in the liver for retinol were $y = 37.83 + 3.68x$ for 1 to 11 days; $y = 145.96 - 5.40x$ for 12 to 17 days; and $y = -128.57 + 10.29x$ for 18 to 21 days. Figure 1 shows an increase in retinol in the pregnant rats

up to day 11 of gestation; a decrease from days 12 to 17; and an increase from days 18 to 21 that exceeded the increment observed on day 11. The changes in the mean levels of retinol ester with time in the pregnant and pseudopregnant controls (fig. 2) were essentially identical to those for liver retinol except in the 18–21 day period of the pregnant group. Regression of y (retinol ester) on x (time) that described the changes observed for retinol ester were $y = 287.56 + 26.89x$ for 1 to 11 days; $y = 823.27 - 24.65x$ for 12 to 17 days; and $y = 152.58 + 15.68x$ for 18 to 21 days. The similarities of changes observed in the retinol and retinol ester concentrations in the pregnant group for 1 to 11 days and 12 to 17 days were apparent, and correlation coefficients which were significant ($P < 0.05$) for these gestational periods were 0.586 and 0.633, respectively. No correlation was found in the 18- to 21-day period of gestation and differed markedly from the relationship shown by these components in the earlier periods of gestation. Retinol and retinol ester levels in pseudopregnant animals did not show the relationship observed in the pregnant animals, and extrapolation of both retinol and retinol ester concentrations to zero day of gestation did not differ from those observed in the nonpregnant control animals.

DISCUSSION

The data presented in these experiments confirmed a previous report that showed liver retinol and retinol ester levels were significantly greater in pregnant rats than those for nonpregnant controls; it did not agree, however, in that differences between the groups were larger. This was explained on the basis of group × time interaction as analysis of variance showed retinol concentrations in the pregnant group changed significantly with the time of gestation with the lowest concentrations observed on the seventeenth day which coincided exactly with the time they were determined in the previous study(1). Retinol concentrations in the liver of pregnant animals were greater than those in the pseudopregnant control animals regardless of the gestational time period studied, except in the early period following conception as determined from extrapolation.

Changes observed in liver retinol were associated with concomitant changes in its ester except in the later stages of pregnancy where a marked increment in retinol was observed without comparable changes in retinol ester. This close relationship as shown by the significant correlation was probably a reflection of the metabolic requirements of the rat during pregnancy rather than one of storage (15) as significant changes were not observed in the pseudopregnant controls. Such a relationship was described by Ganguly and Krinsky (6) who showed the early changes in liver retinol and retinol ester in deficient rats fed fish liver oil were proportional before maximal values of retinol were attained, and by McGillivray (16) who created massive reserves of vitamin A in the livers of rats before feeding them a deficient diet. In these experiments, it appeared that a proportional relationship existed and the metabolic demands of the pregnant rat could be assessed when retinol or its ester was determined except in the last 4 days of pregnancy. Furthermore, it was compatible with the concept of retinol formation at the expense of retinol ester (7), and indicated they had not attained their maximal values in the liver (6).

The dichotomy that existed between these entities as pregnancy progressed towards parturition suggested changes in the mobilization of retinol and retinol ester as no correlation between them was found. Such changes could conceivably be mediated by estrogen production (17) in view of the effect of estrogen on liver stores (8), or it could reflect the loss of retinol ester in preference to retinol in preparation for lactation as suggested previously (16). The pseudopregnant controls demonstrated that differences in liver retinol and retinol ester during pregnancy were not caused by endocrine changes in the maternal organism which were reported to be comparable to those of pregnancy (9), but appeared to be brought about by physiological changes in the fetal placenta or fetus, or both, since these represented the main differences between the groups of animals used in these experiments. The difference observed in the concentration of retinol ester in the pseudopregnant and pseudopregnant-traumatized animals was not

known unless it indicated the influence of the decidual cells (18) and as such increased the storage capacity of retinol ester. This increase did not influence the retinol levels nor were the metabolic demands of the animal changed as evidenced from the variation in the pseudopregnant control.

It appeared to be more than a chance relationship that retinol increments were observed in the early and latter part of gestation when embryological development and colostrum formation were critical. Various investigators have shown that congenital abnormalities occurred in the young bred from mothers receiving inadequate amounts of vitamin A (19–21) and similarly, colostrum formation and lactation were suggested to result in small but significant demands in liver retinol (22). If factors responsible for meeting the retinol requirements in pregnancy were dependent upon physiological changes that occurred in the fetal portion of the placenta or the fetus as the evidence in these experiments indicated, then congenital defects or reduced survival of the nursed young would be expected where metabolic errors in these tissues existed.

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Growth Retardation of Day-old Chickens and Physiological Effects at Maturity

MILTON R. McROBERTS
Hy-Line Poultry Farms, Johnston, Iowa

ABSTRACT Three experiments using White Leghorn chickens fed a low protein diet (7.8% crude protein) whose only source of protein was from corn were conducted to retard growth as measured by weight gain. An approximate physiological age of 10 days was attained regardless of the length of time the diet was fed. A sex difference in growth rate of chickens fed the low protein diet was not observed. The restricted chickens consumed small quantities of the low protein diet, therefore consuming little nitrogen in excess of that required for body maintenance. Improved efficiency of utilization of amino acids was not developed by the chickens during the time of feeding the low protein diet. Minerals and vitamins supplied at levels in excess of the recommendations of the National Research Council were not beneficial for better growth and livability of the restricted chickens. Physical maturity of both males and females was delayed by the approximate length of time that the low protein diet was fed to the chickens. When the restricted chickens were changed to a high protein starting diet (18% crude protein), they grew normally and at a rate comparable to that of chickens fed the high protein diet from day-old.

Prepuberty growth has been arrested by maintaining one-week-old chickens with a semipurified, low tryptophan diet for 9 months, arresting the maturation process at the one-week-old state.[1] McCance (1), using a scheme of severe undernutrition and a commercial chick starting ration, maintained cockerels at 90 to 100 g for 6 months without gain in weight.

The objectives of the present experiments were to observe the effects of a low protein diet, whose only source of protein was corn, upon the growth of chickens started at day-old and to observe the effects of this low protein feeding upon these chickens when they were permitted to grow and develop to maturity.

MATERIALS AND METHODS

Experiment 1. Thirty-five one-day-old male and 35 one-day-old female Hy-Line 934H chicks were used in each of 9 dietary treatments, placed in wire-floor, heated battery sections. The males and females were separated to eliminate competition due to differences in body size resulting from a sex-differentiated rate of growth. The dietary treatments are described in table 1. Feed and water were supplied ad libitum.

The birds in each dietary treatment were weighed as a group each week. Feed consumption for each treatment group was recorded at weekly intervals.

Experiment 2. Twenty one-day-old, Hy-Line 934H male chicks were placed in a wire-floor, heated battery section and were fed a commercial chick starting ration (18% crude protein).

Fifty male chicks of similar breeding were placed in a second battery section, and were fed a low protein diet (7.8% crude protein, described as diet 2 in table 1). Feed and water were supplied ad libitum. After 4 weeks of feeding the low protein diet, the chickens were divided at random into 2 groups. One group of the restricted chickens was changed to the commercial chick starting ration. The second group of the restricted chickens was continued with the low protein diet for an additional 2 weeks. At the end of the 2 weeks this group was also changed to the commercial chick starting ration.

The chickens in each dietary treatment were weighed as a group each week. Feed consumption for each treatment group was recorded at weekly intervals. The experiment was carried out for 8 weeks.

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[1] Gordon, R. S. 1963 Growth arrest through tryptophan deficiency in the very young chicken. Sixth International Congress of Nutrition, Edinburgh, Scotland, p. 471 (abstract).

TABLE 1

Composition of experimental diets

Diet no.	Ground corn	Dextrose	Fat	Mineral mixture [1]	Vitamin mixture [2]
	%	%	%	%	%
1	(Commercial chick starting ration (18.0% protein))				
2	93.65		0.50	4.85	1.00
3	90.85		0.50	7.15	1.50
4	91.35		0.50	7.15	1.00
5	93.15		0.50	4.85	1.50
6	83.65	10.00	0.50	4.85	1.00
7	80.85	10.00	0.50	7.15	1.50
8	81.35	10.00	0.50	7.15	1.00
9	83.15	10.00	0.50	4.85	1.50

[1] Mineral mixture contained: (in grams) calcium phosphate, dibasic, 484; Ca carbonate, 316; iodized salt, 105; trace minerals, 21; magnesium carbonate, 21; and potassium phosphate, 53. [Trace mineral analysis: (in per cent) calcium, 24.0; iodine, 0.24; manganese, 12.0; iron, 5.0; copper, 0.4; cobalt, 0.08; and zinc, 0.7.]

[2] Vitamin-antibiotic mixture contained: (in grams) vitamin A (10,000 IU/g), 250; vitamin D_3 (3,000 ICU/g), 225; choline chloride, 875; niacin, 50; riboflavin, 400; folic acid, 15; vitamin K, 10; Ca pantothenate, 10; vitamin B_{12}, 500; ethoxyquin, 75; ground yellow corn, 1840; Aurofac-25 (American Cyanamid Co., New York), 250; NF 180 (Hess and Clark, Ashland, Ohio), 250; amprolium (25%) (Amprol, Merck and Co., Rahway, N. J.), 250; and (in milligrams) thiamine (4 mg/kg), 20; pyridoxine (7 mg/kg), 35; and biotin, 0.10.

Experiment 3. Pedigreed male and female chickens from 6 Leghorn-type inbred lines were hatched over a period of 8 weeks in 5 bi-weekly hatches (A,B,C,D, and E). Chickens of 2 hatches (A and B) were sexed and divided within sex and within breeding pens into 2 groups: (a) those to be fed a commercial chick starting ration (18% crude protein), and (b) those to be fed a low protein diet (7.8% crude protein), as described in experiment 1. The low protein diet was fed to the A-hatch chickens for 6 weeks and to the B-hatch chickens for 7 weeks and 3 days. After the period of low protein feeding, all chickens were changed to the commercial chick starting ration. When the A-hatch chickens were changed to the high protein ration at 6 weeks of age, one-day-old chickens of the same inbred lines (D-hatch) were started with the same high protein ration. Chickens from E-hatch were started with the high protein ration 10 days in advance of changing the B-hatch restricted chickens to the commercial chick starting ration. Thus the controls for the A- and B-hatch restricted chickens were of similar calendar age and of somewhat similar physiological age. Chickens of C-hatch were discarded because they were not of an age at any time during the experiment comparable to chickens of the A-or B-hatch.

The chickens of A-hatch which were fed the low protein diet were held in battery brooder facilities for 6 weeks, at which time they were transferred to the floor of the brooder house to begin the feeding of the high protein diet. The chickens of the B-hatch that were fed the low protein diet were held in battery brooder facilities for the duration of their restricted program plus one week of being fed the high protein diet before they were moved to the floor of the brooder house. All chickens starting from one-day-old with the high protein diet were reared on the floor of the brooder house.

Chickens that were fed the high protein diet from one-day-old received a vaccination for Newcastle disease and bronchitis at 4 days and 4 weeks, fowl pox at 10 weeks, avian encephalomyelitis at 14 weeks, and Newcastle at 16 and 30 weeks of age. Chickens that were fed the low protein diet started their vaccination program when they were changed to the high protein diet, as though they were day-old birds at this time.

All chickens were weighed when one-day-old and at 6, 12 and 18 weeks of age, and at housing (approximately 30 weeks of age). Lines 3 and 5 were discarded between 18 weeks and housing, due to lack of housing facilities for all of the lines.

RESULTS

Experiment 1. The mean accumulated weight gains made by the male and female chickens fed the 9 different diets are summarized in table 2.

Weight gains made with all of the low protein diets at the end of 4 weeks were essentially the same for males and females. However, weight gains made by males was larger than that made by females when the commercial starting ration was fed for 4 weeks. The addition of minerals (diet 3), vitamins (diet 4), or both minerals and vitamins (diet 5) to the basal diet did not alter the weight gain made by chickens fed these diets as compared with chickens fed the basal corn diet (diet 2). When the protein of maize was diluted by adding 10% dextrose to the diet (diet 6), weight gains were similar to those obtained by feeding the basal corn diet (diet 2).

The largest weekly weight gain made by chickens fed the low protein diets was made during the first week of the experiment, a mean of 6.2 g for both males and females fed diets 2 to 9. At the end of the second week the mean weight gain was 1.8 g; third week, 3.2 g; and fourth week, 3.1 g. This weight gain during the first week of low-protein feeding illustrated the effect of the yolk and its utilization to balance the amino acids of the corn protein.

TABLE 2

Mean accumulated weight gain of 934H male and female chicks for 4 weeks fed 9 different diets

Diet no.	Weeks			
	1	2	3	4
	g	*g*	*g*	*g*
	934H males			
1	39.1	100.9	183.3	270.0
2	7.0	9.0	12.4	15.4
3	7.7	9.1	14.8	16.8
4	6.5	8.9	11.8	15.8
5	7.3	10.4	14.8	16.6
6	6.6	8.0	12.2	17.2
7	5.5	6.5	7.8	10.3
8	6.3	6.9	10.1	14.3
9	4.7	6.0	8.1	12.3
	934H females			
1	38.9	86.6	155.1	225.7
2	6.9	8.2	11.4	14.7
3	6.5	7.3	9.7	12.3
4	6.1	9.0	12.1	15.2
5	7.1	10.9	16.9	20.0
6	5.2	7.6	10.3	12.5
7	4.9	8.2	10.2	13.3
8	5.6	5.6	7.9	11.1
9	4.5	5.9	8.3	11.2

TABLE 3

Feed consumption per chick for 934H male and female chickens fed high and low protein diets (exp. 1)

Diet no.	Males	Females
	g feed consumed/chick	
1	523	463
2–9	124	108

TABLE 4

Mortality for 934H males and females fed 9 different diets for 4 weeks

Diet no.								
1	2	3	4	5	6	7	8	9
934H males								
2 [1]	3	15	17	7	21	13	24	32
934H females								
0	10	6	11	11	7	16	8	11

[1] Number of chickens.

The only evidence of feather growth was in the primary and secondary wing feathers; other areas of the bodies of the chickens maintained the appearance of day-old down.

The grams of feed consumed per chick for male and female chickens fed a high protein (diet 1) and low protein diets (diets 2–9) for 4 weeks are summarized in table 3. Of outstanding interest was the small amount of feed consumed by the chickens fed the low protein diets.

High mortality was recorded in both males and females fed the low protein diets (table 4). No explanation of the mortality could be associated with any specific diet. Diets giving high mortality in male chickens did not have any association with mortality for female chickens.

Experiment 2. Table 5 summarizes the weight gains made by the 934H male chickens fed 4 different diets for 8 weeks. Weight gain after feeding the 7.8% protein diet for 4 weeks was 13.9 g, whereas chickens that were fed the 18.0% protein diet gained 209.5 g in 4 weeks. At 6 weeks of age the restricted chickens (fed the 7.8% protein diet) gained 21.8 g/

chick, slightly less than the weight gain made in one week by day-old chickens fed the 18.0% protein diet. After feeding the 7.8% protein diet for 4 weeks, followed by the 18.0% protein diet for one week, the restricted chickens gained more in one week of feeding the 18.0% protein diet than did the chickens fed the high protein diet during the first week. However, the weight gain of the restricted chickens in one week was not as much as the weight gain of day-old chickens fed the 18.0% protein diet for 2 weeks. Therefore, the restricted chickens, when supplied with an adequate protein diet at 4 weeks of age, had a growth advantage over day-old chickens during the first week.

When the second half of the restricted chickens were changed from feeding the 7.8% protein diet to the 18.0% protein diet at 6 weeks of age, the weight gains of these chickens during the first and second weeks were very similar to the weight gains of chickens that had been restricted for 4 weeks and then fed the adequate protein diet.

The grams of feed consumed to produce 1 g of weight gain by male chickens fed the 4 different diets are shown in table 6. Feed efficiency values for the first 5 weeks were comparable for the 18.0% protein diet to those observed in experiment 1 (2.0 g feed/g weight gain). Continued feeding of the 18.0% protein diet during 6,7 and 8 weeks resulted in feed efficiency values higher than those observed in the earlier weeks. When the chickens were changed from the low protein diet to the high protein diet, the feed efficiency values were somewhat similar to those values obtained with day-old chickens.

Total mortality in this experiment was 2 chickens.

Experiment 3. Table 7 summarizes the weight gains at 6, 12, 18 and approximately 30 weeks of age of male and female chickens of 6 inbred lines that were fed 2 starting diets. Feeding the low protein starting diet gave weight gains similar to those observed in experiments 1 and 2 of this study. Male and female chickens of all 6 inbred lines had similar mean weight gains, indicating that a sex difference in growth rate with the low protein diet did not exist. When the chickens were fed the low protein diet for 6 weeks and then

TABLE 5

Mean accumulated weight gain of 934H male chickens fed 4 diets for 8 weeks

Dietary protein level	Weeks							
	1	2	3	4	5	6	7	8
%	*g*	*g*	*g*	*g*	*g*	*g*	*g*	*g*
18.0	25.4	72.1	104.4	209.5	318.1	431.1	534.8	660.0
7.8 [1]	4.1	6.3	9.6	13.9	16.1	21.8		
7.8 [2]					74.0	162.3	260.5	366.9
7.8 [3]							89.0	170.0

[1] Fed the 7.8% protein diet for 6 weeks.
[2] Fed the 7.8% protein diet for 4 weeks; one-half of the group changed to 18.0% protein diet at 4 weeks of age to recover.
[3] Fed the 7.8% protein diet for 6 weeks; second half of the group changed to the 18.0% protein diet at 6 weeks of age to recover.

TABLE 6

Feed efficiency of 934H male chickens fed 4 different diets for 8 weeks

Dietary protein level	Weeks							
	1	2	3	4	5	6	7	8
%	*g feed/g weight gain*							
18.0	1.8	2.1	3.2	2.0	2.0	2.7	2.8	4.4
7.8 [1]	6.6	13.0	8.8	8.6	13.8	12.6		
7.8 [2]					2.3	2.0	2.5	2.7
7.8 [3]							1.1	2.1

[1] See footnote 1, table 5.
[2] See footnote 2, table 5.
[3] See footnote 3, table 5.

TABLE 7

Mean weight gain at 4 age periods of male and female chickens of 6 inbred lines fed 2 starting diets

Starting dietary treatment	Hatch	Age	Inbred lines												Mean of all lines	
			1		2		3		4		5		6			
			♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
		weeks	*g*	*g*	*g*	*g*	*g*	*g*	*g*	*g*	*g*	*g*	*g*	*g*	*g*	*g*
High protein	ABDE	6	273	238	345	291	373	306	367	320	354	300	346	301	343	293
		12	831	647	1055	833	1029	797	981	801	970	748	1070	863	969	782
		18	1236	907	1611	1255	1561	1103	1472	1110	1498	1141	1692	1267	1512	1130
		housing	1769	1270	2158	1605	—	—	2057	1464	—	—	2334	1801	2230	1535
Low protein	A	6	9	9	10	9	16	16	14	15	14	16	12	11	12	13
		12	343	384	504	456	572	465	494	456	526	454	568	459	501	446
		18	932	795	1233	937	1158	850	1095	804	1087	846	1243	913	1125	858
		housing	1415	1119	2203	1558	—	—	1848	1282	—	—	2177	1504	1911	1366
Low protein	B	7+3 days	17	16	14	13	22	27	21	19	21	26	18	18	19	20
		12	248	259	320	293	376	341	329	293	355	325	381	334	335	308
		18	832	618	981	790	962	797	938	776	947	751	1079	853	886	764
		housing	1613	1097	1998	1444	—	—	1690	1209	—	—	1983	1498	1821	1312

changed to the high protein diet for 6 weeks (low protein, A-hatch), both male and female chickens of all inbred lines made a greater weight gain during the first 6 weeks of receiving the high protein diet than the controls that received the high protein diet as day-old chicks for 6 weeks. Therefore the chicks that were restricted for 6 weeks had a weight gain advantage over day-old chicks when both groups were fed the high protein diet. This weight gain advantage of A-hatch restricted chickens continued to exist at 18 weeks of age as compared with the control chickens (A, B, D, E hatch) at 12 weeks of age.

When chickens were fed the low protein diet for 7 weeks and 3 days, 10 days after the control chickens were started with the high protein diet, and then fed the high protein diet for 6 weeks, the weight gains of these chickens (low protein, B-hatch) were very similar to the weight gains of the control chickens (high protein, A, B, D, E hatch) at 6 weeks of age. Weight gains of the restricted chickens of the B-hatch at 18 weeks of age continued to be similar to the weight gains of the control chickens at 12 weeks of age. Therefore, the restricted chickens had a growth advantage (measured by weight gain) of approximately 10 days as compared with day-old chickens when both groups were supplied the high protein diet.

The percentage livability of males and females of the 6 inbred lines of chickens at 6 weeks of age and at housing, that were fed low protein and high protein starting diets, is summarized in table 8. Livability of both males and females during the first 6 weeks of feeding the high protein diet was slightly better than the livability of chickens fed the low protein diet during the same period. Heavy mortality was experienced when the chickens of the A-hatch, that were fed the low protein diet, were placed on the floor of the brooder house and fed the high protein diet. An attempt was made to reduce mortality at the change-over of dietary treatments at 7 weeks and 3 days in the B-hatch chickens that had been fed the low protein diet by feeding the high protein diet for one week in the battery facilities before these chickens were placed on the

floor of the brooder house. Immediate mortality as experienced in the A-hatch did not occur in B-hatch chickens at this change to the floor facilities; however, at housing the total mortality of these 2 hatches was similar. Livability in the control chickens was not good, when evaluated at approximately 30 weeks of age, but livability of the restricted chickens at this age was worse.

The start of lay was observed carefully in each hatch group (restricted and control) of chickens in which all inbred lines were intermingled. The first egg from the pullets of A-hatch that were started with the low protein diet was laid 38 days after the first egg from the pullets of A-hatch that were started with the high protein diet. Pullets of D-hatch, day-old chicks when A-hatch restricted chickens were changed to the high protein diet, laid their first egg 12 days after the first egg from the pullets of A-hatch that were started with the low protein diet. The first egg from the B-hatch pullets that were started with the low protein diet was laid 21 days after the first egg from the pullets of B-hatch that were started with the high protein diet. Pullets of E-hatch, chickens that were 10 days old when B-hatch restricted chickens were changed to the high protein diet, laid their first egg 22 days after the first egg from pullets of B-hatch that were started with the low protein diet. Egg production at housing for the mixed inbred lines of the 4 hatches of chickens is summarized in table 9. Egg production records indicated that feeding a low protein diet to starting chickens for 6 weeks could delay sexual maturity of pullets, and that the chickens fed the low protein diet were physiologically approximately 10 days of age at the end of the low protein feeding periods.

Table 10 summarizes the percentage egg production, egg weight, fertility, and hatchability of these pullets by inbred lines during the third, fourth, and fifth months of lay. The number of housed pullets, which as day-old chickens fed the low protein diet, was small in number. In all inbred lines the mean percentage production of the restricted hens was below that of the control hens. Weight of eggs from hens fed the low protein diet in some lines was larger and in other lines slightly smaller than the egg weight of hens fed the high protein diet. The small number of restricted hens that were housed did not permit a statistical evaluation of the

TABLE 8

Mean percentage livability of males and females of 6 lines of chickens at 6 weeks of age and at housing, fed 2 different starting diets

Sex	A Hatch		B Hatch [1]		D Hatch	E Hatch	Mean [2]	
	R [3]	C	R	C	C	C	R	C
			Six weeks of age					
Male	64	78	71	81	81	81	68	80
Female	81	77	76	86	84	85	79	83
			At housing					
Male	26	51	22	43	62	39	24	49
Female	31	45	30	51	50	46	31	48

[1] Seven weeks and three days.
[2] R indicates restricted (A hatch + B hatch); C indicates control (A hatch + B hatch + D hatch + E hatch).
[3] R indicates restricted (low protein diet); C indicates control (high protein diet).

TABLE 9

Production of 4 hatches of pullets (a mixture of 4 inbred lines)

Hatch and age	A, 30 weeks		B, 28 weeks		D, 24 weeks	E, 22 weeks
Starting dietary treatment	Low protein	High protein	Low protein	High protein	High protein	High protein
Production of all mixed lines of pullets, %	32.6	55.6	8.3	58.3	41.7	13.2

TABLE 10

Egg production, egg weight, fertility, and hatchability during the third, fourth, and fifth months of lay of 4 inbred lines of hens fed high and low protein diets as day-old chickens

Inbred line	Day-old feeding treatment	No. of hens	Egg production	Egg wt	Fertility	Hatchability of fertile eggs
			%	g	%	%
1	High protein	17	57.3	20.9	18.0	78.1
	Low protein	4	42.4	22.0	75.1	70.2
2	High protein	47	66.0	24.0	93.9	86.2
	Low protein	14	48.2	22.4	64.5	85.8
4	High protein	31	54.4	24.5	83.6	72.0
	Low protein	5	42.6	25.4	84.8	56.4
6	High protein	46	51.9	22.4	71.2	82.2
	Low protein	16	40.1	20.6	57.3	77.4

percentage egg production and the egg weights. Fertility and hatchability of eggs from matings of restricted males and females was good; no indications of adverse effects of feeding the low protein diet to the parents were evident in their offspring.

DISCUSSION

Prolonged undernutrition of immature mammals has been shown to severely restrict growth; however some bone growth continued despite the weight of the animal not increasing (2–6). With the restoration of unlimited food to animals that have been undernourished, rapid growth was resumed (1, 7); however, full stature was not always attained during recovery if the period of undernutrition was long and the animals were very immature. In these studies, attempts were made to understand better the effects of undernutrition, particularly resulting from inadequate and improperly balanced diets.

Although undernutrition studies have been conducted using chickens as the experimental animal (1, 8–10), these studies have used a limited intake of a diet of complete nutrients. The present investigation has eliminated the tedious feature of individual feeding of limited daily amounts of food by using a diet of the natural proteins of corn plus minerals and vitamins.

Studies conducted by McCance (1) permitted cockerels to reach a weight between 90 and 100 g before a restricted feeding program of 8 to 10 g of food/day was initiated; however, in the present investigation the restriction program began with day-old chickens. The loss of feathers and nakedness of some areas of the chicken as reported by McCance (1) were not observed in the present investigation. Wing feathers did grow slightly, but all chickens maintained their day-old down over the entirety of their bodies.

The weight gains and physical appearance (figs. 1 and 2) of the restricted chickens indicated that a physiological age of 10 days was attained regardless of the length of time that the low protein diet was fed. Ten days of age would correspond to the approximate time that the yolk within the chicken would be completely utilized (11–13). When the restricted chickens were changed to a high protein diet, they had a growth rate advantage over chickens started with the high protein diet at one-day-old. The rate of growth of restricted chickens was not observed to be more rapid when fed the high protein diet than chickens that had been continuously fed the high protein diet. Growth rates of the 3 treatment groups of chickens in experiment 3 indicated a remarkably similar rate of gain in weight for the time of being fed the high protein diet regardless of different periods of feeding the low protein diet. At 30 weeks of age, the restricted chickens had not reached body weights comparable to those of the chickens that were fed a good diet during the entire experiment.

The poor efficiency of total feed and also nitrogen utilization of the low protein diet may erroneously suggest some form of impairment in nitrogen metabolism in the re-

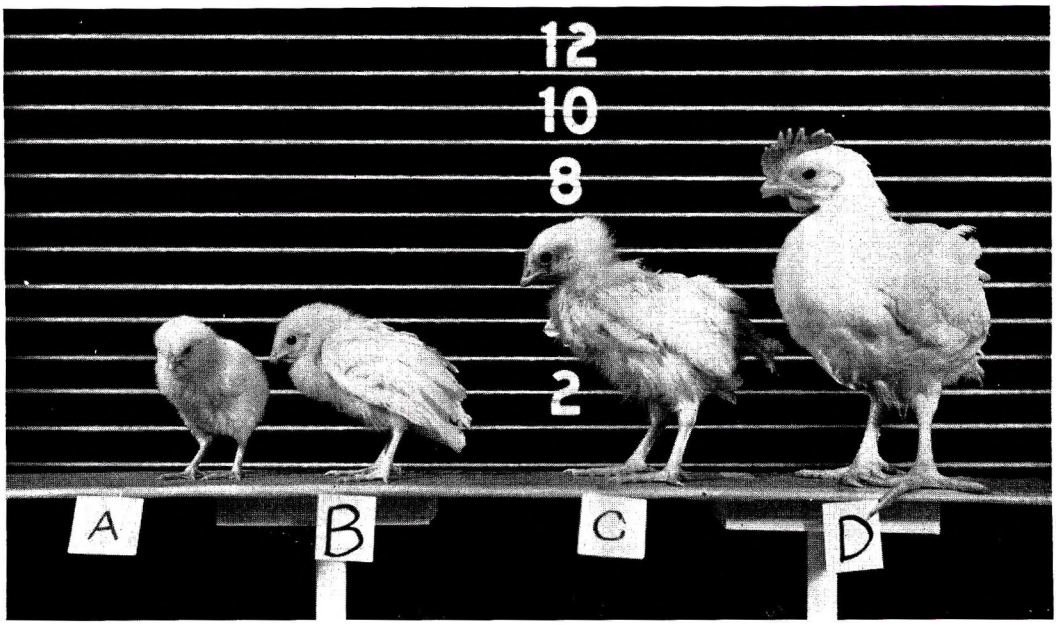

Fig. 1 Experiment 2: A, day-old chick; B, 6 weeks of low protein feeding; C, 4 weeks of low protein feeding followed by 2 weeks of high protein feeding; D, 6 weeks of high protein feeding.

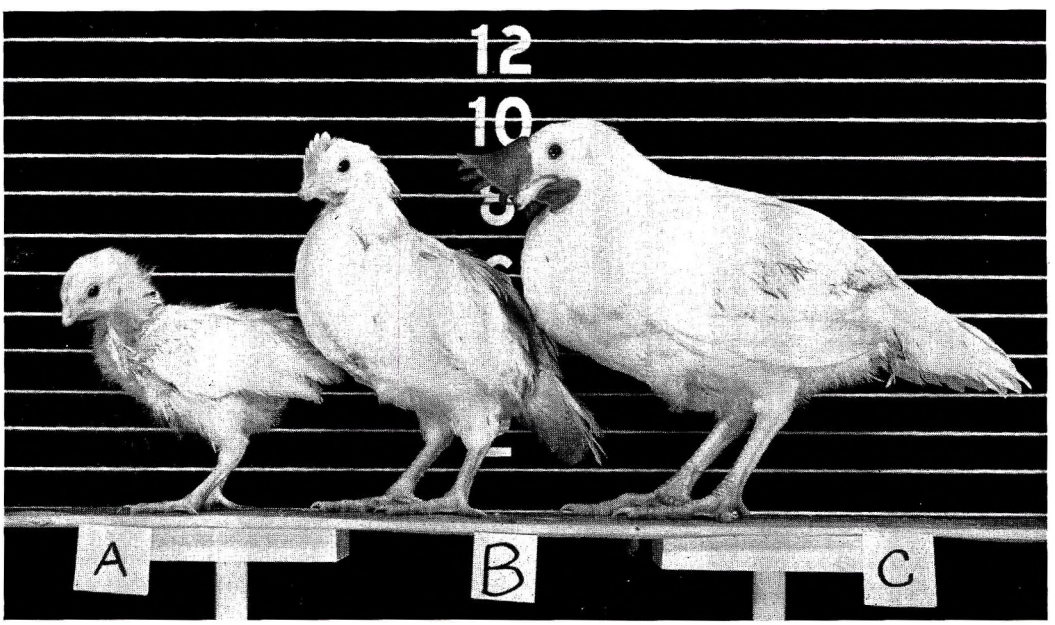

Fig. 2 Experiment 2: A, 6 weeks of low protein feeding followed by 2 weeks of high protein feeding; B, 4 weeks of low protein feeding followed by 4 weeks of high protein feeding; C, 8 weeks of high protein feeding.

stricted chickens. Since the protein intake of these chickens was small due to both a low total feed consumption and a low protein level of the diet, the chickens were probably consuming only little nitrogen in excess of that required for maintenance. The validity of this conclusion was strengthened by the good performance in weight gains and therefore amino acid utilization when they were fed the high protein diet. The failure of the restricted chickens to make better weight gains than their comparable controls indicated that an improved efficiency of utilization of amino acids was not developed by the chickens during the time of feeding the low protein diet.

Nalbandov (14) has reported that acidophilic cells of the anterior lobe of the pituitary gland secrete a protein specific for somatic growth. Somatotrophin has been reported to be of importance in bone growth and also in affecting the rate of retention of ingested nitrogen essential for the formation of protein-containing tissues. At the cellular level, somatotrophin stimulated cell wall permeability to amino acids and activated enzyme systems responsible for protein formation (15). Therefore reduced growth rate and eventually growth stasis may be due to the reduction of hormones which are predominantly proteinaceous in structure. Nalbandov (14) concluded that vigorous growth could occur only when the circulating somatotrophin was sufficiently high to stimulate protein synthesis for bone and muscle growth. Both male and female chickens that were fed the low protein diet in experiment 3 showed that physical maturity had been delayed by the low protein level and imbalanced amino acids of the diet (fig. 3). Chickens that had been fed the low protein diet from one-day-old to 6 weeks of age followed by a high protein diet, had a physical maturity at 18 weeks of age comparable to control chickens that were 12 weeks of age and that had been fed the high protein diet throughout their entire growing period.

The unusually high mortality observed in experiment 3 when the chickens were transferred from the battery facilities to the floor of the brooder house may have been due to changes in temperatures of the 2 facilities. McCance (1) reported that temperature was responsible for most of his fatalities, as undernourished chickens were very susceptible to a decrease in temperature. In the present investigation, keeping the restricted chickens in the battery facilities for one week after the change

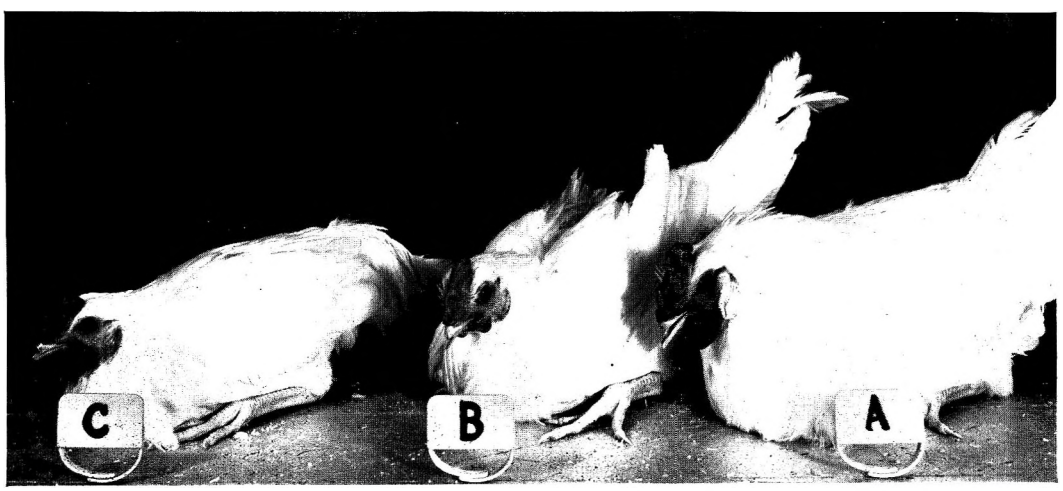

Fig. 3 Experiment 3: A, 18-week-old male, continuously fed a high protein diet; B, 18-week-old male, fed a low protein diet for 6 weeks followed by a high protein diet for 12 weeks; C, 12-week-old male, continuously fed a high protein diet.

to the high protein diet reduced the mortality observed when the chickens were transferred to the floor facilities.

Supplying a high protein diet quickly eliminated the characteristics of the chickens whose growth had been restricted. Once the chickens were fed the high protein diet they gained weight rapidly and at a rate comparable to that of the control group. These results were not in agreement with those of McCance (1) whose cockerels did not grow quite as rapidly as the controls. Other workers (4–6, 16) studying growth rehabilitation concluded that animals that had been undernourished for long periods never attained the full size of their littermates that had been allowed to grow freely from birth. Fabry and Hruza (17, 18) observed that the administration of growth hormone aided restricted animals to attain normal size.

In experiments 1 and 3, weight gains of males and females were similar, indicating that a sex-differentiated rate of growth had been eliminated by feeding the low protein diet. When the restricted chickens were fed the high protein diet, male chickens made larger weight gains than females. Therefore the low protein diet, with its imbalance and deficiency of amino acids, influenced hormone production associated with growth in chickens. The present investigation of growth restriction was not a dietary starvation of total nutrients required for normal growth in chickens as used by McCance (1).

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Absorption of Vitamin B_{12} from the Large Intestine of Rats

DAVID MERZBACH [1] AND NATHAN GROSSOWICZ [2]

Department of Bacteriology, Rambam Government Hospital, Haifa, and Department of Bacteriology, The Hebrew University-Hadassah Medical School, Jerusalem, Israel

ABSTRACT Labeled vitamin B_{12} (17 to 180 of mμg ^{57}Co- or ^{60}Co-labeled vitamin B_{12} corresponding to 15,000 to 400,000 count/min) was injected into the colon ascendens or cecum of 104 rats (3 groups). The animals were killed at different time intervals (from 20 minutes to 29 days) and the tissue radioactivity was determined in a well-type scintillation counter. The total uptake, calculated for each animal, was expressed as percentage of the injected dose; large intestine (injection site) radioactivity was excluded. Thirty rats fed a stock diet containing non-labeled vitamin B_{12} (subdivided in 2 series) showed an average total uptake of 2.9% (range zero to 17.3) and of 2.0% of the injected dose (range 0.9 to 4.3), respectively. Animals fed vitamin B_{12}-deficient diets showed a higher uptake of the labeled vitamin. In one series of 24 rats a total uptake of 8.3% of the injected dose (range 2.2 to 17.7) was obtained. Thirteen rats of a similar experiment showed an uptake of 5.0% of the injected dose (1.3 to 13.4) and 12 rats of 2 additional series showed an uptake of 6.8% (range 4.8 to 9.6) and 11.4% of the injected dose (range 4.0 to 17.3), respectively. The mechanism of the uptake of the radiovitamin was studied in the third group of 25 animals (series 7). In 23 of these rats the passage at the ileocecal valve was occluded by ligation prior to injection. The average uptake was similar to that obtained in a parallel series. No evidence for leakage of the injected vitamin, either intraperitoneally or into the small intestine (by anti-peristaltic movement), could be found. It appears therefore that the labeled vitamin was absorbed from the large intestine, perhaps by non-specific passive diffusion. Similarly, vitamin B_{12} synthesized in the large intestine by microbial flora could gain access to the tissues by such a mechanism. The possible role of intestinal vitamin B_{12} of microbial origin in rats, and perhaps in man, and factors affecting the utilization of this vitamin are discussed.

Uptake of vitamin B_{12} in the animal depends on several factors: extrinsic factor (vitamin B_{12}) supplied by the food, intrinsic factor secreted by the stomach and absorption at specific sites.

On the basis of in vitro and in vivo experiments and observations, it is generally accepted that the small intestine is the main site of dietary vitamin B_{12} absorption; however, the exact anatomical site has not been unequivocally defined. In man, the site of absorption has been reported to be the ileum (1), the upper part of the small intestine (2) or the small intestine as a whole (3); no absorption was observed in the large intestine, either with or without intrinsic factor (3). Similar results were obtained in rats and other animals (4).[3]

The microbial flora exhibits diverse effects with respect to vitamin B_{12}. It has been reported to interfere with absorption of the vitamin in the small intestine (5), whereas other workers have observed that considerable amounts of the vitamin are synthesized by the microbial flora in the large intestine (6). It is generally assumed that the vitamin synthesized in the large intestine is not absorbed and therefore not utilized by the human body. However, a limited utilization of the microbially formed vitamin cannot be excluded. Infrequent remissions observed in untreated cases of pernicious anemia (7) could be attributed in part to such a vitamin supply. The quantitative contribution of the intestinally synthesized vitamin in experimental animals has been studied less.

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[1] Chief, Department of Bacteriology, Rambam Government Hospital.

[2] Professor of Bacteriology, Hebrew University—Hadassah Medical School.

[3] Moertel, C. G., H. H. Scudamore, C. A. Owen and J. L. Bollman 1959 Observations on the site of intestinal absorption of Cobalt^{60}-labeled vitamin B_{12} in the male albino rat. J. Lab. Clin. Med., 54: 926 (abstract).

In the present study labeled vitamin B_{12} was introduced into the large intestine of rats with the aim of establishing the fate of the injected vitamin.

MATERIAL AND METHODS

Male and female albino rats weighing 50 to 120 g were used in this study; they were kept in metabolic cages. The animals received ^{60}Co- or ^{57}Co-labeled vitamin B_{12} by injection into the large intestine and were killed at various intervals thereafter for determination of the tissue radioactivity.

Mode of injection. Since intra-rectal injections were difficult to reproduce, the vitamin was injected with the aid of surgery. The animals were anesthetized with ether and the area of surgery shaved. A longitudinal incision of 1.5 to 2.5 cm was made in the mid-area of the abdomen and the intestine was exteriorized. An intradermal needle was inserted carefully 2-cm deep into the distal part of either the cecum or colon ascendens pointing isoperistaltically (fig. 1). The intestine was then returned to the peritoneal cavity and the wound sutured; all animals regained consciousness shortly after the operation and appeared to have withstood the surgery well. A day after the operation the unligated rats showed a normal behavior both with respect to food and water intake and as well as in excretion of urine and feces. The rats were killed from 20 minutes to 29 days after the injection.

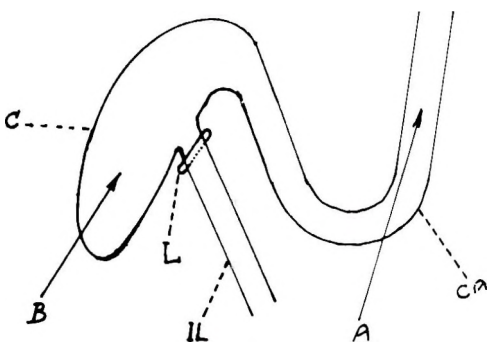

Fig. 1 Mode of injection. Schematic drawing of the distal part of the ileum (IL), the cecum (C) and the colon ascendens (CA) of a rat. The arrows indicate position of the needle for injection into the CA (arrow A) and into C (arrow B). In most animals of series 7 the passage at the ileocecal valve was occluded by ligation prior to injection; L shows area of ligation.

Labeled vitamin. Four vitamin preparations[4] were used: (a) and (b) ^{60}Co-labeled vitamin B_{12} (specific activity 0.93 mμc/mμg and 1.01 mμc/mμg); (c) and (d) ^{57}Co-labeled vitamin B_{12} (specific activity 4.5 mμc/mμg and 16.9 mμc/mμg). Measurements of radioactivity (of ^{60}Co- or ^{57}Co-labeled vitamin B_{12}) were carried out on solutions to be injected. Moreover, each new batch of the labeled vitamin was also determined microbiologically by *Escherichia coli* bioassay (8).

Injected dose. A dose of 0.1 to 0.2 ml of labeled vitamin solution in saline containing 17 to 180 mμg vitamin B_{12} (corresponding to 15,000 to 400,000 count/min) was injected. Larger volumes (0.5 ml) were avoided as they caused intestinal pressure and escape of fluid; no such complications were observed with the small volumes injected.

Experimental set-up. The uptake of the injected vitamin was followed in 3 groups of animals: 1) animals fed a stock diet containing 8 μg vitamin B_{12}/kg dry weight (30 rats); 2) animals fed vitamin B_{12} deficient diets for 2 weeks prior to the injection and during the observation period (49 rats); and 3) animals fed a stock diet. In most of them (23 out of 25 rats) the passage at the ileocecal valve was occluded by ligation prior to the injection. In these rats the mechanism of uptake of the injected vitamin was studied.

Measurement of radioactivity. As the labeled vitamin B_{12} molecule does not degrade in the body (9, 10), the tissue radioactivity was taken to indicate the extent of vitamin uptake.

Radioactivity measurements were made with the aid of a well-type scintillation counter. Determinations were usually performed on the whole organ (liver, kidneys, spleen, stomach, small intestine, cecum and large intestine) without any treatment. Organs that were too large to be counted (exceeding a volume of 3 ml) were divided into segments and their

[4] The authors are grateful to Dr. Charles W. Mushett of Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey, for the generous gift of the ^{60}Co- and ^{57}Co-vitamin B_{12}.

radioactivity summed up. Radioactivity of muscle, skin and skeleton was measured after digestion in 5 volumes of 5 N NaOH and boiling for 10 minutes; 3-ml aliquots of the digests were counted. When low radioactivity was obtained, i.e., in blood, samples were counted until values of 3,000 to 5,000 above background were reached. Blood radioactivity was calculated on the assumption that a 100-g rat contains 6.7 ml of blood (11); the count of the measured sample was multiplied accordingly.

The total uptake of labeled vitamin B_{12} for a given rat was obtained by adding up the counts of the various tissues and excluding the large intestine (the site of injection); the latter values are shown separately. Results are expressed in percentages of the injected dose.

RESULTS

Rats fed stock diet. Twenty-four rats fed the stock diet (containing 8 μg vitamin B_{12} per kg diet) were injected into the colon ascendens with 17 mμg ^{60}Co-labeled vitamin B_{12} (about 15,000 count/min). The rats were divided into subgroups of six and were killed 2, 5, 7 and 12 days after the injection; the radioactivity of the organs was then measured. The results are shown in table 1. The average uptake after 2 and 5 days was 1.8% of the injected dose; percentage values for the individual animals are shown in figure 2. The average radioactivity in the large intestine after 2 and 5 days was 0.5 and 0.2% of the injected dose, respectively. A higher total radioactivity of 2.2 and 5.8% of the injected dose, respectively, resulted in animals that were killed 7 and 12 days after injection; the colon values did not change significantly and averaged 0.5 and 0.3% of the injected dose. The average radioactivity excluding the injection site values was 2.9% of the injected dose.

To assess the reliability of these results a similar experiment was set up, using a vitamin B_{12} preparation with higher specific activity. Six rats were injected into the cecum with 38 mμg of ^{57}Co-labeled vitamin B_{12} (about 400,000 count/min); the animals were killed 3 days after the injection. The total average uptake was 2.0% of the injected dose, a value closely similar to that obtained with the former series (table 1, fig. 2). The colon radioactivity was significantly higher (10.9% of the injected dose).

Animals fed vitamin B_{12}-deficient diets. For reliable measurement of the uptake of the radioactive vitamin B_{12}, methods which favor absorption of the vitamin were used. Therefore, a vitamin B_{12}-deficient diet was employed (12). Preliminary experiments with different deficient diets showed that supplementation with sorbitol in-

TABLE 1

Uptake of labeled vitamin B_{12} injected into the large intestine of rats [1,2]

Series	Injected site	Injected dose		Days after injection	Radioactivity of organs			
					All organs except injected site		Large intestine [3]	
					Range	Avg	Range	Avg
		mμg	count/min		% of injected dose		% of injected dose	
				^{60}Co-labeled vitamin B_{12}				
1	Colon ascendens	17	15,000	2	0 – 9.0	1.8	0.2– 1.3	0.5
				5	0.8– 4.2	1.8	0.1– 0.3	0.2
				7	0.4– 6.0	2.2	0.3– 0.6	0.5
				12	0.5–17.3	5.8	0 – 1.1	0.3
	Avg			2–12	0 –17.3	2.9	0 – 1.3	0.4
				^{57}Co-labeled vitamin B_{12}				
2	Cecum	38	400,000	3	0.9– 4.3	2.0	0.1–60.0	10.9

[1] Rats were fed a stock diet containing 8 μg vitamin B_{12}/kg dry weight.
[2] Six rats/group.
[3] The considerable fluctuations in the values for the large intestine are due to the slow excretion within the first days after administration of the vitamin into the cecum.

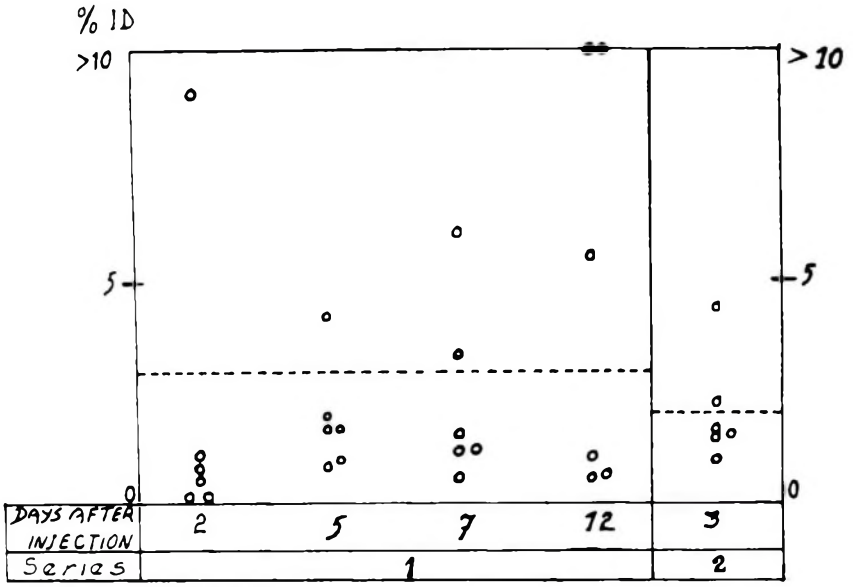

Fig. 2 Uptake of labeled vitamin B_{12} injected into the large intestine of rats. The rats were fed a stock diet containing 8 μg vitamin B_{12}/kg dry weight. The vitamin (17–180 mμg of ^{60}Co- or ^{57}Co-labeled vitamin B_{12} corresponding to 15,000 to 400,000 count/min) was injected into the colon ascendens (series 1) and into the cecum (series 2). The animals were killed at intervals and radioactivity of the various organs measured in a well-type scintillation counter. The uptake in each rat, obtained by adding the counts of all organs (excluding injection site), was calculated as percentage of the injected dose (% ID). Each circle represents the uptake in one animal. The broken line represents the average vitamin uptake in animals of one series. For further data see text and table 1.

creased the absorption of the vitamin in accordance with observations reported by others (13). A diet containing casein and sorbitol was therefore used in the following experiments without investigating whether the increased uptake was due to lack of the vitamin from the diet, addition of sorbitol or to both. The exact composition of the casein-sorbitol diet is shown in table 2.

The animals were fed the diets for at least 2 weeks prior to the intra-cecal injection of the labeled vitamin and until the termination of the experiment. Detailed results of experiments with 49 rats are shown in table 3 and in figure 3.

An average uptake of 8.3% of the injected dose (range 2.2 to 17.7) was obtained in 20 rats (series 3b) killed 29 days after the injection of 40 mμg ^{60}Co-labeled vitamin B_{12}. Similar results were obtained in 4 rats of this series (3a) killed after 3 days. The colon values (injection site) of these animals after 3 and 29 days were 3.6 and 0.2% of the injected dose, respectively.

A similar experiment was set up with 13 rats (series 4) which were injected with a ^{57}Co-labeled vitamin B_{12} preparation of higher specific activity (35 mμg, 157,000 count/min). The animals, which were killed after 2, 6 and 14 days, showed an average uptake of 5.0% of the in-

TABLE 2

Composition of vitamin B_{12}-deficient diets [1]

	Casein-sorbitol	Soya-sorbitol
	%	%
Vitamin-free casein	18	—
Soya flour	—	35
Edible oil	5	5
Salt mixture (USP 13 or 2)	4	4
Starch	33	21
Dextrose	30	25
Sorbitol	10	10

[1] The following vitamins were added/100 g dry weight of diet: (in milligrams) thiamine, 0.2; riboflavin, 0.3; pyridoxine, 0.3; Ca pantothenate, 1.6; choline chloride, 100.0. Vitamin A (50 units) was given orally to each rat once a week.

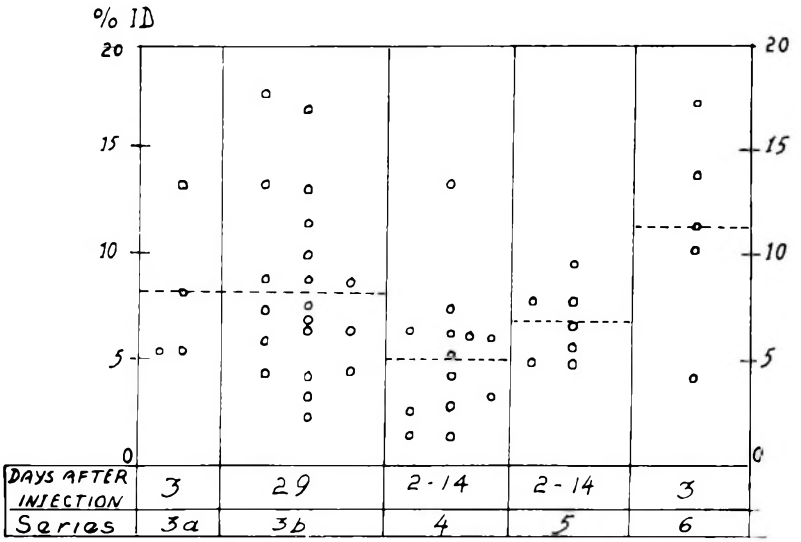

Fig. 3 *Uptake of labeled vitamin B_{12} injected into the cecum of vitamin B_{12}-deficient rats.* Rats were fed vitamin B_{12}-deficient diets (table 2); 40 mμg ^{60}Co-labeled vitamin B_{12} (42,750 count/min) were used for the animals of series 3; in series 4 to 6, 35 to 38 mμg ^{57}Co-labeled vitamin B_{12} (157,000 to 400,000 count/min) were injected. The animals were killed at intervals and the radioactivity measured. The uptake in each rat was calculated as the percentage of the injected dose (% ID). Each circle represents the uptake in one animal. The broken line represents the average vitamin uptake in animals of one series. For further data see text and table 3.

TABLE 3

Uptake of labeled vitamin B_{12} injected into the cecum of vitamin B_{12}-deficient rats [1,2]

Series	Diet	Injected dose		Days after injection	Radioactivity of organs: All organs except injected site		Radioactivity of organs: Large intestine [3]		No. of rats
					Range	Avg	Range	Avg	
		mμg	count/min		% of injected dose		% of injected dose		
				^{60}Co-labeled vitamin B_{12}					
3a	Casein-sorbitol	40	42,750	3	5.4–13.3	8.2	0.5– 6.5	3.6	4
3b				29	2.2–17.7	8.3	0 – 0.5	0.2	20
				^{57}Co-labeled vitamin B_{12}					
4	Casein-sorbitol	35	157,000	2	6.2– 7.3	6.8	70 –87.1	78.5	2
				6	1.3– 6.3	2.9	0.3– 6.8	3.0	4
				14	2.3–13.4	5.7	0.2– 1.1	0.5	7
	Avg (series 4)			2–14	1.3–13.4	5.0	0.2–87.1	13.3	13
5	Soya-sorbitol	35	157,000	2	6.6– 9.6	8.1	2.8– 4.4	3.6	2
				6	4.8– 7.8	6.3	0.75–0.84	0.8	2
				14	4.9– 7.9	6.1	0.2– 0.4	0.3	3
	Avg (series 5)			2–14	4.8– 9.6	6.8	0.2– 4.4	3.1	7
6	Casein-sorbitol + 2% sulfaguanidine	38	400,000 [4]	3	4.0–17.3	11.4	8.6–47.3	18.3	5

[1] For composition of vitamin B_{12}-deficient diets see table 2.
[2] Student's *t* tests: series 3–4: *t*, 2.4; series 3–5: *t*, 1.0; and series 4–5: *t*, 1.0.
[3] The considerable fluctuations in the values for the large intestine are due to the slow excretion within the first days after administration of the vitamin into the cecum.
[4] Specific activity of the ^{57}Co-labeled vitamin B_{12} was reduced by adding 13 mμg of unlabeled vitamin B_{12} to 25 mμg of the labeled vitamin.

jected dose; the colon values averaged 13.3%.

Seven rats (series 5) fed a soya-sorbitol diet (table 2) and injected with the above ^{57}Co-labeled vitamin B_{12} preparation showed an average uptake of 6.8% of the injected dose after 2, 6 and 14 days. The values obtained did not differ significantly from those fed the casein-sorbitol diet.

In 5 other rats (series 6), sulfaguanidine (2%) was added to the casein-sorbitol diet. No detectable effect of the sulfaguanidine addition was noted (average uptake after 3 days, 11.4% of the injected dose; range 4.0 to 17.3).

The calculation of averages of the results presented is shown merely to indicate the magnitude of the absorption pattern of the vitamin under the different conditions used, rather than to represent exact averages. Since, however, the experiments were carried out on a total of about 80 rats, it appears that considerable deviation from the average did not occur, although it is possible that in some experiments the averages were distorted by single high or low values.

The uptake of radioactivity in rats fed a vitamin B_{12}-deficient diet (series 3) was considerably higher (8.3% of the injected dose) than in those (series 1) fed the stock diet (2.9%; table 1). The difference is highly significant ($P < 0.01$). Student's t test for the results of series 3, 4, and 5 are shown in the footnote to table 3.

Distribution of radioactivity in organs of rats. The relative uptake of the labeled vitamin by the different organs is summarized in table 4. Similar values were observed for liver and kidneys; the radioactivity of both organs ranged from 10 to 35% of the total uptake. However, the vitamin concentration (per gram of tissue) in the kidneys was 2 to 3.5 times higher than that of the liver. Usually 50% or more of the total uptake was observed in the carcass (skeleton, muscle and skin). The stomach and small intestine also contained significant amounts of radioactivity (6 to 20%).

Mechanism of vitamin uptake. Since the injected vitamin could be detected, at least in small amounts, in the organs of most rats (77 out of 79) studied, the mechanism of uptake was investigated.

Intraperitoneal leakage of the injected vitamin: The question arose whether the surgical procedure used caused leakage of the vitamin from the injection site into the peritoneal cavity. If this were the case an absorption pattern would be expected similar to that after parenteral administration of the vitamin (14).

Several radioactivity measurements were performed to test this possibility. In 5 rats of series 6 the injection site at the cecum was covered with cotton for as long as 5 minutes after the injection, in order to absorb any leaking fluid. No radioactivity was noted in the cotton pads.

One rat was injected into the cecum with 38 mμg of ^{57}Co-labeled vitamin B_{12} (about 400,000 count/min) contained in a volume of 0.1 ml. After 1 hour, 3 ml of physiological saline were injected intraperitoneally to rinse out any leaking radioactivity. No radioactivity was detected in the saline sample (1.5 ml) which was aspirated from the peritoneum 2 minutes later. Thus, no signs of leakage at the injection site were detected.

TABLE 4

Distribution of radioactivity in organs of rats injected into the large intestine with labeled vitamin B_{12}

Series	No. of rats	Days after injection	Radioactivity in organs				Total uptake, avg
			Liver	Kidneys and spleen	Stomach and small intestine	Carcass	
			% of total uptake		% of total uptake		% of injected dose
2[1]	6	3	5.8	6.2	19.8	68.2	2.0
3a	4	3	5	5	16	74	8.2
3b	20	29	15	9	6	70	8.3
4	13	2–14	16	14	18	52	5.0
5	7	2–14	21	16	18	45	6.8

[1] Except for series 2, all rats were fed a vitamin B_{12}-deficient diet.

Anti-peristaltic movement of the injected vitamin: The next problem considered was whether, as result of the injection, an abrupt antiperistaltic movement was initiated, by which the radiovitamin reached sites in the small intestine, where absorption normally takes place. If this were the case, lower radioactivity, if any, is to be expected in organs of rats in which the passage between the small and large intestine was artificially closed, prior to the injection of the vitamin.

Twenty-three rats, that were fed a stock-diet (containing vitamin B_{12}), had the passage at the ileocecal valve ligated prior to the injection (into the cecum or colon ascendens) of the labeled vitamin (fig. 1). The injected dose was 45 to 90 mμg ^{60}Co-labeled vitamin B_{12} (42,000–84,000 count/min). The animals were killed either after 20 to 180 minutes or after 17 to 36 hours. The ligated rats showed a distended, but intact, small intestine; no perforation occurred during the observation period (fig. 4). The results of the uptake in these animals (series 7) are summarized in table 5.

The average total absorption of the 4 rats injected into the colon ascendens (all tissues excluding carcass and large intestine), killed within 20 to 180 minutes after injection, was 0.3% of the injected dose (range 0.2 to 0.4); the count at the injection site (large intestine) corresponded to a value of 99.2% of the injected dose. Four other rats, killed within 18 to 24 hours, showed an average uptake of 1.6%.

Rats injected into the cecum showed a considerably higher uptake of radioactivity than that of animals injected into the colon ascendens; average values of 1.2% of the injected dose (range 0.3 to 2.5) within 20 to 180 minutes, and 7.9% after 17 to 36 hours were obtained. Radioactivity of the large intestine corresponded to values of 100 and 28.8% of the injected dose respectively.

Values obtained with the animals injected into the colon ascendens (series 7c) were similar to those of unligated rats of series 1 with an uptake of 1.6 and 1.8% of the injected dose, respectively.

Distribution of the labeled vitamin in the organs of ligated rats. Radioactivity values in liver and kidneys of ligated rats did not differ significantly from those of unligated rats; the carcass radioactivity was not measured in this series. The small intestine was cut into 2 to 4 segments which were measured for radioactivity separately. In 7 rats the segment adjacent to the injection site showed a slightly higher radioactivity than the distal ones; in these rats an anti-peristaltic movement cannot be excluded. However, in eleven other rats of the same series very low or no radioactivity was observed in the segments adjacent to the injection site; thus, no evidence for leakage of the vitamin into the small intestine, e.g., by anti-peristaltic movements, could be found. Furthermore, one unlig-

TABLE 5

Uptake of ^{60}Co-labeled vitamin B_{12} [1] injected into the large intestine of rats ligated at the ileocecal valve [2]

Series	Injected site	Time after injection	Radioactivity of organs: All organs except carcass and injected site		No. of rats	Large intestine [3]		No. of rats
			Range	Avg		Range	Avg	
			% of injected dose			% of injected dose		
7a	Colon ascendens	20–180 min	0.2– 0.4	0.3	4	83–113 [4]	99.2	6
7b	Cecum	20–180 min	0.3– 2.5	1.2	6	100–130 [4]	114 [4]	7
7c	Colon ascendens	18–24 hr	0.6– 2.6	1.6	4	0.2– 3.4	1.0	5
7d	Cecum	17–36 hr	0.5–29.7	7.9	5	5.6–79.5	28.8	4

[1] The injected dose varied between 45–90 mμg vitamin (42,000–84,000 count/min).
[2] Twenty-three rats fed a stock diet containing 8 μg vitamin B_{12}/kg dry weight; for further experimental details, see text.
[3] The considerable fluctuations in the values for the large intestine are due to the slow excretion within the first days after administration of the vitamin into the cecum.
[4] This excessive recovery represents a technical error due to the small volumes (0.1–0.2 ml) of the labeled vitamin injected.

Figure 4A

Fig. 4 (A), normal (undistended) small intestine of a rat; (B), distended small intestine of a rat; 36 hours after ligating the passage at the ileocecal valve.

ated rat was injected into the cecum with 38 mμg ^{57}Co-labeled vitamin B_{12} (400,000 count/min). It was killed after one hour, and the small intestine cut into 17 segments of 2 to 4 cm each. In the first 2 segments adjacent to the injection site, 0.02 and 0.015% of the injected dose was observed. All the other 15 segments showed no radioactivity.

Correlation between vitamin absorption and time after injection. With the lapse of time after the injection of the vitamin, the radioactivity at the injection site (colon) decreased, whereas that of the tissues increased (table 5). In rats injected into the colon ascendens, vitamin B_{12} level of the colon decreased rapidly from values of 100% of the injected dose to as little as 1%. Rats injected into the cecum showed a slower decrease in the colon radioactivity (to 28.8%) and a higher uptake in the organs (7.9%) than those injected into the colon ascendens (1.6%).

Blood radioactivity was measured in 19 rats, within 3 hours of injection. In some rats, measurements were taken two to four times within 3 hours. Radioactivity in the blood of 10 rats appeared as early as 5 to 15 minutes and up to 3 hours (range between 0.06 to 4.1% of the injected dose). Injection of higher doses of the radiovitamin (90 to 180 mμg ^{60}Co-labeled vitamin B_{12}, 84,000–168,000 count/min) did not increase the blood levels. Nine out of 19 rats showed no blood radioactivity.

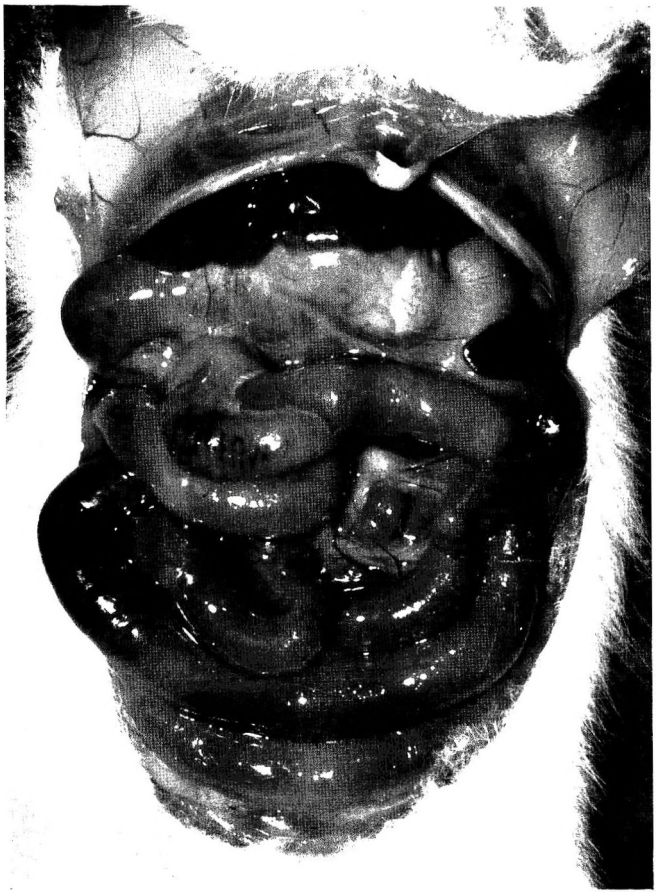

Figure 4B

COMMENTS

This study deals with the fate of physiological doses of labeled vitamin B_{12} injected into the large intestine of rats.

In 77 out of 79 rats injected with labeled vitamin B_{12}, varying amounts of radioactivity were observed in the tissues depending upon the experimental conditions used. Since the vitamin was injected into the large intestine, possible degradation of the vitamin B_{12} molecule by the microbial flora had to be considered. Parenterally administered labeled vitamin B_{12} was shown not to be degraded in the dog (9). Furthermore, simultaneous administration into dogs of ^{57}Co- and ^{60}Co-labeled vitamin B_{12} by oral and parenteral route showed that the vitamin was incorporated into a common pool of cobalamins regardless of the route of administration and that a constant ratio between the 2 labels was obtained in all tissues for as long as 31 days (10). Moreover, since in our experiments the tissue distribution of the radioactivity was similar to that obtained after parenteral injection of the vitamin, it is very likely that degradation of the vitamin in the intestine was negligible, if it occurred at all.

As to the mechanism of uptake, it appears unlikely that the labeled vitamin reached the tissues in a manner similar to that when using parenteral administration; experimentally no leakage into the peritoneal cavity was observed. It is also unlikely that the injection initiated an abrupt anti-peristaltic movement (see below) thereby transporting the labeled vitamin to the small intestine where it could have been absorbed (series 7, table 5, 23 rats). Furthermore, no intestinal pressure was observed during or after the in-

jection, provided the volume of the injected dose was very small (0.1 to 0.2 ml).

On the contrary, it appears to us that absorption of the injected vitamin occurred in the large intestine itself: 1) uptake of the labeled vitamin in the various tissues was of the same order both in ligated and in unligated rats; had injection caused an anti-peristaltic movement, unligated rats would have shown higher vitamin B_{12} uptake than ligated ones; 2) the longer the vitamin remained in the large intestine (e.g., the cecum-injected rats), the higher the uptake of the vitamin by the tissues.

With respect to the increase of tissue radioactivity with time after injection of the vitamin, our results are in accordance with those of others (12, 15). The uptake of a given dose of labeled vitamin B_{12} by the liver continues for several days or weeks, particularly in rats fed a vitamin B_{12}-deficient diet (12). The delayed uptake, as obtained also in our experiments, may be due to transfer of the vitamin to the liver from extra-hepatic sources (12).

Absorption of the injected vitamin by gradient diffusion appears likely. A dual mechanism for vitamin B_{12} absorption in the small intestine has been described (16, 17): specific absorption dependent on intrinsic factor, and absorption by passive diffusion, not requiring intrinsic factor. In the latter case, the absorption was found to be proportional to the concentration of the vitamin in the small intestine. The specific absorption was characterized by blood radioactivity which appeared 8 to 12 hours after oral administration of the vitamin, whereas the passively absorbed vitamin was detected in the blood within one hour (18). In our experiments on rats, as in those of Doscherholmen and Hagen in humans (16, 18), vitamin B_{12} was rapidly absorbed from the large intestine and distributed to the various tissues. In blood, the radioactivity, although very low, probably represents a dynamic value (16).

Role of intestinal vitamin B_{12} of microbial origin. Passive absorption may depend upon many factors, such as degree of saturation of tissues (and serum) with the vitamin, composition of the diet, concentration of the vitamin synthesized by the intestinal flora and interaction between the bacterial flora and the vitamin.

The intestinal flora was shown to synthesize vitamin B_{12} (6);[5,6] however, the bacterial flora was implicated in causing pathological conditions by utilizing the vitamin (5, 19–21). Despite some interfering factors (peristaltic movement, destruction of the vitamin by intestinal microorganisms) uptake of physiological doses of labeled vitamin B_{12} introduced by injection, was observed in a large series of rats. It appears plausible, therefore, that a part of the vitamin synthesized in the large intestine of the rat gains access to the tissues. The low absorption in many rats of this study might be related to the dilution of the injected labeled vitamin with the non-radioactive vitamin of intestinal origin. If a similar mechanism operates in man, it would explain the very slow development of vitamin B_{12} deficiency in vegans [7] eating diets very deficient in vitamin B_{12} and in persons with pernicious anemia after total gastrectomy (22). Dyke et al. (6) have attributed the source of vitamin B_{12} supply in vegans to microbial synthesis in the large intestine, in addition to the vitamin B_{12} stores in liver and other tissues. According to data on man (6) and on rats and rabbits,[8] it may be calculated that 10 to 50 μg of cobalamins are synthesized daily in the large intestine of man. An absorption rate from the large intestine of as low as 1 to 5% of the synthesized vitamin could suffice to supply the body with the minimal maintenance dose (23–25). Spontaneous remissions in pernicious anemia (7, 26), may be due not only to fluctuations in intrinsic factor secretion but also to increased utilization of vitamin B_{12} synthesized in the large intestine. However, since spontaneous remissions of pernicious anemia are rather infrequent, it must be concluded that passive diffusion of microbial vitamin B_{12}, although of potential importance, is

[5] Kaleja, E., and P. S. R. Latvijas 1956 Zinatim Akad. Vestis, *10*: 85 (abstract).

[6] Grossowicz, N., and F. Mandelbaum 1957 M.Sc. Thesis (of M.F.) Department of Bacteriology, The Hebrew University, Jerusalem, Israel.

[7] Vegans is used to denote vegetarian purists who do not consume any animal product, including milk, eggs, etc.

[8] See footnote 6.

variable and negatively influenced by factors, most of them as yet unknown.

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Effect of Dietary Cholesterol on Man's Serum Lipids [1]

FRANCISCO GRANDE, JOSEPH T. ANDERSON,
CONSTANTINE CHLOUVERAKIS,[2] MARCELLO PROJA [3] AND ANCEL KEYS
Laboratory of Physiological Hygiene, University of Minnesota, Minneapolis, Minnesota

ABSTRACT Serum cholesterol levels in man were found to be proportional to the square root of dietary cholesterol. The change of serum cholesterol (mg/100 ml) produced by substituting a diet containing Z_2^2 mg of cholesterol/1000 kcal for another containing Z_1^2 mg of cholesterol/1000 kcal is given by the formula Δ Chol $= 1.5 (Z_2 - Z_1)$. This formula is based on experiments in which the daily cholesterol supplement was dissolved in 100 g of oil which was in turn incorporated into the diet or given in the form of dry egg yolk mixed with the same amount of oil, but it also applies to other results reported in the literature with dietary cholesterol ranging from zero to about 3000 mg/day. Smaller effects were obtained when the cholesterol supplement was given with only 6 g of fat incorporated into cookies. The effect of dietary cholesterol on serum cholesterol and phospholipids was independent of the degree of saturation of the dietary fat. Conversely, the effect on the serum lipids of changing the composition of the dietary fat was independent of the cholesterol content of the diet at either 50 or 1500 mg/day.

Dietary administration of large amounts of cholesterol has been used for many years as a means of producing hypercholesterolemia and atherosclerosis in the rabbit. In contrast with the rabbit, man responds with only minimal changes of serum cholesterol concentration to changes of the cholesterol content of the diet (1). Changes of cholesterol intake within the range of usual natural human diets have been claimed to have no effect on serum cholesterol level (2–4) and a lack of relationship between dietary cholesterol intake and serum cholesterol level in man has been widely accepted until lately. More recent publications, however, have reported that there is some relationship between cholesterol intake and serum cholesterol in man (5–9) and for this reason it was decided to re-examine the problem.

The studies to be reported here consist of 3 experiments. In the first experiment (AE), a comparison was made of diets identical in composition and of fat content similar to that of the usual American diet, but with 3 different levels of cholesterol intake. Furthermore, a comparison was made in experiment AE between the effects of similar amounts of cholesterol when added to the diet as a solution of crystalline cholesterol in oil and in the form of egg yolk. In the second experiment (AF), 2 different levels of cholesterol intake were compared, the cholesterol being dissolved in 2 fats of different degrees of unsaturation. In the third experiment (AM), the effects of 2 different levels of dietary cholesterol were compared using cholesterol baked into cookies which contained only 4 parts of fat for each part of cholesterol. This comparison was made both with a diet containing the usual proportion of fat (40% of the total calories), and with a low fat, high carbohydrate diet.

SUBJECTS, METHODS AND PROCEDURE

The subjects were physically healthy schizophrenic men, 35 to 65 years old, stable in their mental state and free from signs of metabolic abnormality. They were maintained with a standard diet for a stabilization period of 3 weeks before being assigned to groups matched with respect to serum cholesterol level, age, psychiatric diagnosis and relative body weight. Thereafter, for successive 3-week

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[2] Present address: Guy's Hospital, London, England.
[3] District Medical Officer, Ministry of Health, Rome, Italy, during tenureship of a World Health Organization fellowship.

periods, the groups were assigned the experimental diets in a switch-back or reversal pattern devised to compensate for possible time trends. With some exceptions, the same men participated in the 3 experiments. The experimental design is summarized in table 1.

Throughout the experiments each man was served measured amounts of food at each meal. Regular records of rejections, plate waste and supplements allowed the estimation of the actual amount of food individually eaten. Body weight was measured twice a week and the individual rations were adjusted by adding or removing certain amounts of carbohydrate foods (mainly bread and jelly) in order to maintain constant body weight throughout the experiment.

Blood samples were drawn in the morning, in the fasting state, on two of the last three days of each experimental period. Each sample was analyzed in duplicate for total serum cholesterol using a modification (10) of the method of Abell et al. (11). Equal volumes of serum from the 2 days were combined and the pooled sample was analyzed for phospholipids and triglycerides by methods previously described (12). Triglyceride determinations were omitted in experiment AF. In experiment AM, triglyceride determinations were carried out by a method similar to that of Blankenhorn et al. (13) which we found to be equivalent to the earlier method in results.[4]

The diets. The experimental diets consisted in all 3 experiments of a basic low fat, low cholesterol diet plus supplements of fat or carbohydrate and of the various amounts of cholesterol to be tested. The basic diet was composed of vegetable products except for 3 glasses of skim milk daily and 4 servings of fish weekly. The cholesterol content of the basic diet was measured by analyzing separately the foods of animal origin and was of the order of 50 mg/day.

The diets in experiment AE were designed to test the effect of 3 different levels of crystalline cholesterol dissolved in the fat supplement and the effect of cholesterol added as dry egg yolk. The fat supplement in this experiment consisted of cottonseed oil and a small amount of margarine, making a total of 100 g/man/day. Diet LC had no added cholesterol, diets MC and HC were prepared by adding, respectively, 500 mg and 1,500 mg of crystalline cholesterol/day to the fat supplement. A fourth diet (egg) was prepared by adding to the fat supplement an amount of dry egg yolk powder calculated

[4] Pantulu, G. V. A., J. T. Anderson and A. Keys 1965 A rapid and specific micro method for serum triglyceride determination. Federation Proc., 24: 438 (abstract).

TABLE 1

Diet schedule in experiments AE, AF and AM

Experiment [2]	Group	No. men	Periods [1]			
			1	2	3	4
AE	1	5	LC	HC	egg	MC
	2	6	HC	LC	MC	egg
	3	6	MC	egg	HC	LC
	4	5	egg	MC	LC	HC
AF	1	6	LCE	HCE	HCF	LCF
	2	5	HCE	LCE	LCF	HCF
	3	5	LCF	HCF	HCE	LCE
	4	6	HCF	LCF	LCE	HCE
AM	1	6	LCS	HCS	LCF	HCF
	2	6	HCS	LCS	HCF	LCF
	3	4	HCF	LCF	HCS	LCS
	4	6	LCF	HCF	LCS	HCS

[1] Duration of the periods was 3 weeks for experiments AE and AF and 2 weeks for experiment AM.
[2] Key to diet supplements:
Experiment AE: LC = low cholesterol; HC = high cholesterol; MC = medium cholesterol; egg = egg supplement.
Experiment AF: LCE = low cholesterol, fat E; HCE = high cholesterol, fat E; LCF = low cholesterol, fat F; HCF = high cholesterol, fat F.
Experiment AM: LCS = low cholesterol, starch; LCF = low cholesterol, fat; HCS = high cholesterol, starch; HCF = high cholesterol, fat.

to have a quantity of cholesterol similar to that of diet MC. Since dry egg yolk contains glycerides and phospholipids in addition to cholesterol, the diets were matched in this respect by adding to the fat supplement of diets LC, MC, and HC, a mixture of soybean lecithin and vegetable fat (cocoa butter and olive oil) of composition similar to that of the egg lipid. In this way, the 4 diets were matched with respect to total fat content, phospholipid content and the proportion of saturated, monoene and polyene fatty acids, the only difference between the diets being the cholesterol content. The sample of dry egg used turned out to have a lower cholesterol content than anticipated, and therefore the egg diet did not exactly match the MC diet.

The diets in experiment AF were designed to test the effects of 2 different levels of cholesterol in the presence of 2 fat supplements of different degrees of saturation. The fat supplement (fat E) in diets LCE and HCE was similar to that used in experiment AE except that the mixture of lecithin and fat used in AE to match the egg lipid was omitted. Diets LCF and HCF were prepared by adding a fat supplement (fat F) consisting of a mixture of equal parts of cocoa butter and coconut oil. Diets LCE and LCF had no added cholesterol, whereas 1,500 mg/day of cholesterol were added to the fat supplements in diets HCE and HCF.

In both experiments AE and AF the crystalline cholesterol was dissolved in the fat supplement which in turn was incorporated into the diet by stirring and mixing in with such foods as hot cereals, mashed potatoes, stews and casseroles. The concentration of cholesterol in oil was never greater than 1.5% which is well below the solubility limit of cholesterol in the oils used.[5] The dry egg (exp. AE) was incorporated in a similar way.

The diets in experiment AM were designed to test the effect of adding cholesterol without dissolving it in the fat supplement. The daily cholesterol supplement of 1500 mg in diets HCF and HCS was incorporated into 3 cookies. Each cookie contained 500 mg of crystalline cholesterol and 2 g of shortening. The mixture of cholesterol and shortening was heated at 150° until a clear solution was formed. This solution was incorporated by stirring into the cookie batter which was then divided into portions and baked. During the low cholesterol periods (LCF and LCS) the men received similar cookies without added cholesterol. The effect of adding cholesterol was tested in 2 types of diets. Diets LCF and HCF contained a fat supplement similar to that used in the corresponding diets of experiment AF. Diets LCS and HCS were prepared by adding to the basic diet a supplement of starch and sugar of the same caloric value as the fat supplement of the LCF and HCF diets.

Samples of the diets were collected and analyzed as described in previous reports (12, 14). From the analysis of the diets and the records of food intake, the calorie and nutrient values of the diet actually eaten by each man were calculated. The average values for the various experimental periods are shown in table 2.

The table shows that the total fat intake and the fatty acid composition of the diets eaten were very similar in all the diets of experiment AE and in diets LCE and HCE of experiment AF. Furthermore, the fat intake and the fatty acid composition of diets LCF and HCF of experiment AF matched very closely diets LCF and HCF in experiment AM. However, the diets in experiment AE contained a higher proportion of phospholipid than the diets of either experiment AF or AM.

RESULTS

The mean values of serum cholesterol, phospholipids and triglycerides at the end of each of the dietary periods for the 3 experiments are presented in table 3.

The serum cholesterol values in experiment AE were lowest with the low cholesterol (LC) diet and highest with the high cholesterol diet (HC), with intermediate values for the medium cholesterol diet (MC) and for the egg diet. The serum cholesterol levels with the egg diet were very similar to those with the MC diet despite the cholesterol intake being lower with the former diet than with the latter.

[5] Grande, F., and S. Wada 1961 The solubility of cholesterol in dietary fats. Federation Proc., 20: 96 (abstract).

TABLE 2

Average calorie value and nutrient content of the food eaten daily (22 men)

Diets	Experiment AE				Experiment AF				Experiment AM			
	LC	MC	HC	Egg	LCE	HCE	LCF	HCF	LCF	HCF	LCS	HCS
Kilocalories	2650	2600	2610	2640	2730	2770	2770	2780	2870	2740	2900	2860
Protein, g	70	69	70	74	77	76	77	77	73	72	80	80
Carbohydrate, g	300	290	292	295	320	305	328	333	353	341	576	573
Fat, g	130	130	130	130	127	127	127	127	129	126	27	27
P third of phospholipid,[1] g	1.16	1.12	1.04	1.12	0.47	0.48	0.41	0.42	0.75	0.75	0.08	0.08
Cholesterol added, g	0	0.48	1.42	0	0	1.40	0	1.41	0	1.50	0	1.50
Unsaponifiable, g	1.6	2.1	3.1	2.1	2.9	4.2	2.2	3.7	—	—	—	—
DPLB [2]												
All sources, g	0.42	0.88	1.83	0.71	0.45	1.84	0.36	1.78	—	—	—	—
Foods of animal origin, g [3]	0.05	0.04	0.04	0.38	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Glycerides, g [4]	127	127	126	127	124	122	125	123	124	122	26	26
Glycerides, % of total calories												
Saturated	11	11	11	11	12	12	30	29	27	29	4	4
Monoene	14	15	14	14	13	13	8	8	9	9	2	2
Polyene	18	18	18	18	16	15	3	3	3	2	2	2
Total	43	44	43	43	41	40	41	40	39	40	8	8

[1] Lipid phosphorus × 6.3.
[2] Digitonin precipitable, Liebermann-Burchard reacting substances of the unsaponifiable lipid.
[3] Added cholesterol not included.
[4] Total fat less unsaponifiable lipid and less phosphorus third of phospholipids.

TABLE 3

Serum cholesterol, phospholipids and triglycerides for 22 men

Exp.[1]	Diet cholesterol		Serum concentration		
	Diet	(Z^2)	Cholesterol	Phospholipids	Triglycerides
		mg/1000 kcal	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*
AE	HC	559	223 ± 34.5 [2]	220 ± 24.6	143 ± 38.5
	MC	200	210 ± 32.2	211 ± 26.3	153 ± 54.8
	LC	19	196 ± 27.7	203 ± 22.4	144 ± 46.5
	Egg	144	212 ± 35.4	215 ± 23.9	150 ± 59.8
AF	HCE	523	224 ± 36.5	210 ± 23.6	— —
	LCE	18	194 ± 28.1	196 ± 18.5	— —
	HCF	525	257 ± 49.4	242 ± 30.3	— —
	LCF	18	228 ± 35.7	224 ± 23.4	— —
AM	HCF	566	259 ± 43.1	242 ± 26.9	150 ± 46.2
	LCF	17	239 ± 38.5	235 ± 24.5	151 ± 48.3
	HCS	542	219 ± 39.5	218 ± 28.3	208 ± 65.2
	LCS	17	205 ± 37.6	211 ± 29.0	195 ± 53.1

[1] See footnote 1, table 1, for key to diet supplements.
[2] Mean ± SD.

In experiment AF, the serum cholesterol levels were higher with the high cholesterol diets (HCF and HCE) than with the corresponding low cholesterol diets (LCF and LCE), but for equal cholesterol intake the diets containing the more saturated fat (HCF and LCF) caused higher serum cholesterol than the diets containing the less saturated fat (HCE and LCE).

Similarly, in experiment AM, the high cholesterol diets (HCF and HCS) caused higher serum cholesterol levels than the corresponding low cholesterol diets (LCF and LCS), but for the same cholesterol intake the fat-containing diets (HCF and LCF) were associated with higher levels of serum cholesterol than the high carbohydrate diets (HCS and LCS). The comparisons in experiment AM were made at the end of 2-week dietary periods instead of the 3-week periods used in the previous experiments. However, the final period of experiment AM was extended to 3 weeks and comparisons were made between the serum cholesterol values at the end of two and of three weeks. The changes observed at the end of 3 weeks were not significantly different from those established at the end of 2 weeks.

The serum phospholipid values were closely parallel to the cholesterol values. The triglycerides do not show any striking change with the exception of the higher values observed when the men were eating the high carbohydrate diets of experiment AM (HCS and LCS).

The serum cholesterol changes produced by changing the cholesterol content of the diet are shown in the column headed Δ Chol of table 4. The values are the means of the individual differences for 22 men.

The corresponding differences in cholesterol intake, as mg/1000 kcal, are shown in the same table under the heading ($Z_2^2 - Z_1^2$). All the serum cholesterol differences are statistically significant with the exception of the difference between the egg diet and the medium cholesterol (MC) diet in experiment AE. Changing the cholesterol content of the diet produced a change of the serum cholesterol in the same direction. The quantitative relationship between changes in cholesterol intake and changes of serum cholesterol concentration, as well as the influence of the mode of administration of the cholesterol supplement will be considered in the discussion.

The changes in serum phospholipids are summarized in table 5. They are parallel to the changes in serum cholesterol concentration, but smaller.

No significant changes were observed in the concentration of serum triglycerides in experiments AE and AM, in relation to the changes of cholesterol intake. The only significant change of serum triglyc-

TABLE 4

Changes in serum cholesterol concentration (Δ chol) produced by varying the cholesterol content of the diet; [1] *(mean values for 22 men)*

Exp.	Diet change	Diet cholesterol difference $(Z_2^2 - Z_1^2)$	Δ Chol		P value
			Predicted [2]	Observed	
		mg/1000 kcal	*mg/100 ml*	*mg/100 ml*	
AE	HC *minus* LC	540	28.9	27 ± 5.0 [3]	< 0.0001
	HC *minus* MC	359	14.2	13 ± 4.3	0.007
	HC *minus* Egg	415	17.5	11 ± 3.7	0.007
	Egg *minus* LC	125	11.5	16 ± 3.6	0.0002
	Egg *minus* MC	−54	−3.2	2 ± 3.7	0.6
	MC *minus* LC	181	14.7	14 ± 4.6	0.005
AF	HCE *minus* LCE	505	27.9	30 ± 5.7	< 0.0001
	HCF *minus* LCF	507	28.0	29 ± 5.1	< 0.0001
AM	HCF *minus* LCF	549	29.5	20 ± 4.0	< 0.0001
	HCS *minus* LCS	525	28.7	14 ± 3.6	0.0007

[1] The cholesterol content of the diets (Z^2) in mg/1000 kcal is shown in table 3.
[2] Predicted serum cholesterol change from equation 2: Δ Chol = 1.5 $(Z_2 - Z_1)$.
[3] Mean ± SE.

TABLE 5

Changes in serum phospholipids (Δ PL) produced by varying the cholesterol content of the diet; [1] *(mean values for 22 men)*

Exp.[2]	Diet change	Diet cholesterol difference $(Z_2^2 - Z_1^2)$	Δ PL	P value
		mg/1000 kcal	*mg/100 ml*	
AE	HC *minus* LC	540	17 ± 3.2 [3]	< 0.0001
	HC *minus* MC	359	9 ± 3.4	0.012
	HC *minus* Egg	415	5 ± 3.1	0.12
	Egg *minus* LC	125	12 ± 2.7	0.0002
	Egg *minus* MC	−54	4 ± 2.8	0.16
	MC *minus* LC	181	9 ± 3.3	0.012
AF	HCE *minus* LCE	505	14 ± 4.6	0.006
	HCF *minus* LCF	507	18 ± 4.4	0.0005
AM	HCF *minus* LCF	549	7 ± 3.1	0.03
	HCS *minus* LCS	525	7 ± 3.1	0.03

[1] The cholesterol content of the diets (Z^2) in mg/1000 kcal is shown in table 3.
[2] See footnote 1, table 1 for explanation of supplements to diets.
[3] Mean ± SE.

erides was observed in experiment AM when the starch and sucrose diets were compared with the corresponding fat diets: the serum triglyceride difference between diets HCS and HCF was 58 mg/100 ml (SE ± 5.94, $P < 0.0001$) and that between diets LCS and LCF was 44 mg/100 ml (SE ± 5.70, $P < 0.0001$). The difference between these 2 means (14 mg/100 ml) was not statistically significant (SE ± 10.6, $P = 0.2$).

DISCUSSION

Relationship between dietary cholesterol and serum cholesterol concentration. From a preliminary analysis of experiments AE and AF,[6] we concluded that "other things being equal the serum cholesterol appeared to be a linear function of the square root of the cholesterol in the

[6] Anderson, J. T., F. Grande, C. Chlouverakis, M. Proja and A. Keys 1962 Effect of dietary cholesterol on serum cholesterol level in man. Federation Proc., 21: 100 (abstract).

daily diet." A new analysis of the relationship between the changes in serum cholesterol concentration and the changes of cholesterol intake has been made. The data used in this analysis include comparisons made on 22 men who ate diets with 4 different cholesterol levels in experiment AE, and 2 sets of comparisons with 2 kinds of dietary fat, each using an equal number of men and 2 different levels of dietary cholesterol in experiment AF.

In addition to the results from our laboratory, data from 4 other reports have been used. Other data have been excluded because they are not considered comparable. In some experiments (1, 15) the cholesterol intake was increased by adding egg yolk, thus producing also a change in the fat content of the diet. Another (7) involved dietary periods of unequal length and a change from a formula diet to a diet of natural foods.

The data used in the computation are from the reports of Beveridge et al. (6), Connor et al. (9), Erickson et al. (16) and Steiner and Domanski (17). The data from Beveridge et al. were obtained on 7 groups of men (67 men) eating diets with cholesterol levels ranging from 4 mg/1000 kcal to 1140 mg/1000 kcal. The data from Connor et al. include 2 comparisons with 5 men and cholesterol intakes of zero and about 264 mg/1000 kcal. The data from Erickson et al. included comparisons with 22 men eating various diets with and without cholesterol. The data of Steiner and Domanski were obtained with 5 men who ate a cholesterol-free diet and the same diet supplemented with 1364 mg of cholesterol/1000 kcal. In terms of man-periods the contributions of data were: this laboratory 176; Beveridge et al., 67; Connor et al., 10; Erickson et al., 44; and Steiner and Domanski, 10. The details of the computations are given elsewhere (18). This analysis has confirmed the close relationship between serum cholesterol concentration and the square root of the cholesterol content of the diet and failed to disclose any other function that would serve equally well. If we call Z^2 the cholesterol content of the diet in milligrams per 1000 kcal and Z its square root, the change in serum cholesterol in milligrams per 100 ml (Δ Chol) when changing from a diet with cholesterol content of Z_1^2 to another of cholesterol content Z_2^2 is given by the equation:

$$\Delta \text{ Chol} = a(Z_2 - Z_1) \qquad (1)$$

Least squares calculation of a in this equation as reported by Keys et al. (18) gives the result:

$$\Delta \text{ Chol} = 1.5\,(Z_2 - Z_1) \qquad (2)$$

The correlation between observed and predicted values was $r = 0.96$ and is illustrated in figure 1.

Equation 2 can be used for the prediction of serum cholesterol changes produced by changing the cholesterol content of the diet. The coefficient of correlation between observed and predicted values for the combined data from the 5 laboratories was $r = 0.96$ and is illustrated in figure 1. A comparison between observed and predicted values for the data from this laboratory is presented in table 4. Whether the same relationship holds at higher levels of cholesterol intake is questionable and the data at hand (1) indicate that very large cholesterol intakes may not have more effect than the highest intakes considered here. This may find its explanation in the limited capacity of the

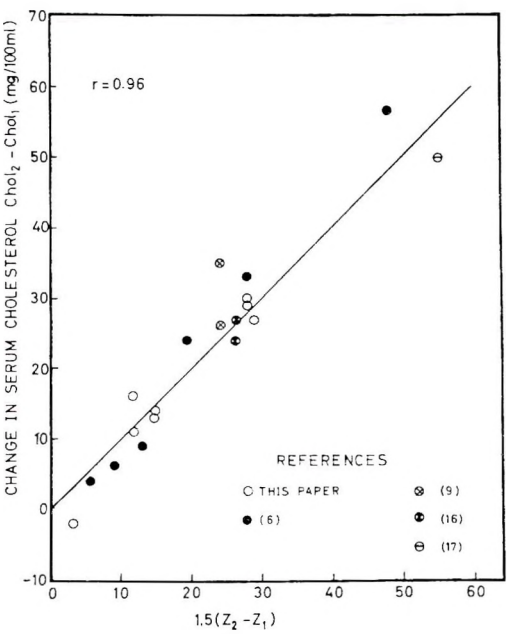

Fig. 1 Observed difference in serum cholesterol plotted against $1.5(Z_2 - Z_1)$ where Z^2 is milligrams of cholesterol per 1000 kcal of diet.

human gastrointestinal tract to absorb cholesterol (19–21).

Crystalline cholesterol versus egg cholesterol. Although Messinger et al. (1) concluded that the hypercholesterolemic effect of egg is not dependent on its cholesterol content, the view that egg cholesterol has a greater effect than crystalline cholesterol has found some acceptance. In our experiments, the egg diets produced somewhat higher serum cholesterol levels than diets containing equal amounts of cholesterol in other forms, but the differences were not statistically significant. Figure 1, in addition to data from our experiments wtih egg and with crystalline cholesterol, includes data from other experiments with egg (9, 16, 17) and with crystalline cholesterol (6). That these different data appear to agree indicates that the difference between the effects of egg cholesterol and crystalline cholesterol is small. The results reported by Connor et al. (8) in 1961 are an exception in that the effect of egg cholesterol was much greater and that of crystalline cholesterol much smaller than the effects illustrated in the figure. This result disagrees not only with the data from the other laboratories but also with more recent values reported by the same authors (9). Examination of the experimental conditions gives no clue to an explanation of this discrepancy.

The observations reported by Wells and Bronte-Stewart (22) have shown that the effect of feeding egg yolk on serum cholesterol concentration can be reproduced by feeding a mixture of cholesterol and a fat of iodine value similar to that of egg yolk fat. Unfortunately, no other data are given to characterize this fat. The results of these authors indicate clearly that the effect of increasing amounts of dietary cholesterol upon serum cholesterol levels tends to be relatively smaller as the cholesterol intake increases. Up to a cholesterol intake of about 500 mg/day the data appear to follow a square root relationship but higher intakes do not produce any further increase in serum cholesterol concentration.

Nature of the dietary fat and response to changes of cholesterol intake. The question of whether the nature of the dietary fat influences the effect of dietary cholesterol on the serum lipids is of considerable importance and we made an attempt to obtain information in this respect in experiment AF in which the effects of 2 levels of dietary cholesterol were compared in the presence of 2 diets differing only in their fatty acid composition (see table 2). The data shown in table 4 indicate that the increase of serum cholesterol caused by increasing the cholesterol intake by 500 mg/1000 kcal was identical for the 2 fats used in experiment AF and very close to the value observed in experiment AE (comparison HC *minus* LC). The fat used in this experiment was similar to that used in diets LCE and HCE of experiment AF but contained a greater proportion of phospholipids because of the addition of lecithin. The experiment recently reported by Connor et al. (9) includes a similar comparison in 5 subjects. They increased the cholesterol content of the diet (as egg yolk and beef fat) from zero to 264 mg/1000 kcal and observed a serum cholesterol increase of 42 mg/100 ml (SE ± 9.2) in the presence of a dietary fat of iodine value 63. The same increase in dietary cholesterol in the presence of more unsaturated dietary fat (iodine value 100) gave a serum cholesterol increase of 29 mg/100 ml. The difference between these 2 changes (13 mg/100 ml) has a standard error of 13.0 and therefore the 2 values cannot be considered significantly different from each other. Their results, therefore, are compatible with the idea that the effect of dietary cholesterol is independent of the nature of the dietary fat.

Dietary cholesterol level and serum lipid response to changes of dietary fat. Equally important is the problem of whether the level of dietary cholesterol influences the response of the serum lipids to changes in the composition of the dietary fat. In experiment AF, the replacement of fat supplement E by the more saturated fat supplement F in the presence of a low level of dietary cholesterol (LCF *minus* LCE) caused a change of serum cholesterol of 34 mg/100 ml (SE ± 4.7). The same dietary change in the presence of the high cholesterol intake (HCF *minus* HCE) caused a serum cholesterol change of 33 mg/100 ml (SE ± 5.1). The same change

of dietary fat caused the same change of serum cholesterol concentration irrespective of the cholesterol content of the diet. From a similar comparison, Connor et al. (9) conclude that "the concentration of serum lipids remained unchanged during the 2 periods of cholesterol-free diets when the fatty acid composition of the dietary fat was made more polyunsaturated and less saturated (from an iodine number of 63 to 100)." Recomputation of their data using the values for the 5 men who completed the experiment shows that the change of dietary fat caused a serum cholesterol change of 4 mg/100 ml (SE ± 7.5) in the presence of the cholesterol-free diet and of 19 mg/100 ml (SE ± 12.0) in the presence of the high cholesterol diet. But these changes are not statistically significant ($P = 0.60$ and 0.13, respectively) and they are not significantly different from each other (mean difference 15.0, SE ± 12.0, $P = 0.28$). Erickson et al. (16) reported "that with a cholesterol-free formula diet the plasma level is unaffected by changing the P/S ratio of the diet from 1.6 to 0.1," but there is no information in their report as to the effect of the more saturated diets at high dietary cholesterol levels, to substantiate whether the lack of serum cholesterol response was really due to the absence of dietary cholesterol.

Admittedly, our low cholesterol diet was not cholesterol-free, but in our experiments the effect of changing the dietary fat was the same regardless of the level of cholesterol intake, and this conclusion is not opposed by the observations of Connor et al. (9) and of Erickson et al. (16). Their failure to obtain changes of serum cholesterol concentration by changing the degree of saturation of the dietary fat appears to be related to the nature of the fats used as we have recently discussed elsewhere (18).

Method of incorporation of crystalline cholesterol into the diet. Difference in the method of incorporation of crystalline cholesterol into the diet may be responsible for some of the discrepancies of the results in the literature. Table 6 summarizes the serum cholesterol changes produced by adding 1500 mg of cholesterol to a low cholesterol diet under different conditions. The table shows that administering cholesterol dissolved in large amounts of oil, as in experiments AE and AF, produced a greater elevation of serum cholesterol than giving the same amount of cholesterol in cookies as a supplement to a low fat diet. The intermediate procedure of administering the cholesterol and the oil in separate foods in the same meal (line 2, table 6) gave an intermediate serum cholesterol increase which is not clearly distinguishable from either of the other 2 extreme cases. Although the cholesterol was dissolved in the shortening when this was stirred into the cookie batter, it undoubtedly crystallized to a large extent upon cooling since the amount of cholesterol far exceeded its solubility in the shortening at room temperature. If the increases of serum levels are caused by increased absorption of cholesterol from the intestine, the results suggest that much cholesterol was absorbed when it was incorporated into cookies given in conjunction with a diet containing only 27 g of fat/day.

TABLE 6

Comparison of different methods of adding 1500 mg of cholesterol to a low cholesterol (950 mg/day) diet [1]

	Method of adding cholesterol	Type of diet	Exp.	Serum cholesterol increment
				mg/100 ml
1	In cookie	starch	AM	14 ± 3.6 [2]
2	In cookie	saturated fat	AM	20 ± 4.0
3	In oil	saturated fat	AF	29 ± 5.1
4	In oil	unsaturated fat	AF	30 ± 5.7
5	In oil	saturated fat	AE	27 ± 5.0

[1] Line 1 is significantly different from combined lines 3, 4 and 5 ($P = 0.006$) but not significantly different from line 2 ($P = 0.32$). Combined lines 1 and 2 are significantly different from combined lines 3, 4 and 5 ($P = 0.005$).
[2] Mean ± SE.

Dietary cholesterol level and the response to an increase of saturated fat in the diet. Experiment AM makes it possible to decide whether the effect on serum cholesterol of substituting fat for carbohydrate is altered by the level of dietary cholesterol. From the values in table 3, the replacement of carbohydrate by fat in the presence of the high cholesterol diet caused an increase of 40 mg/100 ml (SE ± 5.2), whereas the same substitution produced an increase of 34 mg/100 ml (SE ± 3.7) in the presence of the low cholesterol diet. These values are not significantly different from each other (mean difference, 6 mg/100 ml, SE ± 5.5, $P = 0.3$). We, therefore, conclude that the effects on serum cholesterol of changing the amount or nature of the dietary fat were independent of the amount of cholesterol in the diet. This is supported by recent experiments in dogs,[7] showing that addition of coconut oil to the diet produced similar increases of serum cholesterol in the presence of a cholesterol-free diet and in the presence of a diet containing the amount of cholesterol present in a commercial dog feed.

Serum phospholipid and triglyceride changes. The changes in serum phospholipids in all these experiments were parallel to those of serum cholesterol but of smaller absolute value.

The only significant changes of serum triglycerides were observed in experiment AM in which the high carbohydrate diets consistently produced higher serum triglyceride levels than the high fat diets. The changes in serum triglyceride, were not significantly different from each other when the change of the fat to the high carbohydrate diet was made in the presence of low or of high cholesterol intake. We conclude, therefore, that changes in the cholesterol content of the diet do not affect the serum triglyceride level or the changes of serum triglycerides produced by changing the relative content of fat and carbohydrate of the diet.

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Mrs. Helen Williams served as dietitian. Cloyd Thomas, Frank Heatwole and Jon Williams, voluntary assistants assigned by the Brethren Service Committee, cooked and served the food. Assistance was rendered by staff members of the Laboratory of Physiological Hygiene, Mrs. Nedra Foster, Margaret Niebling, Gayle Olson, Dr. Paul Kupchs, David Plum and Mrs. Mary Jane Schweich.

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Effect of Dietary Linoleic Acid, Vitamin E and Ethoxyquin on Fertility of Male Chickens [1]

G. H. ARSCOTT, J. E. PARKER AND E. M. DICKINSON
Departments of Poultry Science and Veterinary Medicine, Oregon State University, Corvallis, Oregon

ABSTRACT Adult male chickens were fed diets low in linoleic acid, vitamin E and ethoxyquin or high in linoleic acid with or without added vitamin E or ethoxyquin for 25 weeks. Fertilizing capacity and sperm concentration of semen were adversely affected with males fed the high linoleic acid diet without vitamin E or ethoxyquin. The addition of vitamin E or ethoxyquin to the high linoleic acid diet overcame these adverse effects. The low linoleic acid diet without vitamin E and ethoxyquin had no adverse effect on fertilizing capacity and concentration of semen. No differences were evident for males fed any of the diets as far as semen volume, hatchability of fertile eggs, body or testes weights or feed consumption were concerned. During the 26th and 27th weeks increasing the number of sperm inseminated failed to improve fertility of the males fed the diet high in linoleic acid without vitamin E. The results indicate that diets high in linoleic acid but without vitamin E affect fertilizing capacity as well as the number of sperm produced.

Adamstone and Card (1) reported testicular degeneration resulting after a vitamin E-deficient diet was fed to Rhode Island Red males for a 2-year period. Although no differences were observed at one year, an adverse effect on fertilizing capacity was noted with a limited number of males after 2 years. More recently Machlin et al. (2) reported adverse effects on fertility as well as egg production of White Leghorn layers fed diets low in vitamin E but containing a high level of linoleic acid. They showed that lowered fertility and egg production were overcome by addition of vitamin E or ethoxyquin to the diet. In the absence of the high level of linoleic acid as well as vitamin E and ethoxyquin no adverse effects on fertility or egg production could be observed during the 6 weeks of their experiment.

The relationship of vitamin E to linoleic acid metabolism has been reviewed by Scott (3). Evidence is cited indicating that vitamin E functions as a biological antioxidant that protects against encephalomalacia in chicks by preventing the breakdown of linoleic acid to 12-oxo-*cis*-9-octadecenoic (keto) acid. Whether or not the same relationship exists with adult poultry has not been established, but the data reported with layers (2) suggest such a possibility.

In view of a previous interest in the area of nutrition as related to male fertility (4), the present experiment was undertaken to determine whether linoleic acid would exert a detrimental effect on fertilizing capacity of adult male chickens fed diets with or without vitamin E or ethoxyquin.

EXPERIMENTAL

Twenty-four dubbed White Leghorn cockerels hatched February 20, 1963, were housed in individual wire-floor cages on July 27, 1963. The present experiment commenced March 31, 1964, at which time the males were divided into 4 comparable groups and fed the experimental diets shown in table 1.[2] The males were distributed into their respective groups in such a way that differences in body weights, as well as semen volume and fertility (based on 3 preliminary ejaculations and inseminations) were minimized. In addition the males on the 4 treatments

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[2] Prior to this date the males had previously been divided into 3 comparable groups beginning November 26, 1963, during which time they were fed rations containing varying protein levels for 7 weeks. For 18 weeks prior to the commencement of the present trial all birds were fed an adequate control ration free-choice.

TABLE 1
Composition of experimental diets

	Diet no.			
	1	2	3	4
	%	%	%	%
Isolated soybean protein [1]	25.0	25.0	25.0	25.0
Coconut oil, air-oxidized [2]	10.0	—	—	—
Safflower oil, air-oxidized [3]	—	10.0	10.0	10.0
Glucose monohydrate [4]	48.7	48.7	48.2	48.4
Cellulose [5]	8.0	8.0	8.0	8.0
Methionine hydroxy analogue [6]	0.4	0.4	0.4	0.4
Vitamin A, dry (10,000 IU/g)	0.2	0.2	0.2	0.2
Vitamin D_3, dry (15,000 ICU/g)	0.025	0.025	0.025	0.025
Vitamin mixture [7]	0.6	0.6	0.6	0.6
Vitamin E (44 IU/g) [8]	—	—	0.5	—
Choline chloride (25%)	1.0	1.0	1.0	1.0
Salts N [9]	6.0	6.0	6.0	6.0
Na_2SeO_3 in glucose monohydrate [10]	0.1	0.1	0.1	0.1
Ethoxyquin [11]	—	—	—	0.3
	100.0	100.0	100.0	100.0
Calculated analysis: [12]				
Linoleic acid, %	0.03	7.3	7.3	7.3
Vitamin E, mg/kg	0.9	4.3	166.3	4.3

[1] ADM C-1 Assay Protein, Archer-Daniels-Midland, Minneapolis.
[2] Konut, Durkee Famous Foods, Glidden Company, Berkeley, California.
[3] Alkaline refined safflower oil, Pacific Vegetable Oil Corporation, San Francisco.
[4] Cerelose 2001, Corn Products Company, New York.
[5] Solka Floc BW-100, Brown Company, Berlin, New Hampshire.
[6] Ca DL-2-hydroxy,4-methylthiobutyrate; provided by Monsanto Chemical Company, St. Louis.
[7] Vitamin mixture supplied: (in mg/kg of diet) vitamin K (menadione), 1.2; vitamin B_{12}, 0.036; thiamine·HCl, 28.8; riboflavin, 19.2; Ca D-pantothenate, 24; niacin, 120; pyridoxine·HCl, 9.6; folacin, 4.8; and biotin, 0.36 (Nutritional Biochemicals Corporation, Cleveland).
[8] Myvamix; provided by Distillation Products Industries, Rochester, New York.
[9] Salts N supplied: (as % of diet) Ca, 1.24; P, 0.8; K, 0.37; Na, 0.384; Cl, 0.58; Mg, 0.06; Fe, 0.00334; Mn, 0.00813; I, 0.0006; Zn, 0.00728; and Cu, 0.0004 (Nutritional Biochemicals Corporation).
[10] Optional trace mineral, with 21.9 mg of Na_2SeO_3 mixed per 100 g glucose monohydrate.
[11] Santoquin (1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline); provided by Monsanto Chemical Company, St. Louis.
[12] Based on values cited for ingredients in table 2.

were placed in the 3 tiers of a battery in such a way as to avoid positional effects as much as possible. Data were collected on all groups for 25 weeks. After this period additional data were obtained for the groups receiving the high level of linoleic acid with and without supplemental vitamin E. All males were killed on October 16, 1964, at which time testes weights were obtained.

The rations used are shown in table 1 and were adapted from the report of Machlin et al. (2) as diet S-25-B modified by reducing the calcium and phosphorus levels to values slightly above those specified for chicks (5) through the use of salts N (6) [3] including an optional level of selenium (0.1 mg/kg of diet). Linoleic acid values were varied by using either coconut [4] or safflower [5] oil (diets 1 vs. 2, 3 and 4). The tocopherol content of the coconut or safflower oil was reduced by bubbling air from an aspirator pump through a 4.54-kg sample of the respective oil contained in a 12-liter round-bottom flask enclosed in an electric heating mantle for 32 hours at 72°. Following this treatment, 0.1% of an antioxidant mixture [6] was added to minimize subsequent development of rancidity (2). The oils were stored in the deep freeze (− 12°) until required. The α-tocopherol and linoleic acid content [7] of selected ingredients used in these rations is shown in table 2. The calculated vitamin E and linoleic acid content of the diets resulting from these analyses is shown in table 1. The addition of 162 mg of vita-

[3] See footnote 9, table 1.
[4] See footnote 2, table 1.
[5] See footnote 3, table 1.
[6] Tenox 6 (Eastman Chemical Products, Inc., Kingsport, Tennessee) which contains: (in per cent) butylated hydroxytoluene, 10; butylated hydroxyanisole, 10; propyl gallate, 6; citric acid, 6; propylene glycol, 12; and mixed glycerides, 56.
[7] The authors are grateful to Mr. R. Bull, Department of Animal Science and Dr. I. J. Tinsley, Department of Agricultural Chemistry for conducting the analyses for α-tocopherol and linoleic acid, respectively.

TABLE 2

α-Tocopherol and linoleic acid content of selected ingredients used in the diets

	α-Tocopherol	Linoleic acid
	μg/g	%
Coconut oil	21.5	0.3
Coconut oil, air-oxidized	7.4	0.3
Safflower oil	490.0	75.0
Safflower oil, air-oxidized	41.1	72.6
Isolated soybean protein	0.8	—
Cellulose	0	—
Glucose monohydrate	0	—

min E[8]/kg of diet (diet 3) or 0.3% of ethoxyquin[9] (diet 4) was made at the expense of glucose monohydrate.[10] The rations were mixed in 45.4-kg lots as needed and stored in the deep freeze. Estimated amounts to be consumed weekly were kept in the room with the males for use during that week.

Care and management of these males have been described previously (8). Both feed and water were provided ad libitum. Body weights and feed consumption data were obtained bi-weekly starting with the first week. Usually, groups of 3 White Leghorn layers each were artificially inseminated every 4 weeks with 0.05 ml of undiluted semen from individual males; however, in a few instances only 1 or 2 females could be inseminated when limited amounts of semen were available or when mortality had occurred among the females. The hens were rotated so that no male's semen was used for insemination of the same group of hens twice. No fertility data were obtained during the seventeenth week of the experiment because the yearling hens were replaced by young pullets which were not yet laying at an adequate rate. A total of 2,153 eggs was incubated during this experiment.

In addition to obtaining data on semen volume, fertility and hatchability of fertile eggs, data on semen concentration were also obtained turbidometrically (9). Semen samples from individual males were stored overnight in a refrigerator (5°), diluted 1:100 using 0.05 ml of semen in 0.85% saline solution and transmittancy readings obtained using tubular absorption cells in a Cenco-Sheard-Sanford photometer with a 640-mμ filter. All such readings were subsequently converted to optical density values (10) for expression on a linear basis.

After 25 weeks, the hens were inseminated on 2 separate occasions with semen (0.03 to 0.1 ml) from males receiving the high level of linoleic acid with and without vitamin E (2 hens/male). This was done to compare fertility resulting from inseminations of more comparable numbers of sperm from the 2 groups since sperm concentration of semen from the high linoleic acid — low vitamin E males was lower than that of the group with vitamin E.

The data were subjected to regression analysis with statistical significance determined by t or F tests.

RESULTS AND DISCUSSION

The results for the first 25 weeks of this experiment are shown in figure 1. A significant decrease ($P < 0.01$) in fertilizing capacity of semen was observed when males were fed diet 2, containing a high level of linoleic acid (7.3%), a low level of vitamin E (4.3 mg/kg) and no ethoxyquin (fig. 1A). A marked decrease occurred after 5 weeks and at 25 weeks all eggs were infertile as determined by macroscopic examination of the germinal disc for all clear eggs candled out. However, diet 3, containing high levels of linoleic acid and vitamin E (166.3 mg/kg) but no ethoxyquin, maintained fertilizing capacity as did diet 4, supplemented with 0.3% ethyoxquin but no vitamin E. Diet 1, containing low levels of linoleic acid (0.03%) and vitamin E (0.9 mg/kg) as well as no ethoxyquin, also maintained fertilizing capacity throughout the experiment.

Semen volume appeared unaffected throughout the test period for all treatments used (fig. 1B). On the other hand, semen density decreased significantly ($P < 0.01$) for the males fed a high level of linoleic acid without supplemental vitamin E or ethoxyquin (fig. 1C). In this group a marked decrease was noted after 13 weeks. A significant difference ($P < 0.05$) in hatchability of fertile eggs (fig. 1D) was noted with males fed at the high level of linoleic acid without supplemental vita-

[8] See footnote 8, table 1.
[9] See footnote 11, table 1.
[10] See footnote 4, table 1.

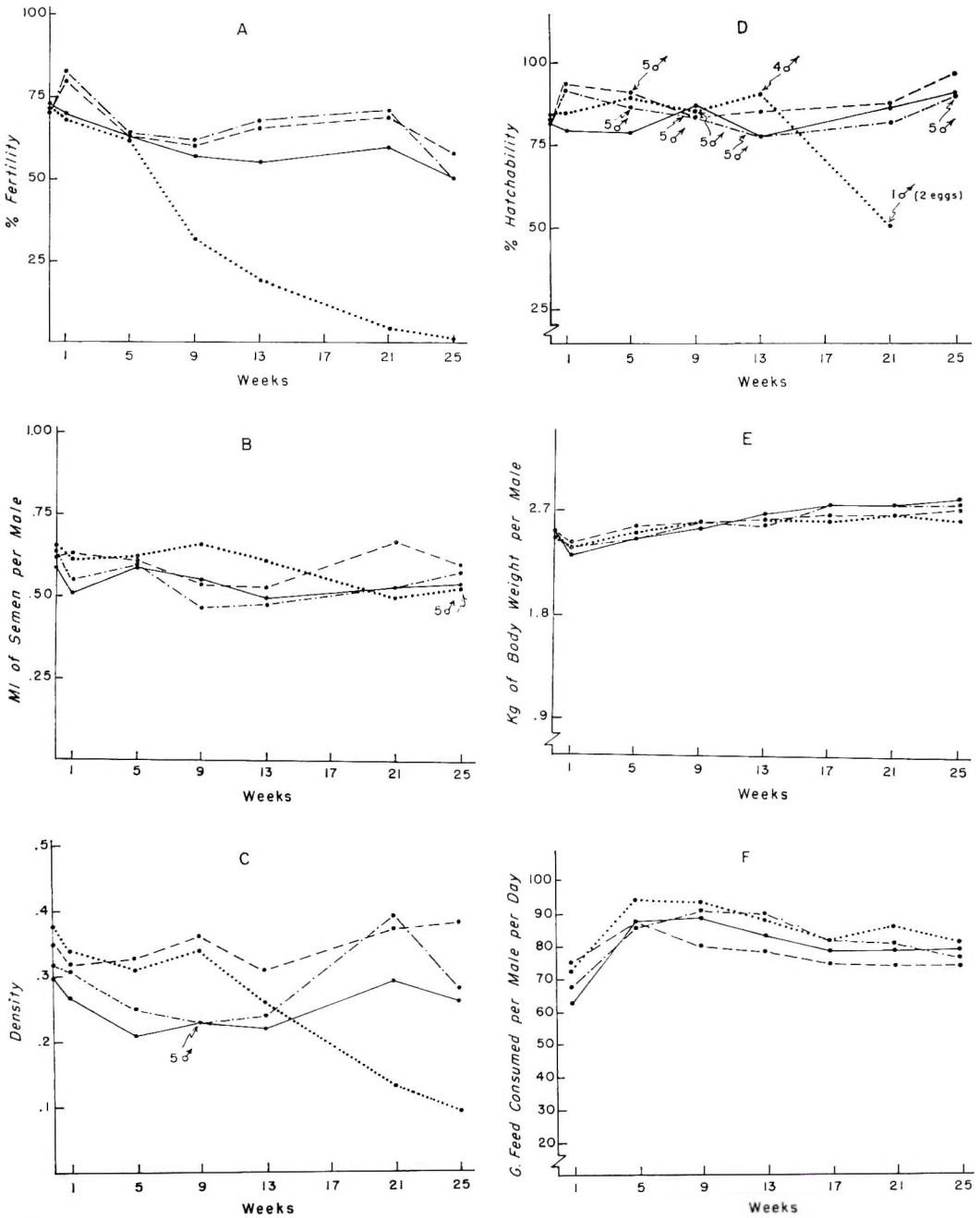

Fig. 1 Influence of linoleic acid (LA), vitamin E (VE) and ethoxyquin (EQ) on (A) fertilizing capacity, (B) volume and (C) concentration of semen; (D) hatchability; (E) body weights and (F) feed consumption in adult male chickens. Legend: low LA-VE-EQ – – –; high LA, low VE-EQ . . . . .; high LA-VE, low EQ ——; high LA-EQ, low VE —·—·—. Numerals indicate number of males from which data were derived when less than 6 males were involved.

TABLE 3

Effect of increasing volume and number of sperm inseminated per hen on fertilizing capacity of males fed diets high in linoleic acid with and without added vitamin E

Treatment	No. males	Volume inseminated	Optical density	Sperm index [1]	Fertility	
					All	Paired
		ml			%	%
		26th Week				
High linoleic acid — low vitamin E (diet 2)	3	0.06(1) [2]	0.046	2.76	0	0
	4	0.06(2)	0.161	9.66	0	0
	8	0.1	—	—	0	—
	12	0.1	0.061	—	0	—
	13	0.07(3)	0.174	12.18	0	0
	17	—	—	—	—	—
Avg			0.111	8.20	0	0
High linoleic acid and vitamin E (diet 3)	7	0.05(1)	0.051	2.55	92.9	92.9
	11	0.04	0.328	—	86.7	—
	15	0.03	0.377	—	92.9	—
	16	0.03	0.420	—	92.3	—
	21	0.04(3)	0.268	10.72	75.0	75.0
	23	0.05(2)	0.215	10.75	23.1	23.1
Avg			0.277	8.01	77.2	63.7
		27th Week				
High linoleic acid — low vitamin E (diet 2)	3	0.1(4)	0.102	10.20	0	0
	4	—	—	—	—	—
	8	0.1(5)	0.076	7.60	0	0
	12	0.1	0.022	—	0	—
	13	0.1(6)	0.215	21.50	0	0
	17	—	—	—	—	—
Avg			0.104	13.10	0	0
High linoleic acid and vitamin E (diet 3)	7	0.03(5)	0.208	6.24	93.8	93.8
	11	0.03(4)	0.319	9.57	100.0	100.0
	15	0.03(6)	0.456	13.68	69.2	69.2
	16	0.03	0.398	—	58.3	—
	21	0.03	0.552	—	100.0	—
	23	0.03	0.456	—	100.0	—
Avg			0.387	9.83	86.9	87.7

[1] To reflect relative numbers of sperm used for insemination, obtained by multiplying the volume of semen used × optical density × 1000.
[2] Numbers in parentheses represent paired males.

min E and ethoxyquin; however, no clear-cut treatment effects on hatchability were observed to 13 weeks and the 21-week figure is based on only one male and 2 fertile eggs, since only one of the 6 males fertilized any eggs at 21 weeks. No differences were observed between treatments for body weight (fig. 1E) and feed consumption (fig. 1F).

No differences in testes size between treatments were noted. Average weight of both testes per male for diets 1, 2, 3 and 4 were 19.3 ± 3.7, 20.5 ± 12.5, 17.3 ± 6.6 and 16.7 ± 2.8 g, respectively.

Increasing the volume of semen used from the group of males fed at the high level of linoleic acid without added vitamin E (diet 2) so that the total number of sperm (sperm index) from this group and the group receiving added vitamin E (diet 3) were more comparable, failed to improve the fertility of eggs during the 26th and 27th week of the experiment (table 3). In all instances where increased amounts of semen were used, fertility was zero from all males fed the diet containing the high level of linoleic acid without vitamin E, indicating that this diet ad-

versely affected the fertilizing capacity of the sperm.

These observations show that a diet containing a high level of linoleic acid in the absence of supplemental levels of vitamin E and ethoxyquin has an adverse effect on fertility of adult male chickens. These observations are interesting in view of those of Machlin et al. (2) showing that high linoleic acid without vitamin E and ethoxyquin resulted in decreased fertility of hens. Contrary to the work of Adamstone and Card (1), feeding a diet to males deficient in vitamin E without a high level of linoleic acid did not adversely affect fertilizing capacity. It is recognized, however, that this experiment did not extend over a 2-year period as did their experiment. These results suggest that vitamin E like ethoxyquin serves primarily as a biological antioxidant. Of particular interest is the observation that semen volume was not adversely affected, whereas sperm concentration was. Furthermore, increasing the number of sperm used for insemination failed to exert any beneficial effect on percentage of eggs fertilized, indicating that feeding rations high in linoleic acid and low in vitamin E and ethoxyquin not only reduced the numbers of sperm but also the fertilizing capacity of those that were produced.

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Interrelationships of Molybdenum and Copper in the Diet of the Guinea Pig

DAVID ARTHUR
Department of Nutrition, Ontario Agricultural College, University of Guelph, Guelph, Ontario, Canada

ABSTRACT Five experiments were conducted to study the effect of molybdenum upon molybdenum and copper metabolism in the guinea pig. An excess of dietary molybdenum resulted in poor growth, excessive mortality and achromotrichia in colored animals. Copper was effective in alleviating the achromotrichia but only partially effective in overcoming growth depression. Molybdenum ingestion increased the molybdenum content of blood, liver, kidney and hair and decreased the copper content of hair. The addition of copper to molybdenum supplemented diets increased the copper content of kidney and hair. There are many similarities between the effects of molybdenum and the molybdenum copper interrelationships observed here for the guinea pig and those reported in the literature for ruminant animals. It is thus suggested that the guinea pig is a reasonably suitable experimental animal for basic work in this area which may have application to ruminants.

The toxicity of excess dietary molybdenum and the therapeutic effect of copper upon molybdenosis has been shown in cattle (1, 2), in rabbits (3) and in rats (4–7). Fairhall et al. (8) studied the effects of molybdenum ingestion upon rats and guinea pigs.

The toxic effects of molybdenum ingestion vary with the species. In cattle the outstanding features of molybdenosis are diarrhea, loss of weight and loss of coat color. In young rabbits the toxic syndrome is characterized by anorexia, loss of weight, alopecia and dermatosis, without diarrhea or achromotrichia. In rats and guinea pigs, anorexia and retardation of growth or loss of body weight appear to be indications of molybdenum toxicity.

A reciprocal antagonism between molybdenum and copper, in which the level of dietary molybdenum affects copper metabolism and the level of dietary copper affects molybdenum metabolism, has been demonstrated in cattle, sheep, rabbits and rats. Such interrelationships have not been studied in the guinea pig.

Molybdenum-induced achromotrichia is characteristic of cattle (2) and of sheep (9) but does not appear in rabbits (3). Although achromotrichia occurs as a result of a simple copper deficiency in rats (10), molybdenum-induced depigmentation does not appear to occur in these animals and its occurrence in guinea pigs has not been reported.

The present paper is a report of 5 experiments conducted in an endeavor to relate the symptoms of molybdenosis observed in cattle to those in a suitable laboratory animal and to increase the knowledge concerning the effect of molybdenum ingestion upon the guinea pig. The effect of molybdenum upon the guinea pig and the interrelationships of copper and molybdenum in this animal were also studied with emphasis being placed upon the composition of blood, liver, kidney and hair.

METHODS AND MATERIALS

Experimental diets were prepared by adding reagent grade sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$) or copper sulphate ($CuSO_4 \cdot 5H_2O$), or both, to each of 2 diets. One basal diet, used in the first 2 experiments, was a commercial laboratory feed [1] which, on analysis, contained 8.9 ppm copper, 0.3 ppm molybdenum, 0.25% sulphate and 23.1% protein. The second basal diet, containing a lower level of copper was used in experiments 3, 4 and 5 and had the following composition: commercial laboratory feed,[2] 2270 g, dry powdered whole

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[1] Guinea Pig Chow, Ralston Purina Company of Canada, Woodstock, Ontario.
[2] See footnote 1.

milk,[3] 2015 g, cellulose,[4] 255 g. This diet contained 5.6 ppm copper, 1.8 ppm molybdenum, 0.13% sulphate and 24.7% protein. Ascorbic acid (0.2 mg/ml) was added to the drinking water in the last 3 experiments to overcome any deficiency which might be induced by diluting the laboratory feed with milk. When compared in a feeding trial, the low copper diet gave equally as good growth as the laboratory feed. In the various experiments, from 3 to 15 individually penned guinea pigs were fed each diet. In all but the first experiment the guinea pigs were weanling animals weighing 250 to 350 g. In the first experiment the animals weighed 300 to 400 g. Both sexes were used and the pigs were randomly allotted to each diet.

The guinea pigs were reared in an air conditioned room, in cages with raised wire-mesh floors. Feed and water were given ad libitum. Criteria in the various experiments included body weight gain, mortality, incidence of grey hair and copper and molybdenum content of hair, blood, liver and kidney. When mortality caused a loss of data, the animals that died during the course of the trial were replaced by others of similar initial weight. Such pigs were maintained with the test diet for the full experimental period.

Whole blood for analysis was obtained by severing the blood vessels of the neck, collecting the blood in a beaker, treating it with sodium oxalate and then digesting a 5-ml aliquot with nitric and perchloric acids before analyzing colorimetrically for copper and molybdenum. When the animal had been exanguinated, liver and kidney tissues were removed, freeze-dried and extracted with diethyl ether before analysis. Samples of hair were washed with 50% ethanol, air-dried and then extracted with diethyl ether before analysis. Copper was determined in feeds and biological material by an adaptation of the 2-2′-biquinoline method of Cheng and Bray (11) and molybdenum by the thiocyanate method of Sandell (12). Sulphate was determined in feeds by the method of Wynne and McClymont (13).

EXPERIMENTAL AND RESULTS

The first experiment was designed to determine the tolerance of guinea pigs to molybdenum. Five guinea pigs, some of which were white and others black, were maintained for 8 weeks with each of 9 diets consisting of commercial laboratory feed[5] to which were added graded levels of molybdenum from 1000 to 8000 ppm. The dietary treatments and results of this experiment are presented in table 1.

Although supplemental molybdenum caused an accumulation of molybdenum in the livers of the animals, it exerted no marked effect upon the copper content of those organs. Weight gains decreased with added molybdenum from zero to 4000 ppm at 1000-ppm increments and losses in

[3] Trumilk, The Borden Company, Toronto, Ontario.
[4] Alphacel, Nutritional Biochemicals Corporation, Cleveland.
[5] See footnote 1.

TABLE 1

Effect of dietary molybdenum upon the copper and molybdenum content of guinea pig livers and upon hair color and growth

Mo added	Liver, dry, fat-free		Greying index [1]	Mortality	Avg 8-week gain
	Copper	Molybdenum			
ppm	*ppm*	*ppm*			*g*
0	30.7 ± 2.6 [2]	7.8 ± 0.6	0/2	0	316 ± 21
1000	34.8 ± 1.5	29.2 ± 1.7	0/1	1	115 ± 15
2000	42.0 ± 1.6	42.4 ± 6.5	2/2	1	73 ± 23
3000	39.7 ± 7.6	52.1 ± 4.2	1/1	1	77 ± 3
4000	36.4 ± 8.2	44.5 ± 8.1	2/2	2	10 ± 2
5000	44.2 ± 8.3	67.2 ± 2.9	0/0	1	−28 ± 20
6000	37.8	82.3	0/0	4	−45
7000	51.7	61.7	1/1	4	−30
8000	31.8 ± 8.8	61.0 ± 3.7	1/1	3	−96 ± 38

[1] Numerator indicates the number of black pigs whose coat turned grey; denominator, the number of black animals fed diet.
[2] SE of mean.

weight occurred beyond the level of 4000 ppm of supplementary molybdenum.

The color of the hair of black guinea pigs, as indicated by the "greying index" of table 1, changed to grey when 2000 ppm or more of molybdenum were added to the commercial diet. The appearance of the grey hair began at the fourth week of the feeding period and appeared over the whole body in individual hairs, giving a "salt and pepper" effect. This phenomenon was observed in red and brown guinea pigs under similar feeding conditions.

Experiment 2 was designed to investigate the effect of additional dietary copper upon the symptoms induced by the feeding of diets supplemented with 2000 ppm molybdenum. The primary interest in this experiment was the effect of the treatments upon the color of the hair. Ten colored guinea pigs, black, black and white or brown were assigned to each diet.

The basal diet in this experiment was the commercial laboratory feed.[6] The experiment was a factorial arrangement involving zero and 2000 ppm molybdenum and zero, 10 and 20 ppm of supplementary copper. The results are shown in table 2.

All of the colored animals whose diets contained 2000 ppm of added molybdenum or 2000 ppm molybdenum and an additional 10 ppm copper, developed grey hair (greying index, table 2). The inclusion of 20 ppm copper, however, overcame this depression of the normal pigmentation in 9 of 10 animals.

Molybdenum accumulated in the liver as a result of supplemental dietary molybdenum in the presence or absence of additional copper in the diet. In agreement with the results of the first experiment, the addition of molybdenum to the diet did not appear to influence the level of copper in the liver. There is an indication, however, that the addition of 20 ppm copper to the diet increased the level of copper in the liver in the absence of supplemental molybdenum and to a lesser extent in the presence of added molybdenum.

The weight gain data indicate that the depression which occurred when molybdenum was fed at a level of 2000 ppm, was only slightly alleviated by the addition of copper to the diet at levels of 10 and 20 ppm.

One of the observations made in the first 2 experiments was the depigmentation of guinea pig hair by molybdenum ingestion. Goss and Green (14) reported that the copper content of normal fleece in sheep was higher than that of fleece which had become grey as a result of molybdenum ingestion. Cunningham and Hogan (15) observed that the molybdenum content of bovine hair and ovine fleece was markedly increased by increased dietary molybdenum. Experiment 3 was conducted to determine whether these phenomena also applied to the hair of the guinea pig. Since it appeared from experiment 2 that the level of copper in the ration influenced the frequency with which the molybdenum-induced depigmentation occurred, it was decided to use the diet containing a low level of copper in com-

[6] See footnote 1.

TABLE 2

Effect of added dietary copper and molybdenum upon hair color, upon the copper and molybdenum content of liver and upon growth of guinea pigs

Diet supplementation		Greying index [1]	Liver composition, dry, fat-free		Avg 8-week gain
Cu	Mo		Cu	Mo	
ppm	*ppm*		*ppm*	*ppm*	*g*
0	0	0/10	23.5 ± 2.8 [2]	6.2 ± 1.0	283 ± 39
10	0	0/10	22.3 ± 2.4	3.4 ± 0.4	242 ± 60
20	0	0/10	59.5 ± 8.4	4.4 ± 0.3	262 ± 46
0	2000	10/10	21.3 ± 3.5	42.4 ± 4.8	92 ± 48
10	2000	10/10	26.0 ± 1.1	28.1 ± 2.9	131 ± 40
20	2000	1/10	34.3 ± 4.3	39.0 ± 4.2	117 ± 39

[1] Numerator indicates the number of colored guinea pigs whose coat turned grey; denominator, the number of colored animals fed diet.
[2] SE of mean.

bination with several levels of supplementary molybdenum.

Fourteen guinea pigs, either black, white, red or brown in color were assigned to each diet. The hair was clipped from the back and sides of the pigs before they were fed the experimental diets and these lots of hair were retained for analysis. After one week the hair was clipped again to insure that any hair resembling the first cut in composition would have passed through the epidermis. This hair was discarded. After 4 weeks, the growth period of guinea pig hair (16), the hair was clipped again and retained for analysis. At this time the control diet was substituted for the molybdenum-supplemented diets. One week later the hair was clipped again and discarded. The pigs were continued with the control diet for a further 3 weeks, during which time the hair grew sufficiently to obtain an adequate sample. The hair was clipped and analyzed. The results of this experiment are shown in table 3.

The data in table 3 show that during the 5-week period when the diets contained supplementary molybdenum, the level of copper in the hair was reduced to less than half and the molybdenum increased markedly as the level of supplementary molybdenum was increased. In the second period of the experiment, when all of the pigs received the basal diet, the copper content of the hair was largely restored and there was a marked reduction in the molybdenum content of new hair of the pigs which had been fed molybdenum in the first phase of the experiment. Examination of the data, grouped according to hair color, showed that color was not consistently related to the copper or molybdenum content either in the control or experimental diets.

The greying indexes in table 3 show that during the period of molybdenum ingestion the developing hairs of some of the colored guinea pigs were grey, the effect being pronounced at levels of 500 ppm and greater. With the change to the basal diet, normal pigmentation of the new hair took place.

Weight gains of the animals fed the molybdenum-supplemented diets were less than the gains of those fed the control diet. In the second phase of the experiment

TABLE 3

Effect of molybdenum ingestion and the subsequent ingestion of a normal diet upon the composition of guinea pig hair and upon weight gains

Mo added	Initial composition of hair		Experimental diets [1]				Control diet resumed [1]			
			Composition of hair		Greying index [2]	Weight gain	Composition of hair		Greying index [2]	Weight gain
	Cu	Mo	Cu	Mo			Cu	Mo		
ppm	*ppm*	*ppm*	*ppm*	*ppm*		*g*	*ppm*	*ppm*		*g*
0	9.8 ± 0.3 [3]	2.8 ± 0.1	11.2 ± 0.5	3.1 ± 0.2	0/11	163 ± 9	10.9 ± 0.5	2.9 ± 0.3	0/11	120 ± 12
200	9.6 ± 0.2	2.4 ± 0.1	6.3 ± 0.6	25.8 ± 2.6	1/14	123 ± 14	10.4 ± 0.5	4.2 ± 0.3	0/14	121 ± 12
500	10.7 ± 0.3	2.6 ± 0.1	4.4 ± 0.4	48.7 ± 3.3	7/12	106 ± 22	10.3 ± 0.3	5.2 ± 0.4	0/12	105 ± 13
1000	10.3 ± 0.4	2.7 ± 0.1	5.1 ± 0.8	66.8 ± 3.3	6/9	56 ± 17	10.0 ± 0.5	6.5 ± 0.4	0/9	155 ± 13
2000 [4]	9.7 ± 0.7	2.8 ± 0.2	4.1 ± 0.3	96.4 ± 5.1	9/11	−18 ± 3	8.4 ± 1.0	7.8 ± 0.4	0/11	175 ± 9

[1] Mo-supplemented diets fed for first 5 weeks and the unsupplemented diet from the fifth to the ninth week.
[2] Numerator indicates the number of colored pigs whose coat turned grey; denominator, the number of colored animals fed diet.
[3] SE of mean.
[4] Mortality — Seven black and white pigs and one brown pig died with diet supplemented with 2000 ppm molybdenum.

there was a compensatory improvement in growth in those animals whose weight gains had been depressed by molybdenum feeding, with these animals tending to catch up to those fed the lower levels of the mineral.

Since the ingestion of molybdenum increased the molybdenum content of hair and lowered its copper content, in experiment 3, the following experiment was conducted to determine the effect upon other tissues, namely, blood, liver and kidney. In this preliminary trial 3 weanling guinea pigs were assigned to each of 5 low copper diets, supplemented with zero to 2000 ppm molybdenum, for 8 weeks. One animal, fed at the highest level of molybdenum, died during the experimental period and was not replaced. The results of this experiment are presented in table 4.

There was an increase in the level of molybdenum in the blood, liver and kidney with increasing levels of molybdenum in the diet. The addition of molybdenum to the diet resulted in an increase in the copper level in the blood and kidney. These elevated levels of copper were relatively constant, being independent of the level of molybdenum from 200 to 2000 ppm added to the diet. The copper content of the liver decreased as the level of molybdenum in the diet was increased above 500 ppm. As in previous experiments, weight gain decreased with an increase in dietary molybdenum.

Previous experiments indicated that molybdenum ingestion resulted in changes in the copper and molybdenum content of guinea pig hair and in a depression of growth. The results of experiment 2 showed that the addition of copper to a molybdenum-supplemented diet could overcome molybdenum-induced achromotrichia. Experiment 5 was designed to study the effect of additional dietary copper upon the composition of guinea pig hair and other tissues and upon the growth of animals suffering from molybdenosis.

Fifteen weanling guinea pigs of mixed colors were assigned at random to each of 6 diets for 9 weeks. The basal diet contained the low level of copper and served as a control. To the other 5 diets were added 500 ppm molybdenum and graded levels of copper from 3 to 24 ppm. The hair was clipped from the back and sides of the pigs before they were fed the diets and it was analyzed for copper and molybdenum. One week later the hair was clipped again and discarded. At the end of the fifth and ninth weeks hair samples were obtained and analyzed. The animals were killed at the end of 9 weeks, when samples of blood, liver and kidney were obtained for analysis. The results of this experiment are shown in table 5.

When 500 ppm molybdenum were added to the control diet, the copper content of the hair was decreased and the molybdenum content increased. These changes were evident in both the fifth- and ninth-week samples which were almost the same in their content of these minerals. The addition of copper to the molybdenum-supplemented diets resulted in an increase in the copper content of the hair and 17 ppm overcame the depression in copper content of the hair which resulted from the addition of 500 ppm molybdenum to the diet. Copper was without effect in altering the high molybdenum content (50 to 60 ppm) of the hair, brought about by the ingestion

TABLE 4

Effect of dietary molybdenum upon the composition of blood, liver and kidney and upon the growth of guinea pigs

Mo added	Blood [1]		Liver		Kidney [1]		Avg 8-week gain
	Cu	Mo	Cu	Mo	Cu	Mo	
ppm	*μg/100 ml*	*μg/100 ml*	*ppm*	*ppm*	*ppm*	*ppm*	*g*
0	28	45	12.5±0.9 [2]	3.7±0.3	24.6	4.5	235±13
200	259	600	16.3±2.7	17.0±3.1	77.3	72.6	145±32
500	207	828	12.2±1.5	18.2±1.7	68.4	77.1	128±7
1000	252	1101	9.7±2.9	28.3±3.3	—	84.3	99±18
2000	231	1440	6.7±1.5	31.3±1.9	84.4	127.5	6±2

[1] Analyses of blood and kidney on pooled samples.
[2] SE of mean.

TABLE 5

Effect of added dietary molybdenum and copper upon the molybdenum and copper content of hair, blood, liver, kidney and upon the growth of guinea pigs

Treatment		Hair						Blood		Liver		Kidney		9-week gain
		Initial		5 Weeks		9 Weeks								
Cu	Mo	Cu	Mo	Cu	Mo	Cu	Mo	Cu	Mo	Cu	Mo	Cu	Mo	
ppm	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*µg/100 ml*	*µg/100 ml*	*ppm*	*ppm*	*ppm*	*ppm*	*g*
0	0	9.6 ± 0.5 [1]	2.4 ± 0.4	10.9 ± 0.6	1.4 ± 0.2	12.9 ± 0.5	1.3 ± 0.1	50 ± 6	25 ± 3	14.4 ± 0.4	4.2 ± 0.1	19 ± 1	2.5 ± 0.2	278 ± 17
0	500	9.3 ± 0.4	1.8 ± 0.1	4.4 ± 0.4	49.6 ± 3.8	4.7 ± 0.3	45.4 ± 3.0	221 ± 20	551 ± 14	16.8 ± 2.7	16.0 ± 2.1	75 ± 22	62 ± 11	175 ± 17
3	500	9.6 ± 0.3	1.8 ± 0.2	5.4 ± 0.5	50.1 ± 3.0	6.2 ± 0.4	48.9 ± 3.3	235 ± 2	583 ± 19	18.2 ± 2.2	17.5 ± 0.7	96 ± 15	74 ± 13	195 ± 15
10	500	9.8 ± 0.4	2.3 ± 0.5	7.0 ± 0.7	49.6 ± 4.1	7.1 ± 0.7	48.9 ± 4.8	224 ± 1	512 ± 20	29.1 ± 5.8	24.2 ± 4.4	130 ± 3	103 ± 9	228 ± 14
17	500	10.5 ± 0.7	1.0 ± 0.2	10.7 ± 0.6	60.0 ± 5.2	11.2 ± 0.8	54.2 ± 4.4	255 ± 6	561 ± 35	42.4 ± 10.3	24.3 ± 2.9	162 ± 39	140 ± 13	236 ± 17
24	500	10.3 ± 0.5	1.9 ± 0.3	12.1 ± 0.5	50.0 ± 4.7	12.3 ± 0.8	47.8 ± 4.4	257 ± 8	501 ± 12	81.5 ± 8.9	26.7 ± 4.4	185 ± 9	123 ± 9	246 ± 23

[1] SE of mean.

of a diet containing 500 ppm molybdenum. The coats of the colored guinea pigs that were fed 500 ppm molybdenum but no additional copper lost color at 4 to 5 weeks. No greying was observed in the coats of those animals fed the control diet or fed the diets supplemented with 3 to 24 ppm copper.

The levels of copper and molybdenum in the blood (table 5) increased as the molybdenum in the diet was increased by 500 ppm. The addition of copper to the diet did not change appreciably either of these elevated levels of copper or molybdenum.

The inclusion of 500 ppm molybdenum in the diet increased the molybdenum content of the liver and it did not change the level of liver copper appreciably. However, increasing the copper content of the diets which contained supplemental molybdenum increased both the copper and molybdenum content of the liver.

Molybdenum supplementation of the diets increased both the molybdenum and copper content of the kidneys. Increasing the copper in high molybdenum diets further increased both the copper and molybdenum observed in the kidneys.

The addition of 500 ppm molybdenum to the control diet resulted in reduced weight gain. Increasing the copper content of the molybdenum-supplemented diets tended to overcome this depression but the highest level of copper added (24 ppm) did not entirely restore the growth of the guinea pigs (table 5).

DISCUSSION

The results of these experiments indicate that dietary molybdenum interferes with the utilization of copper by the guinea pig and that part of this interference can be overcome by the addition of copper to the diet. In some respects the observations made here are similar to those reported for the effects of molybdenum in ruminant animals.

The addition of molybdenum to the guinea pig diets (exps. 1, 2 and 3) resulted in achromotrichia, a condition which disappeared when the molybdenum was withdrawn (exp. 3) and which could be reduced by the addition of copper to the diet (exp. 2). The phenomenon of varying degrees of achromotrichia noted in our experiments has also been observed in cattle (2) and sheep (9) under similar conditions.

An increase in dietary molybdenum resulted in an increased deposition of molybdenum in guinea pig hair and a decrease in its copper content (exps. 3 and 5). Further additions of copper to the diets overcame the effect of molybdenum upon the copper content but did not alter the molybdenum content, indicating that although the molybdenum was still present, its effect upon the copper composition of the hair was limited. Similar effects have been observed in cattle and sheep (15).

When the concentration of copper in the diet was low, the ingestion of molybdenum decreased the amount observed in the liver (exp. 4) but in the presence of sufficient dietary copper the level of copper in the liver was maintained (exps. 1 and 2).

Dick (18) showed that in sheep there was an increase in the copper content of liver as the total daily copper intake was increased. He reported that dietary molybdenum limited the amount of copper stored in the liver and that there was a quantitative relationship between the copper intake and the amount of molbydenum which had to be given to prevent the accumulation of copper in the liver.

The increase in the copper content of the blood with molybdenum consumption (exps. 4 and 5), as observed in this study indicates a probable withdrawal of copper from other tissues. Dick (17) reported that at very high levels of molybdenum and of sulphate in the diet there was an immediate increase in the copper content of the blood. Cunningham et al. (19), however, reported that Jersey cattle fed a diet low in copper and containing 0.1% sulphate showed a decrease in the concentration of copper in the blood when additional molybdenum was included in the diet. The effect of varying the sulphate content of guinea pig diets is presently being studied.

Analyses of guinea pig kidney tissue in cases where excess molybdenum was fed indicated a marked increase in both the molybdenum and copper content. The increased molybdenum content may be a reflection of greater excretion in the urine of the ingested molybdenum as this is the

normal excretory pathway. Whether or not elevated levels of copper in the kidney are an indication of copper excretion in the urine as a result of molybdenum ingestion is a matter for further study.

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Urinary Nitrogen and Sulfur Excretion in Dogs under Different Dietary Treatments [1,2]

RICARDO BRESSANI, J. EDGAR BRAHAM, LUIZ G. ELÍAS AND RAÚL BALCONI [3]

Institute of Nutrition of Central America and Panama (INCAP), Guatemala, Central America

ABSTRACT Nitrogen balance studies with dogs were carried out to determine the relationship between urinary nitrogen and sulfur excretion, under different dietary treatments. The results indicate that the variation of urinary sulfur is slightly higher than that of urinary nitrogen excretion. Although urinary nitrogen and sulfur excretion varied among individual dogs, the N/S ratio was similar for all animals. A parallelism between urinary nitrogen and sulfur excretion was noted when the protein of the diet fed was of relatively high nutritional value, during a decrease in nitrogen intake and during protein depletion. Urinary nitrogen and sulfur excretion did not parallel each other when the animals were fed at a low level of a methionine-deficient protein and its supplementation with the amino acid. A decrease in the intake of calories caused an increase in urinary nitrogen excretion but did not affect urinary sulfur excretion. It was also observed that the supplementation of a protein deficient mainly in lysine and tryptophan with small amounts of good quality proteins decreased urinary nitrogen excretion but did not affect sulfur excretion. It is suggested that the determination of the urinary sulfur excretion helps in the interpretation of nitrogen balance studies, as related to diet and physiological condition of the animal.

Relatively few studies have reported the factors affecting excretion of urinary sulfur. Since methionine is an essential amino acid and component of proteins, investigations of urinary sulfur excretion would be helpful in obtaining more information on nitrogen metabolism as related to diet. Eckert (1) observed a linear relationship between absorbed sulfur and urinary inorganic sulfate sulfur, similar to that described by Allison and Anderson (2) for absorbed and urinary nitrogen and nitrogen balance. It has been suggested, therefore, that sulfur excretion can be used as a measure of protein quality. Other workers (1, 3–5), studying the relationship between urinary nitrogen and sulfur excretion, noted a close relationship between the 2 elements. Bressani et al.[4] also studied sulfur excretion in comparison with nitrogen excretion in amino acid imbalance feeding conditions. In these studies a difference in the pattern of excretion between sulfur and nitrogen in urine was reported. Recently, Miller and Naismith (6) and Miller and Donoso (7) studied the sulfur content of foods, as an indicator of the deficiency of total sulfur-containing amino acids in proteins and of the protein value of diets. They reported that the sulfur content of food could be used to predict protein value. More information on sulfur metabolism and its relation to nitrogen metabolism is needed to interpret more efficiently the newer modifications of protein quality evaluation (8, 9). Therefore, the results of several experiments in dogs carried out during the last 3 years are presented in this paper to show the behavior of urinary sulfur excretion in comparison with nitrogen excretion under different dietary treatments.

MATERIAL AND METHODS

In a series of experiments, factors affecting nitrogen balance were studied to determine whether urinary sulfur excretion would behave as urinary nitrogen excretion. The experiments were carried

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[3] Mr. Balconi is presently a Rockefeller Foundation Fellow in the Department of Animal Science, North Carolina State of the University of North Carolina at Raleigh.

[4] Unpublished data, R. Bressani, L. G. Elias and J. E. Braham.

out in healthy, deparasitized dogs 3 to 8 months of age. The animals were housed in individual metabolism cages, weighed daily before feeding, and fed once a day at 8:00 A.M. Feeding was performed by weighing daily the amount of food according to body weight and adding to it 600 cm^3 of hot water and allowing to cool to 32° before feeding. Balance periods lasted 4 days. Urine was collected in dark bottles containing 1 cm^3 of concentrated acetic acid and both urine and feces were collected twice a day and stored at 4° until analysis was performed. Each 4-day collection of urine and of feces was homogenized, volume and weight noted and representative aliquots withdrawn for nitrogen analysis by the Kjeldahl method. Total inorganic sulfur was measured in urine using the gravimetric method of Folin (10). No attempts were made to determine ethereal sulfate and sulfur and organic sulfur excretion, since they have been shown to be independent of sulfur intake, are not influenced by diet and tend to remain nearly constant (1, 3, 11, 12).

Variability of urinary nitrogen and sulfur excretion. In the first experiment the variability in urinary nitrogen and sulfur excretion under constant nitrogen and calorie intake was studied. Four male dogs, 3 months of age, from the same litter were used. The initial weights were 4.35, 4.17, 4.76 and 4.01 kg. The dogs were fed approximately 6 g of protein and 130 kcal/kg body weight/day for 32 days with a casein diet and 32 days on a diet made with INCAP vegetable mixture 9 (13). The casein diet fed consisted of: (in per cent) casein, 25; DL-methionine, 0.3; hydrogenated vegetable fat, 10; mineral mixture (14), 2; cod liver oil, 1; cellulose, 2.7; sucrose, 15; dextrin, 7.4; and dextrose, 36.6; plus 5 ml of a complete vitamin solution (15) per 100 g. This diet contains around 22% protein and 418 kcal/100 g. To adjust the intake of calories to the required level, a nitrogen-free diet was fed in which casein and methionine in the diet described above were replaced by dextrin; the nitrogen-free diet contained 418 kcal/100 g. Vegetable mixture 9 was fed as such together with specific amounts of the nitrogen-free diet. In this experiment, 8 consecutive balance periods of 4 days' duration each, were analyzed for each protein source.

Effect of supplementation of casein with methionine at 3 levels of nitrogen intake. In the second experiment, 9 dogs divided in groups of three, were used. One group received 3.0 g of protein, the second 6.0 g and the third 7.2 g of protein/kg body weight/day. Calories were set at 120 kcal/kg/day. Data on three 4-day balance periods were obtained from each group of dogs with a casein diet equal in composition to the one described above but without added methionine, and also three 4-day balance periods for each group of dogs with the casein-plus-methionine diet described above. The group fed low protein had an average initial weight of 2.70, the second group averaged 4.02 and the third 8.08 kg and the ages ranged from 5 to 7 months.

Effect of decreasing nitrogen intake. In a third experiment, 3 dogs with an initial weight of 9.05, 9.48 and 9.04 kg were used. The dogs were fed the casein-plus-methionine diet described above. The protein intake for three 4-day balance periods was about 1.1 g/kg/day. It was then decreased to approximately 0.5 g, whereas the intake of calories was adjusted to 120 kcal/kg/day, by feeding small amounts of the nitrogen-free diet. The lower level of nitrogen intake was maintained for 8 additional 4-day balance periods.

Effect of protein depletion. The fourth experiment was designed to study urinary nitrogen and sulfur excretion during protein depletion followed by protein repletion. Four dogs, initially weighing 16.7, 11.4, 13.4, and 10.6 kg, were used. The animals were fed a nitrogen-free diet of the following percentage composition: cornstarch, 20; mineral mixture (14), 2; cod liver oil, 1; and 5 ml of a complete vitamin solution (15) per 100 g of diet. The diet contained 436 kcal/100 g and the dogs received an amount of food providing 130 kcal/kg of body weight/day. The nitrogen-free diet was fed for 6 balance periods of 4 days duration each. After the sixth period, or 24 days, the dogs were repleted by feeding vegetable mixture 9 (13) as the source of protein, and the nitrogen-free diet described above as

the source of additional calories and other nutrients for 3 more 4-day balance periods.

Effect of calorie restriction. The effect of calorie intake was studied in the fifth experiment, in which 4 dogs were used, fed at a constant level of protein at 2 levels of intake of calories, 140 and 85 kcal/kg body weight/day. At the beginning of the study the animals weighed 8.41, 6.89, 8.69, and 6.64 kg. Vegetable mixture 9 (13) was fed as the source of protein, and the intake of calories was adjusted with the nitrogen-free diet described above. Protein intake at the low and high intake of calories was approximately 6.0 g/kg body weight/day. At the higher intake of calories, eight 4-day balance periods were analyzed, whereas at the lower level five 4-day balance periods were examined.

Effect of the supplementation of poor quality protein on urinary sulfur. Finally, to test the sensitivity of sulfur as a measure of supplementation of poor quality protein, a sixth experiment was carried out. In this study, 4 dogs weighing at the start 6.94, 5.73, 6.57, and 6.22 kg were used. For two 4-day balance periods, they were fed about 3.0 g of protein/kg body weight/day of a diet made up of the following: (in per cent) lime-treated corn flour, 78.0; corn gluten, 4.0; skim milk, 5.0; mineral mixture, 2.0; hydrogenated vegetable fat, 6.0; cod liver oil, 1.0; and vitamins. The milk in the diet was then replaced by an equivalent amount of corn gluten and the diet fed to the dogs for two 4-day periods. In the last stage, 4% fish flour replaced an equivalent amount of corn gluten protein and the diet was fed for two more 4-day balance periods. In all experiments, the handling of the dogs was the same.

RESULTS

Variability of urinary nitrogen and sulfur excretion. Table 1 presents the results of the first experiment. The coefficient of variation in urinary sulfur excretion was higher than that of urinary nitrogen excretion for both sources of protein. However, there was no statistical evidence that this difference was significant. It is probable that the higher coefficient of variation for sulfur excretion is due to the lower accuracy of the sulfur determination, as compared with the analysis of nitrogen in urine. Although urinary nitrogen and sulfur excretion varied among individual dogs, the N/S ratio was similar for all animals, within the diet fed.

TABLE 1

Variation [1] in urinary nitrogen and sulfur excretion in 4 dogs fed 2 types of protein [2]

Dog no.	Nitrogen			Urinary sulfur	Urinary N/S ratio
	Intake	Urine	Retention		
	mg/kg/day	*mg/kg/day*	*mg/kg/day*	*mg/kg/day*	
			Casein		
1	954	450	422	34.4	13.3
2	959	462	397	35.0	13.7
3	956	518	383	36.7	14.7
4	958	554	310	36.4	15.6
Avg	959	491	378	36.7	13.5
SD	34.5	36.4	35.8	3.5	2.5
C [3]	3.6	7.4	9.5	9.1	18.5
			Vegetable mixture 9		
1	936	335	320	19.7	17.0
2	940	443	245	24.5	18.0
3	943	340	322	19.5	17.7
4	940	450	267	25.0	18.1
Avg	940	392	288	22.2	17.8
SD	25.4	32.9	50.1	2.3	2.1
C	2.7	8.4	17.4	10.4	11.8

[1] Estimates of variability on a within dog basis.
[2] Each value represents the average of eight 4-day balance periods.
[3] Coefficient of variation.

TABLE 2

Effect of feeding casein at 3 levels with and without methionine on urinary nitrogen and sulfur excretion in 9 dogs

Diet fed	Balance no.[1]	Nitrogen			Urinary sulfur	Urinary N/S ratio
		Intake	Urine	Retained		
		mg/kg/day	*mg/kg/day*	*mg/kg/day*	*mg/kg/day*	
Casein	1	497	453	− 4	16.8	26.9
	2	505	437	+ 7	13.3	32.8
	3	511	427	+47	9.7	44.0
	Avg	504 ± 12.0	439 ± 53.0	+16 ± 49.3	13.3 ± 3.6	
Casein + DL-methionine	1	534	361	126	10.3	35.2
	2	558	295	241	11.1	26.7
	3	561	269	250	12.8	21.0
	Avg	551 ± 57.7	308 ± 63.7	206 ± 69.7	11.4 ± 3.6	
Casein	1	998	578	375	16.1	35.9
	2	1006	563	400	14.5	38.7
	3	973	554	389	15.2	36.5
	Avg	992 ± 19.0	565 ± 37.0	388 ± 38.3	15.3 ± 1.4	
Casein + DL-methionine	1	953	539	384	23.4	23.0
	2	994	499	452	23.7	21.1
	3	976	518	426	22.7	22.8
	Avg	974 ± 20.0	519 ± 29.3	420 ± 40.3	23.3 ± 2.9	
Casein	1	1161	617	504	17.3	35.6
	2	1148	776	294	22.7	34.1
	3	1130	750	348	24.7	30.4
	Avg	1146 ± 23.0	714 ± 79.3	382 ± 105.0	21.6 ± 3.1	
Casein + DL-methionine	1	1134	705	375	24.2	29.1
	2	1120	746	323	34.0	21.9
	3	1106	642	418	27.2	23.6
	Avg	1120 ± 20.3	698 ± 72.0	372 ± 71.3	28.5 ± 7.8	

[1] Each balance period was of 4 days' duration.

Effect of supplementation of casein with methionine at three levels of nitrogen intake. Table 2 summarizes the results of the second experiment. At the low and intermediate level of nitrogen intake when no methionine was added, urinary nitrogen and sulfur excretion decreased with respect to time. At the high level, urinary nitrogen and sulfur excretion increased with respect to time. Addition of methionine at the low and intermediate level of nitrogen intake decreased urinary nitrogen excretion, whereas urinary sulfur remained essentially the same at the low level of intake and increased at the intermediate level. When methionine was added at the high level of nitrogen intake, urinary nitrogen and sulfur excretion showed no trend, although on the average, nitrogen excretion was less than with the unsupplemented diet, whereas sulfur excretion was higher. The N/S ratio with the unsupplemented diet increased at the low level of nitrogen intake and decreased at the high, with no change observed at the intermediate level. When the supplement was added, the N/S ratio decreased with respect to time at the low level of nitrogen intake but showed no trend at the other 2 levels studied. When averages per treatment were compared, the N/S ratios were higher when the diet was without the methionine supplement than when it was added.

Effect of decreasing nitrogen intake. Table 3 shows the effect of decreasing the level of nitrogen intake from approximately 1.0 to 0.5 g/kg/day. Urinary nitrogen and sulfur excretion was high at the higher level of nitrogen intake and decreased when the intake of nitrogen was decreased. From the fourth period on, both urinary nitrogen and sulfur tended to

increase. The urine N/S ratio, however, remained constant.

Effect of protein depletion. Table 4 presents the results of protein depletion on urinary nitrogen and sulfur excretion. Both urinary nitrogen and sulfur excretion decreased as depletion progressed, from the third to sixth period of depletion when they became almost constant. Feeding of protein increased both urinary nitrogen and sulfur excretion. The N/S ratio, however, decreased during nitrogen deprivation, but was similar before and after protein depletion.

Effect of calorie restriction. The results on the effect of the intake of calories on urinary nitrogen and sulfur excretion is shown in table 5. At the high level of intake of calories both nitrogen and sulfur excretion were parallel and did not differ between periods. At the lower level of calorie intake, urinary nitrogen and sulfur excretion were also similar between periods, except in period 2 in which excretion of both was less. On the average, the low level of calorie intake resulted in increased nitrogen excretion in urine, but in no change in urinary sulfur when compared with the higher caloric intake level. The N/S ratio was also higher for the lower intake of calories.

Effect of the supplementation of poor quality protein on urinary sulfur. Table 6 presents the data obtained on the effect of

TABLE 3

Effect of decreasing nitrogen intake on the urinary nitrogen and sulfur excretion in dogs

Balance no.[1]	Nitrogen		Urinary sulfur	Urinary N/S ratio
	Intake	Urine		
	mg/kg/day	*mg/kg/day*	*mg/kg/day*	
Initial	1120 ± 20 [2]	698 ± 62	28.5 ± 7.2	24.5
1	543 ± 8	460 ± 40	19.4 ± 1.1	23.7
2	539 ± 6	393 ± 15	18.6 ± 2.2	21.1
3	532 ± 6	370 ± 23	14.4 ± 2.7	25.7
4	540 ± 8	334 ± 16	13.0 ± 0.9	25.7
5	550 ± 14	370 ± 10	12.8 ± 0.9	28.9
6	603 ± 20	408 ± 20	16.8 ± 0.9	24.3
7	607 ± 22	447 ± 3	18.4 ± 2.8	24.3
8	564 ± 24	390 ± 16	15.8 ± 0.2	24.7

[1] Average of the 4-day balance periods of 3 dogs.
[2] SD.

TABLE 4

Effect of protein depletion and repletion on urinary nitrogen and sulfur excretion in 4 dogs

Balance no.[1]	Nitrogen		Urinary sulfur	Urinary N/S ratio	Avg wt
	Intake	Urine			
	mg/kg/day	*mg/kg/day*	*mg/kg/day*		*kg*
			Before depletion		
1	979 ± 4	497 ± 86	23.6 ± 1.8	21.0	12.30 ± 2.39
2	978 ± 1	501 ± 80	26.4 ± 5.1	19.0	12.56 ± 2.46
3	975 ± 6	453 ± 101	25.4 ± 6.6	17.9	12.86 ± 2.57
			Depletion		
1	—	143 ± 21	11.6 ± 1.4	12.3	13.05 ± 2.72
2	—	114 ± 21	9.9 ± 1.5	11.5	12.93 ± 3.34
3	—	89 ± 20	8.1 ± 2.3	11.0	12.76 ± 2.70
4	—	84 ± 19	7.8 ± 1.1	10.8	12.71 ± 2.68
5	—	82 ± 5	7.7 ± 0.9	10.7	12.70 ± 2.76
6	—	87 ± 29	7.1 ± 1.5	12.2	12.63 ± 2.52
			Repletion		
1	986 ± 42	329 ± 96	14.4 ± 4.4	22.9	12.54 ± 2.08
2	951 ± 39	342 ± 115	14.4 ± 5.3	23.8	12.69 ± 1.06
3	964 ± 58	360 ± 114	16.9 ± 5.1	26.6	13.12 ± 1.90

[1] Average of the 4-day balance periods of 4 dogs.

TABLE 5

Effect of intake of calories on urinary nitrogen and sulfur excretion in dogs (average 4 dogs)

Balance no.	Nitrogen			Urinary sulfur	Urinary N/S ratio
	Intake	Urine	Balance		
	mg/kg/day	*mg/kg/day*	*mg/kg/day*	*mg/kg/day*	
	Intake of calories, 140 kcal/kg/day				
1	940 ± 28	404 ± 71	276 ± 53	23.2 ± 13.1	17.5
2	939 ± 23	386 ± 68	312 ± 41	22.4 ± 3.6	17.4
3	939 ± 23	397 ± 51	284 ± 40	21.9 ± 3.2	18.1
4	941 ± 28	382 ± 72	282 ± 65	21.3 ± 3.2	17.9
Avg	940 ± 24	392 ± 65	288 ± 50	22.2 ± 5.8	17.7
	Intake of calories, 85 kcal/kg/day				
1	1005 ± 4	485 ± 69	305 ± 61	21.2 ± 3.7	22.9
2	1006 ± 24	423 ± 155	358 ± 112	18.9 ± 7.7	22.4
3	1014 ± 17	536 ± 131	241 ± 94	23.4 ± 5.8	23.0
4	1016 ± 22	594 ± 120	179 ± 87	24.1 ± 4.8	24.7
5	1014 ± 17	581 ± 95	219 ± 66	23.3 ± 3.3	24.9
Avg	1011 ± 17	524 ± 114	260 ± 84	22.2 ± 5.1	23.6

TABLE 6

Effect of supplementing a corn protein diet with skim milk and fish flour on urinary nitrogen and sulfur excretion (average for 4 dogs)

Additions to basal diet	Nitrogen			Urinary sulfur	Urinary N/S ratio
	Intake	Urine	Balance		
	mg/kg/day	*mg/kg/day*	*mg/kg/day*	*mg/kg/day*	
5% skim milk	484 ± 14	272 ± 16	95 ± 19	20.4 ± 1.9	13.3
None	494 ± 10	307 ± 38	60 ± 97	19.8 ± 1.7	15.5
4% fish flour	480 ± 6	219 ± 8	151 ± 11	18.9 ± 1.7	11.9

supplementing poor quality protein with proteins of good quality on the excretion of urinary nitrogen and sulfur. The addition of skim milk and of fish flour resulted in lower excretion of nitrogen, but urinary sulfur did not change. The N/S ratio for the unsupplemented diet was slightly higher than the N/S ratio resulting from supplementation.

DISCUSSION

The results observed in most of the experiments reported indicate that, in general, urinary sulfur excretion parallels the urinary excretion of nitrogen. In all phases of metabolism there appears to be a parallelism between nitrogen and sulfur. They occur together in the proteins of food and are stored together in body proteins. Although there are many compounds of significance in body economy which contain only one or the other of these 2 elements, the preponderance of both nitrogen and sulfur storage goes into the synthesis of protein. From the results presented, however, it is evident that factors which affect nitrogen excretion in the urine do not necessarily affect the excretion of sulfur in the same manner. This difference may be useful in understanding the metabolic interaction between different nutrients, and in the physiological state of the organism with respect to nitrogen and sulfur needs.

In the studies presented, only inorganic sulfate sulfur was determined in the urine, since it has been demonstrated in dogs (1, 11, 12) that excretion of inorganic sulfate sulfur is directly proportional to absorbed sulfur and that ethereal sulfate and sulfur and organic sulfur excretion are independent of sulfur intake. Similar results have been reported by Beach et al. (3) in studies of 8 normal children. These authors reported that inorganic sulfate sulfur in urine accounted for 84% of the total urinary sulfur which is similar to the value reported by Eckert (1). Beach et al. (3)

also stated that inorganic sulfate sulfur in urine depends primarily on the level of sulfur intake and the ethereal and organic or neutral sulfur are influenced less by diet and tend to remain more nearly constant.

Fecal loss of sulfur also appears to remain constant according to the results of Eckert (1), Beach et al. (3) and Wright et al. (5), and it is equal, or nearly so, to the inorganic sulfur content of the diet. Eckert (1) showed that fecal sulfur excretion did not increase in magnitude when casein sulfur was added to the diet, a result which suggested that all the protein sulfur was absorbed by the animal.

The N/S ratios in the present study under normal protein feeding conditions were similar to those reported by Lewis (4) in dogs, and Beach et al. (3) and Wright et al. (5) in children.

Lewis (4) observed in growing dogs that the N/S retention ratio is around 14.5 and showed that during fasting and consequent tissue catabolism a urinary N/S ratio of 13 to 16 was obtained. In the present investigation, decreasing nitrogen intake decreased the nitrogen and sulfur excretion and a parallelism between urinary nitrogen and sulfur excretion was observed. Feeding a protein-free diet, however, did not affect the N/S ratio and the decrease in absolute amounts of N and S were essentially the same proportion. Again a parallelism between the 2 elements was observed.

A difference in behavior between urinary nitrogen and sulfur excretion was also observed when calories became limiting. A reduction in calories increased nitrogen excretion but did not affect sulfur excretion, suggesting an economy by the animal of sulfur-containing amino acids, which are limiting in muscle protein. This observation was in agreement with the results of Allison et al. (16) and Swanson (17) who reported that methionine supplementation to protein-free diets fed to dogs and rats with full stores of nitrogen resulted in decreased excretion of nitrogen in urine.

Finally, urinary sulfur excretion was not affected significantly when a poor quality protein was supplemented with skim milk or fish meal. There are two possible explanations for this. 1) The poor quality protein, which in this case was corn protein, is not limiting in sulfur-containing amino acids, but rather is limiting in lysine and tryptophan. Therefore, nitrogen was more important than sulfur. 2) The supplements added are themselves relatively limiting in sulfur containing amino acids. When added to the low quality protein, even if they supplied small excesses of lysine and tryptophan, sulfur-containing amino acids were relatively low, and thus no change in sulfur excretion was observed.

The determination of sulfur excretion in urine is, therefore, important since it shows, not only the parallelism to N excretion, but also a difference in behavior which may explain differences in needs of nitrogen and sulfur as affected by dietary conditions and physiological states of the animal. The use of sulfur analysis in urine has been used by Bressani et al.[5] in studies of amino acid imbalance and niacin deficiency and by Bressani and Braham (18) in investigations of the effect of water on nitrogen metabolism. In cases of amino acid imbalances and niacin deficiency, sulfur in urine did not parallel nitrogen excretion, whereas changes in water intake did not affect sulfur excretion but affected urinary nitrogen.

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Absorption of Dietary Fats by the Rat in Cholestyramine-induced Steatorrhea [1]

ROBERT W. HARKINS, LARRY M. HAGERMAN AND HERBERT P. SARETT

Department of Nutritional Research, Mead Johnson Research Center, Evansville, Indiana

ABSTRACT Cholestyramine, a bile acid-sequestering resin, impaired fat absorption in weanling rats fed a diet containing 15% corn oil. With no cholestyramine, 94% of the fat was absorbed. Absorption decreased with increasing amounts of resin, and with 10% cholestyramine in the diet, only 35% of the fat was retained. In a second study, 5% cholestyramine decreased the retention of a variety of dietary fats as follows: (in per cent) medium-chain triglycerides, 2; coconut oil, 7; safflower oil, 16; soy oil, 17: corn oil, 20; olive oil, 27; butterfat, 28; and lard, 37. Two per cent cholestyramine had little effect upon net calcium absorption although 5% decreased absorption with all dietary fats. The type of dietary fat had little effect on the fecal excretion of bile acids. Two per cent cholestyramine increased the fecal excretion of bile acids 30-fold over control levels although 5% cholestyramine did not further increase bile acid excretion. Net retention of the medium-chain fatty acids and linoleic acid was high even with marked steatorrhea, whereas the long-chain saturated fatty acids and oleic acid were poorly absorbed.

Triglycerides composed of the C_8 and C_{10} fatty acids, the so-called medium-chain triglycerides (MCT), are absorbed via the portal vein (1). The MCT is well utilized in a number of syndromes in which other dietary fats are not well absorbed or properly metabolized, such as chylothorax and chyluria (2–4), tropical sprue (5), exudative enteropathy (6), pancreatogenous steatorrhea (7), and a number of other steatorrheas of varied etiology (8). Fatty acids with a chain length of C_{12} and C_{14} are partitioned between the lymphatics and the portal circulation, whereas long-chain fatty acids, which usually make up the largest part of the dietary glyceride fatty acids, reach the systemic circulation from the intestinal lacteals via the thoracic duct.

In the present studies on fat absorption, cholestyramine, a bile acid-sequestering resin, was used to produce experimental steatorrhea. Hashim et al. (9) have previously reported that fat absorption was normal when 15 g of cholestyramine, a dose which decreases plasma cholesterol levels, were given in man each day, although 30 g a day produced frank steatorrhea. Fat absorption was also decreased in rats when cholestyramine was given at high levels; this effect was influenced by the age of the animals, and by the type and level of fat in the diet (10).

In the present work, the effects of graded levels of dietary cholestyramine on the absorption of dietary corn oil were first determined. A second study was conducted to compare the absorption and utilization of MCT and other dietary fats, using levels of cholestyramine which had slightly or moderately inhibited the utilization of corn oil. The results indicate that MCT was well absorbed, whereas there were variable effects on the absorption of the other fats; coconut oil was more completely absorbed than the highly unsaturated vegetable oils, and these were more completely absorbed than olive oil, butterfat or lard.

EXPERIMENTAL

In both experiments, male weanling rats of the McCollum-Wisconsin strain were housed in individual screen-bottom cages in an air conditioned animal room. In the first experiment, 6 groups of 8 animals each were fed diets containing zero, 2, 4, 6, 8 or 10% cholestyramine added at the expense of carbohydrate. The basal diet consisted of the following: (in grams) partially hydrolyzed cornstarch, 55.65; casein, 20; corn oil, 15; non-nutritive fi-

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TABLE 1

Effect of graded levels of dietary cholestyramine on growth, caloric efficiency and fat retention in male weanling rats

Cholestyramine intake		Three weeks			Second week				Third week				
		Wt gain	Caloric intake	Caloric efficiency	Fecal wt	Fecal fat			Fat retention [1]	Fecal fat			Fat retention [1]
						Free	Neutral	Total		Free	Neutral	Total	
% of diet	*g*	*g*	*kcal*	*g gain/ 1000 kcal*	*g*	*g*	*g*	*g*	*%*	*g*	*g*	*g*	*%*
0	0	91 ± 18 [2]	860 ± 125	105 ± 9	16 ± 2	0.4	0.1	0.5 ± 0.4	95 ± 3	0.5	0.2	0.7	94
2	4.1	86 ± 12	804 ± 74	107 ± 9	27 ± 5	2.0	0.2	2.2 ± 1.1	80 ± 8	1.6	0.2	1.8	86
4	8.5	79 ± 13	819 ± 95	97 ± 7	37 ± 5	3.2	0.2	3.4 ± 1.2	68 ± 8	3.1	0.2	3.3	74
6	15	90 ± 8	939 ± 72	97 ± 8	55 ± 2	5.9	0.6	6.5 ± 1.2	47 ± 8	6.0	0.9	6.9	56
8	18	73 ± 19	848 ± 86	86 ± 18	59 ± 7	6.8	0.5	7.3 ± 1.6	40 ± 8	6.6	0.8	7.4	48
10	24	63 ± 12	881 ± 57	71 ± 12	74 ± 7	8.3	0.8	9.1 ± 0.9	31 ± 4	8.5	1.0	9.5	35

[1] Calculated from dietary intake and fecal excretion.
[2] Mean ± SD.

ber, 4; mineral mixture (11), 4; and vitamins (12), 0.35. The diet supplied 620 mg of calcium and 500 mg of phosphorus/100 g of diet. Feces were collected from all animals for 2 periods of 7 days each beginning on the eighth day, and analyzed for free and esterified fatty acids by the method of Van de Kamer et al. (13). Fat retention was calculated from dietary fat intake and fecal fat loss.

The second experiment was carried out in 2 parts to accomodate the large number of animals. At weaning, all animals were given a diet similar to that used in experiment 1 but containing 15% safflower oil for a 4-day pretest period. This supplied the animals with sufficient essential fatty acids to meet their requirements during the test period since some of the fats contained little or no essential fatty acids. Groups of 10 rats each were then selected on the basis of body weight and litter and given diets containing zero, 2 or 5% cholestryamine and 15% fat for 17 days. Cholestyramine was added to the diets at the expense of a portion of the dietary fiber, included in the control diet at a level of 6%. The dietary fats in the first part were MCT, corn oil, butterfat, lard and safflower oil. In the second part, MCT and corn oil were again studied, as well as coconut, olive and soy oils.

Feces were collected from all animals beginning on the fourth day of experimental feeding, weighed, and pooled for determination of free and esterified fatty acids (13), calcium (14) and fecal fat fatty acid pattern (15). Bile acids were extracted from the feces using the procedure of Kuron and Tennent,[2] and were measured individually and quantified by triangulation after gas-liquid chromatography of the methyl esters-trimethylsilyl ethers prepared according to Makita and Wells (16).

EXPERIMENTAL PROCEDURE

Experiment 1. The effects of the addition of graded levels of cholestyramine to a 15% corn oil diet on the rats' growth, caloric efficiency and fat utilization are shown in table 1. The inclusion of 8% dietary cholestyramine decreased 3-week

[2] Kuron, G. W., and D. M. Tennent 1961 An ion-exchange procedure for separating bile acids from feces and serum. Federation Proc., 20: 268 (abstract).

weight gains, although not significantly ($P > 0.05$), whereas 10% cholestyramine significantly restricted weight gains ($P < 0.005$). The weight gains of the groups receiving lower levels of cholestyramine were not significantly affected nor were caloric intakes of any of the groups. Caloric efficiency values were decreased in the groups receiving 4 and 6% cholestyramine and were significantly lower ($P < 0.025$) than the controls with 8 and 10% dietary cholestyramine.

Increasing the dietary level of cholestyramine markedly increased the excretion of both free and esterified fat, as shown in table 1. Fat retention, calculated on the basis of fat intake and fecal loss, averaged 95% in the control group receiving no cholestyramine and decreased to approximately 35% with 10% cholestyramine. The decrease in caloric efficiency values paralleled the decreased absorption of fat and the concomitant loss of dietary calories in the feces as fat.

Experiment 2. The effects of the addition of zero, 2 and 5% cholestyramine on the utilization of several different dietary fats as measured by weight gains, food intakes and caloric efficiency values are shown in table 2. Two per cent cholestyramine had little effect on weight gains, whereas 5% cholestyramine decreased weight gains by zero to 11 g. Although weight gains were consistently lower in the groups receiving the higher level of cholestyramine, the differences were not significant ($P < 0.05$) nor was there any effect on caloric intake. Although 2% cholestyramine had no marked effect on caloric efficiency values, 5% decreased ca-

TABLE 2

Effect of the type of dietary fat and cholestyramine intake on 17-day weight gains, food intakes and food efficiency values of male weanling rats

Dietary fat	Cholestyramine	Part 1			Part 2		
		Wt gain	Caloric intake	Caloric efficiency	Wt gain	Caloric intake	Caloric efficiency
	% of diet	*g*	*kcal*	*g gain/ 1000 kcal*	*g*	*kcal*	*g gain/ 1000 kcal*
Medium-chain triglycerides	0	74 ± 12 [1]	724 ± 63	102 ± 10	74 ± 12	755 ± 117	99 ± 8
	2	66 ± 9	694 ± 105	96 ± 6	73 ± 9	720 ± 111	102 ± 10
	5	63 ± 11	682 ± 64	91 ± 7 [2]	65 ± 8	709 ± 66	92 ± 6
Coconut oil	0				84 ± 12	778 ± 74	107 ± 6
	2				80 ± 9	794 ± 107	101 ± 52
	5				75 ± 9	769 ± 68	97 ± 5
Safflower oil	0	80 ± 11	792 ± 105	101 ± 9			
	2	84 ± 13	806 ± 81	104 ± 92			
	5	71 ± 15	842 ± 70	84 ± 13			
Soy oil	0				79 ± 15	772 ± 108	102 ± 7
	2				82 ± 16	796 ± 104	102 ± 11
	5				79 ± 8	854 ± 76	93 ± 6
Corn oil	0	76 ± 8	761 ± 58	99 ± 6	85 ± 13	813 ± 114	105 ± 10
	2	80 ± 9	759 ± 113	106 ± 11	80 ± 9	774 ± 46	104 ± 7
	5	75 ± 14	814 ± 101	92 ± 8	80 ± 8	835 ± 117	97 ± 10 [2]
Olive oil	0				81 ± 15	795 ± 98	101 ± 9
	2				78 ± 10	775 ± 101	101 ± 10
	5				72 ± 12	810 ± 77	88 ± 10
Butterfat	0	77 ± 7	774 ± 70	100 ± 7			
	2	79 ± 9	802 ± 78	99 ± 5			
	5	68 ± 13	850 ± 100	80 ± 10 [2]			
Lard	0	82 ± 14	829 ± 85	99 ± 11			
	2	85 ± 5	830 ± 41	103 ± 5			
	5	71 ± 15	836 ± 121	84 ± 9 [2]			

[1] Mean ± SD.
[2] $P < 0.05$ compared with control.

loric utilization by 4 to 8 g gain/1000 kcal with an average decrease of 5 g gain. The decreased caloric efficiency values resulted, in part, from the impairment of fat absorption. Even though fat absorption was essentially normal in the groups receiving MCT, caloric efficiency values were decreased.

In the absence of cholestyramine, net fat absorption varied from a high of 99% with MCT to a low of 86% with the butterfat and lard diets in the first collection period (table 3). The administration of 5% cholestyramine decreased the absorption of MCT by only 3%, whereas absorption of the other dietary fats was more markedly affected. Five per cent cholestyramine decreased net absorption of coconut oil by 15%, the highly unsaturated vegetable oils by 19 to 40%, olive oil by 40% and butter and lard by 47 and 55%, respectively. Absorption values for the following week paralleled those of the first week but the effect of cholestyramine was decreased.

Fecal calcium losses were measured to determine whether the steatorrhea induced by cholestyramine impaired calcium absorption (table 3). With 2% cholestyramine there was no marked or consistent

TABLE 3

Fat retention and fecal bile acid excretion by weanling rats receiving cholestyramine and various dietary fats

Dietary fat	Cholestyramine	Days 4 to 10			Days 11 to 17						
		Fecal fat	Net absorption		Fecal fat	Net absorption		Fecal bile acids			
			Fat	Calcium		Fat	Calcium	Lithocholic	Deoxycholic	Cholic	Total
	% of diet	*g*	*%*	*%*	*g*	*%*	*%*	*mg/week*			
Medium-chain triglycerides	0	0.1	99	65	0.1	99	60	1.6	1.3	0.32	3.2
	2	0.3	97	60	0.1	99	53	18	27	11	56
	5	0.4	96	65	0.2	98	51	21	35	9	65
Medium-chain triglycerides	0	0.1	100	62	0.1	99	60				
	2	0.3	98	63	0.3	98	58				
	5	0.3	97	63	0.3	97	61				
Coconut oil	0	0.8	93	65	1.3	89	59	1.7	1.5	0.50	3.7
	2	1.8	83	60	2.0	84	55	21	34	9	64
	5	2.2	78	60	2.2	82	54	19	31	10	60
Safflower oil	0	0.5	96	54	0.5	96	54	2.3	1.0	1.8	5.1
	2	1.4	86	57	1.2	91	46	42	11	17	70
	5	3.8	68	49	2.6	80	50	39	6	16	61
Soy oil	0	0.6	94	55	0.9	92	53	0.68	1.2	0.51	2.4
	2	1.4	87	58	1.4	88	54	16	40	11	67
	5	2.9	75	48	3.3	75	48	17	41	9	67
Corn oil	0	0.4	96	58	0.7	94	50	1.4	0.17	1.0	2.5
	2	2.0	81	53	1.5	88	53	18	42	12	72
	5	4.8	56	44	3.0	76	49	16	39	9	64
Corn oil	0	0.6	94	58	0.9	93	55				
	2	1.3	88	58	1.7	86	51				
	5	4.0	64	54	3.8	70	51				
Olive oil	0	0.9	96	60	1.2	90	58	0.97	1.5	0.48	3.0
	2	2.6	75	56	2.2	82	55	16	30	22	68
	5	4.7	56	47	4.7	63	49	18	32	14	64
Butterfat	0	1.5	86	54	1.5	88	52	1.4	1.4	0.51	3.3
	2	3.4	70	51	2.3	82	56	23	33	12	68
	5	7.1	39	45	5.1	60	31	19	34	6	59
Lard	0	1.5	86	53	1.6	88	59	2.0	2.0	1.1	5.1
	2	3.6	67	55	5.0	62	54	17	27	14	58
	5	7.8	31	45	6.4	51	47	16	29	13	58

TABLE 4

Distribution of dietary and fecal fatty acids excreted by weanling rats receiving cholestyramine and various dietary fats during days 4 to 10

Dietary fat	Cholestyramine	Fatty acids									
		8:0	10:0	12:0	14:0	16:0	18:0	16:1	18:1	18:2	Other
	% of diet	*mg/week*									
Medium-chain triglycerides	diet	4730	3320	250							
	0					10	10		20	10	20
	2		10			60	80	10	50	10	70
	5		20		10	90	120	10	80	20	60
Coconut oil	diet	810	720	3780	1710	900	270		630	180	
	0			220	270	200	70		30	10	10
	2			460	520	460	180		150	10	10
	5			550	600	570	250		190	20	20
Safflower oil	diet					740	210		1050	8510	
	0					80	100		180	100	40
	2				20	330	170		510	270	110
	5				30	1150	1010	60	1270	160	120
Soy oil	diet					970	390		2430	5340	680
	0					180	190		130	40	40
	2			10	10	470	430		340	50	90
	5				10	990	660		860	190	160
Corn oil	diet					1290	200		2570	5740	100
	0					90	80		110	70	50
	2				10	470	310	20	720	310	170
	5				20	1070	530		1960	940	280
Olive oil	diet					960	100	290	7100	1150	100
	0					350	190		300	20	30
	2					700	350		1380	90	70
	5					1290	700		2490	120	90
Butterfat	diet	210	420	320	950	2630	1260	420	2630	420	1160
	0			10	90	580	550		180		90
	2			10	150	1140	810	120	940	60	170
	5			20	300	2440	2400		1490	60	400
Lard	diet				110	2630	1580	320	4730	950	320
	0				10	350	850		220	20	50
	2					670	600		1590	510	230
	5				40	2460	3080	30	1740	90	360

effect on the fecal excretion of calcium, as noted previously in this laboratory.[3] But with 5% cholestyramine net calcium absorption values were decreased. Calcium absorption was higher in the groups receiving MCT or coconut oil than in the other groups.

The excretion of bile acids, shown in table 3, was compared both as a function of the type of dietary fat and as a function of the level of dietary cholestyramine. The total amounts of bile acids excreted on each of the control diets were similar. The addition of 2% cholestyramine increased the excretion of bile acids about 30-fold but 5% cholestyramine had no further effect. The type of dietary fat had no apparent effect on the total excretion of bile acids, either in the absence or presence of dietary cholestyramine.

Palmitic, stearic and oleic acids were the major fecal fatty acids with all of the dietary fats except coconut oil (table 4). With coconut oil, approximately equal amounts of lauric, myristic and palmitic acids were lost in the feces.

No caprylic acid (C_8) was excreted in the feces although MCT, butterfat and co-

[3] Unpublished data, R. W. Harkins and H. P. Sarett.

conut oil contain varying amounts of this fatty acid. Only trace amounts of capric acid (C_{10}) were excreted by the animals fed MCT. The large amounts of lauric acid (C_{12}) in coconut oil were quite well absorbed although the administration of cholestyramine increased lauric acid excretion from 220 mg with no cholestyramine to 550 mg with 5% cholestyramine, compared with an average dietary intake of 3780 mg. In the groups fed coconut oil, the fecal loss of myristic acid (C_{14}) was similar to that of lauric acid, although the dietary intake was only half as great.

Utilization of palmitic and stearic acids was poorer than the utilization of any of the other fatty acids derived from the dietary triglycerides. Increasing the dietary level of cholestyramine decreased the apparent absorption of both palmitic and stearic acids markedly, although palmitic acid was better utilized than stearic acid.

In the absence of cholestyramine, all of the dietary fats were relatively well absorbed, and retention of oleic acid was high. However, with 2 and 5% cholestyramine the net absorption of this fatty acid was markedly decreased. In contrast, linoleic acid was relatively well absorbed even with levels of cholestyramine which markedly decreased the overall retention of fat.

DISCUSSION

The use of dietary cholestyramine, a bile acid sequestrant which increases fecal bile acid excretion (17), for the experimental production of steatorrhea is a useful technique for studies of fat absorption. Steatorrhea produced by high levels of cholestyramine is thought to be due to the lowering of the effective bile acid concentration in the intestinal tract. Du Bois et al. (18) recently demonstrated that polyoxyethylene sorbitan monooleate (Tween 80) [4] improved fat absorption in patients with experimental steatorrhea induced by cholestyramine and suggested that Tween 80 was substituting for the emulsifying action of bile acids.

Playoust and Isselbacher (19) reported that trioctanoin can enter the mucosal cell without prior hydrolysis and were able to show extensive lipolysis of trioctanoin when incubated with subcellular fractions of rat intestinal mucosa. An earlier study by Dawson and Isselbacher (20) reported the essentiality of conjugated bile acids for the esterification of free fatty acids within the intestinal mucosa. Since two-thirds of the decanoic acid secreted into the portal circulation is present in the unesterified form (21), reduced concentrations of bile acids in the intestinal mucosal cell presumably have little effect on the absorption, esterification or transport of MCT across the mucosal cell.

The recent report of Valdivieso and Schwabe (22), which suggests that bile acids are necessary for absorption of trioctanoate, does not appear to be in agreement with the present observations or with the report of Playoust and Isselbacher (19). The use of a relatively large amount of olive oil as a vehicle for administering ^{14}C-labeled glyceryl trioctanoate may account for the discrepancy between the results of Valdivieso and Schwabe and those in the present report. As shown here, MCT was well absorbed when bile acids were bound by cholestyramine but the absorption of olive oil was markedly impaired. The data of Playoust and Isselbacher also show that bile acids are not needed for absorption of trioctanoin.

Triglycerides containing large amounts of linoleic acid (safflower, soy and corn oils) were better absorbed than triglycerides containing predominantly palmitic, stearic and oleic acids (butterfat, lard and olive oil). Even with high levels of cholestyramine, most of the linoleic acid was absorbed. In contrast, palmitic and stearic acids were poorly absorbed; increasing amounts of dietary cholestyramine markedly increased the fecal excretion of these long-chain, saturated fatty acids.

The total excretion of bile acids, both in the absence and presence of cholestyramine was similar with all of the dietary fats. Lithocholic and deoxycholic acids were present in the fecal samples in approximately equal amounts along with smaller amounts of cholic acid. The animals ingesting the safflower oil diets excreted more lithocholic acid than the other groups, although the total excretion of bile acids was similar. Spritz et al. (23) have previously reported that synthesis of cho-

[4] Atlas Powder Company, Wilmington, Delaware.

lesterol and bile acids was not significantly affected by the type of sterol-free dietary fat, whereas Gordon et al. (24) reported an increased production of cholic acid in a patient changed from a diet containing 45% of the calories as butterfat to a similar diet containing corn oil. In the present studies, there were no marked differences in the excretion of bile acids between any of the diets.

Huff et al. (17) demonstrated that bile acid excretion was proportional to the cholestyramine intake with diets containing from zero to 2% cholestyramine. However, in this study, 5% cholestyramine did not further increase bile acid excretion over the levels noted with 2% resin. Bile acid synthesis from cholesterol may be limiting bile acid secretion and hence excretion.

Five per cent cholestyramine resulted in a greater loss of fat in the stool than did 2% cholestyramine, but did not further increase bile acid loss. The higher level of cholestyramine in the diet might decrease the intraluminal concentration of bile acids enough to account for these differences in fat losses; subsequent elution of some of the bile acids from cholestyramine, as shown by Billiau and Van den Bosch (25), may result in similar fecal bile acid levels. Alternatively, cholestyramine may directly bind fatty acids which are generated during triglyceride digestion.

The dietary fatty acids influenced the composition of the fecal fatty acids, which include endogenous fat as well as the end products of microbiological activity. Only those groups fed coconut oil excreted appreciable amounts of lauric acid, whereas the groups receiving olive oil excreted large amounts of oleic acid. Correlations between overall fat absorption and the net absorption of individual fatty acids indicate the very high absorption of the medium-chain fatty acids and of linoleic acid. However, the long-chain, saturated fatty acids were not as well absorbed as the total dietary fat.

Fecal loss of calcium was not markedly increased by 2% cholestyramine but was increased with 5% resin. The increased loss of calcium confirms previous observations that impaired calcium retention is frequently associated with steatorrhea. There was no indication that cholestyramine administration increased calcium absorption in contrast with the observations of Briscoe and Ragan (26).

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Antithyrotoxic Activity of Hemoglobin in the Rat [1]

BRUCE E. BAKER, DAN A. RICHERT, DARWIN MIRISOLOFF AND W. W. WESTERFELD
Department of Biochemistry, State University of New York, Upstate Medical Center, Syracuse, New York

ABSTRACT Dietary hemoglobin and the acid-insoluble precipitate obtained from an alkaline hydrolysate of hemoglobin had good antithyrotoxic activity, as measured by the α-glycerophosphate dehydrogenase (GPD) assay. Both interfered with the intestinal absorption of thyroxine, as measured by the level of radioactivity in blood after feeding ^{131}I-labeled thyroxine to rats. Globin was inactive in both test systems, whereas crystalline hemin was very active in the absorption test, but only moderately active in the antithyrotoxic (GPD) assay. Hemin interfered with the catalytic role of thyroxine in the oxidation of DPNH by the horseradish peroxidase enzyme system; this indicated the formation of a complex between thyroxine and hemin. At least a part of the antithyrotoxic activity of hemoglobin can be attributed to an inhibition of the intestinal absorption of thyroxine by its iron porphyrin moiety.

In a previously published assay procedure (1), hemoglobin was used as the reference standard for antithyrotoxic activity because of its high potency; a 10% hemoglobin diet essentially nullified the effects of 0.025% dietary iodinated casein. All of the antithyrotoxic activity of liver residue, cottonseed meal, lactalbumin, soy protein or hemoglobin could be recovered in the water-insoluble acid precipitate of an alkaline hydrolysate obtained from each of these substances, but the activities in such concentrates did not appear to be due to the same chemical substance.

The additional evidence presented herein shows that at least a part of the antithyrotoxic activity of hemoglobin is related to its iron porphyrin moiety. Free hemin bound thyroxine in vitro and interfered with the intestinal absorption of thyroxine in vivo. The antithyrotoxic actiivties of both hemoglobin and of hemin in the presence of globin were enhanced greatly by alkaline hydrolysis.

EXPERIMENTAL

Separation of globin and hemin (modified procedure of London et al. (2)). Beef erythrocytes, washed with 0.9% NaCl, were frozen and thawed twice, and then stirred with one-half volume of cold benzene. After centrifugation, the benzene layer and emulsion products were removed by suction, and the aqueous solution containing the hemoglobin was poured with rapid stirring into 10 volumes of cold acetone containing 5% HCl. The insoluble globin fraction was filtered, extracted repeatedly with acetone until free from iron porphyrin, and dried in air. The acetone extracts containing the iron porphyrin were combined, the solvents were distilled, and the residue was dried in air.

Alkaline hydrolysis. Dry commercial hemoglobin (800 g) was suspended in 8 liters of 0.5 N NaOH and autoclaved at 120° for 7 hours. In an alternate procedure the hemoglobin was refluxed for 22 hours with 8 liters of 1 N NaOH but this gave a less active product. Autoclaving for longer periods in more concentrated alkali also decreased the activity. The alkaline hydrolysate was filtered hot with a filter aid [2] (1), washed with hot water, and the insoluble residue was discarded. After cooling to room temperature, the filtrate was acidified to a pH of 3.5 to 4. The acid-insoluble precipitate was filtered off, washed with water and dried in air. The

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[2] Hyflo Super-cel Filter Aid was obtained from Johns Manville Products Corporation, Manville, New Jersey; hemoglobin powder was obtained from General Biochemicals, Chagrin Falls, Ohio; DPNH, crystalline hemin, L-thyroxine, keratin and bile acids from Nutritional Biochemicals Corporation, Cleveland; horseradish peroxidase, B grade from Calbiochem, Los Angeles; rats from Holtzman, Madison, Wisconsin; chitin and deacylated chitin from Mann Research Laboratories, Inc., New York.

dry fraction weighed 137 g and contained a relatively large quantity of iron porphyrin.[3] Relatively little of the iron porphyrin could be removed from this acid-insoluble fraction by repeated extractions with chloroform-pyridine, (20:1), ethyl acetate-acetic acid, (3:1), acetone-HCl, (20:1), or glacial acetic acid, and no procedure has yet been found which will remove the iron porphyrin from this fraction.

Absorption of dietary thyroxine. Male rats weighing approximately 120 g were fasted for 24 hours and then fed 2 g of the basal diet[4] with or without the test substance; 0.5 μg of thyroxine (T_4) labeled with 0.5 μc of ^{131}I was also added to the diet. Whole blood samples were collected in oxalate from the decapitated rats 10 hours later, and 2-ml samples were counted in a scintillation well counter. Preliminary time studies showed that the blood radioactivity in such experiments was maximal at about 10 hours post-feeding. The blood radioactivity obtained with each test diet was calculated as a percentage of the radioactivity observed in the blood of the control rats fed the basal diet and tested simultaneously. The results obtained with such control rats ranged from 910 to 1240 count/min in 9 repeat experiments, and averaged 1075 ± 37 (SE) count/min.

RESULTS

Antithyrotoxic activity. The antithyrotoxic activities of the fractions prepared from hemoglobin were determined by the α-glycerophosphate dehydrogenase assay procedure (1), and the results are shown in table 1. Most of the original activity of the hemoglobin was lost when the latter was separated into its protein and porphyrin constituents by the acetone-HCl extraction. By contrast, the acid precipitate obtained from the autoclaved alkaline hydrolysate was more active than the original hemoglobin. Alkaline hydrolysis of the globin or commercial hemin separately did not increase their antithyrotoxic activities, but such hydrolysis of a mixture of the two again gave an acid precipitate with enhanced activity. The hemin-containing acetone-HCl extract and commercial crystalline hemin produced both diarrhea and a diminished growth rate. Neither the original hemoglobin, nor the acid-insoluble precipitates obtained from the alkaline hydrolysis of hemoglobin, hemin, or globin plus hemin had any such toxic effects. The antithyrotoxic effect of hemin was more pronounced when its toxic effects were eliminated through a combination with protein or protein fragments, but not by alkaline hydrolysis of the hemin alone.

Thyroxine binding by hemin. Thyroxine stimulates the oxidation of DPNH by the peroxidase enzyme system (4, 5); an inhibition of this effect was used by Tritsch et al. (6) to measure the binding of thyroxine by serum albumin. We have utilized the manometric DPNH-peroxidase-Mn^{++}-oxygen system (5) to measure the binding of thyroxine by hemoglobin and iron porphyrin. In such a system and with a constant amount of peroxidase, the rate of oxygen consumption was directly proportional to the thyroxine concentration. The amount of free thyroxine remaining in a solution to which iron porphyrin had also been added was determined from such oxygen consumption measurements.

The standard curve was determined as follows. The main body of a Warburg vessel contained 0.2 ml (about 200 μg) of peroxidase, 0.1 ml (0.2 μmole) of $MnSO_4$, 0.2 ml of varying amounts (zero to 0.02 μmole) of thyroxine and 2.1 ml of 0.2 M potassium phosphate buffer, pH 7.0. The side arm contained 0.4 ml (10 μmoles) of DPNH. Water was substituted for the thyroxine in a blank vessel and any manometric reading obtained with this vessel was subtracted from the ones containing the thyroxine. After a 10-minute equilibration at 30°, the DPNH was tipped in, and

[3] The sample was dissolved in a minimal amount of 1 N NaOH, diluted with pyridine and water to give a 10% pyridine solution of desired color intensity, and read in the Beckman D.U. spectrophotometer at 400 mμ. A solution of crystalline hemin, prepared in the same way was used as the reference standard. Assuming that all of the absorption at 400 mμ was due to iron porphyrin and that the molar absorbancy was the same as for the standard hemin, then the iron porphyrin content was estimated to be equivalent to about 8.5% hemin. At least the bulk of the pigment appeared to be iron porphyrin as indicated by the absorption spectrum in the region of 400 mμ.

[4] The basal diet was the same as used in the antithyrotoxic assay method (1) and consisted of the following: (in per cent) crude casein, 30; corn oil, 10; Phillips and Hart salt mixture, 4; sucrose, 56; choline chloride, 0.1; and inositol, 0.05. Vitamins were added as follows: (in mg/kg of diet) riboflavin, 25; thiamine·HCl, 50; niacin, 100; pyridoxine·HCl, 15; folic acid, 5; Ca pantothenate, 200; *p*-aminobenzoic acid, 100; menadione, 1; mixed tocopherols, 600; vitamin B_{12}, 0.1; vitamin A, 40; a vitamin D preparation containing 200,000 USP units/g (Charles Bowman Company, Holland, Michigan), 20; and biotin, 0.5.

TABLE 1

Antithyrotoxic activities of various fractions of hemoglobin

Exp.	Fraction tested	% in diet	GPD	Units	Activity relative to the original Hb [1]
	None (basal diet with iodinated casein)	—	30	0	0
	Hemoglobin	2	16	20	100
		4	12	40	100
		6	7	60	100
		10	5	100	100
1	Acetone-HCl extract of Hb [2]	0.88	18	16	16
2	Globin fraction (acetone-HCl precipitate)	10	24	7	7
3	Acid-insoluble precipitate of hydrolyzed Hb [3]	0.50	8	55	190
4	Acid insoluble-precipitate of hydrolyzed Hb [4]	1.23	8	55	67
5	Acid insoluble-precipitate of hydrolyzed globin	1.40	24	7	7
6	Commercial crystalline hemin [2]	0.24	17.5	18	29
7	Hemin refluxed with 1 N NaOH	0.32	19.5	14	17
8	Hemin + globin refluxed with 1 N NaOH (acid-insol. ppt)	3.50	8	55	60
9	Hemin + globin autoclaved with 0.5 N NaOH (acid-insol. ppt)	0.66	6	70	140

[1] A standard α-glycerophosphate dehydrogenase (GPD) reference curve was established by feeding to weanling male rats the 30% casein diet containing 0.025% iodinated casein (1% thyroxine equivalent) plus zero, 2, 4, 6 or 10% hemoglobin for 11 to 14 days (1) and such reference points are shown in the table. Other diets contained the substances to be tested for antithyrotoxic activities. Liver GPD activity was determined manometrically as described previously (1) and recorded as μl of O_2/10 min/25 mg of fresh liver. The values have also been translated to hemoglobin (Hb) units, i.e., the relative activity of a test fraction in comparison with a 10% hemoglobin diet (= 100 Hb units). For example, the acetone-HCl extract of hemoglobin was assayed at 0.88% in the diet yielding 16 units. Since 0.88 g of this fraction was obtained from 10 g of hemoglobin, it had retained 16% of the original hemoglobin activity. Similarly, 0.5 g of the acid-insoluble precipitate of hydrolyzed hemoglobin, yielding 55 units, was derived from 2.9 g of hemoglobin and hence had a relative activity of 190.

[2] Caused diarrhea and diminished growth rate.

[3] Hydrolyzed with 0.5 N NaOH in the autoclave for 7 hours.

[4] Hydrolyzed with 1 N NaOH by refluxing for 22 hours.

3 to 5 manometer readings were recorded at 5-minute intervals. The enzyme concentration was adjusted to give an oxygen consumption of about 15 μliters/5 minutes with 0.02 μmoles of thyroxine, and this value is shown as 100% activity in figure 1. The rate of oxygen consumption decreased linearly with smaller amounts of thyroxine, and became zero (when corrected for the small blank) in the absence of any thyroxine. Crystalline hemin was dissolved in a minimal volume of NaOH, diluted with 0.2 M potassium phosphate buffer, pH 7.0, and added to a peroxidase assay system which contained 0.02 μmole of thyroxine. The relative oxygen consumption in the presence and absence of hemin was determined and plotted against the hemin concentration to give the curve in figure 2. The values in figure 2 were then translated into the corresponding amounts of free thyroxine by reference to the standard curve in figure 1, and these values are recorded in table 2. All such values are based upon the assumption that the hemin-T_4 complex had no activity in the peroxidase assay system. If it is further assumed that hemin reacts in a 1:1 molar ratio with T_4, it is possible to calculate from these data a dissociation constant (K) of approximately 2.2×10^{-5} for the reaction:

$$\text{Hemin:}T_4 \rightleftarrows \text{hemin} + T_4$$

If all the T_4 was completely bound in the presence of 0.5 μmole of hemin, the maximal activity that such a complex could exhibit in the peroxidase assay would be 14% of an equivalent amount of T_4. If the data in table 2 are recalculated on the assumption that the "free T_4" activity is partly due to the complex, no dissociation constant can be calculated to fit the revised data. Hence, the results are consisent with the assumption that the hemin:T_4 complex is inactive in the peroxidase assay system.

Hemin had no inhibitory effect on peroxidase itself when the latter was assayed by the pyrogallol method (7) (which is performed in the absence of thyroxine). The Mn concentration was not critical in

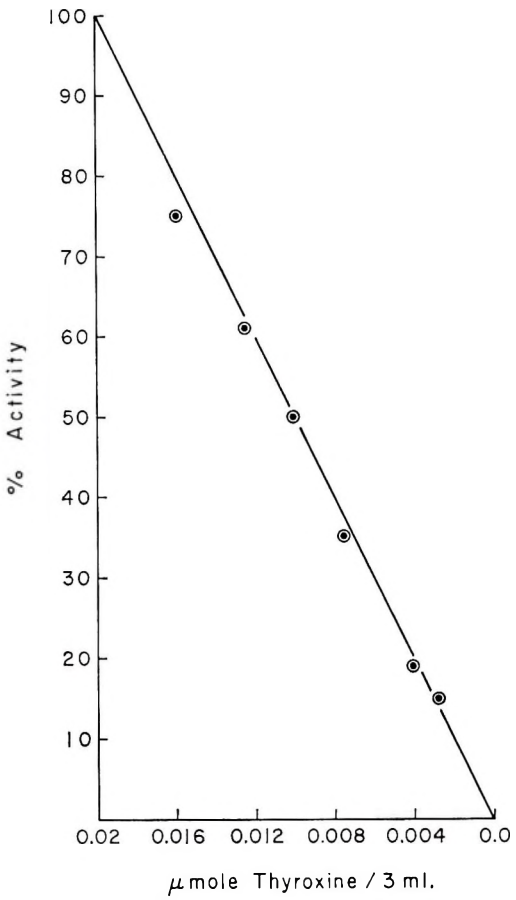

Fig. 1 Standard curve showing the effects of thyroxine concentration on the rate of oxidation of DPNH in the peroxidase system. The rate of oxygen consumed with 0.02 μmole of thyroxine = 100% activity.

the Warburg assay procedure, and excess Mn or DPNH did not counteract the hemin inhibition. These results showed that the hemin reacted with the thyroxine and not with the enzyme or some other component of the assay system.

Crystalline hemoglobin was not as effective as the free iron porphyrin in this test, since 0.1 μmole of hemoglobin (containing 0.4 μmole of heme) reduced the activity of 0.02 μmole of thyroxine by only 40%. Hematoporphyrin (0.55 to 2.75 μmoles/3 ml) had no effect, whereas 0.5 μmole of protoporphyrin decreased the T_4 activity to 40%. Of the soluble proteins tested, gelatin had no effect, but 0.01 μmole of albumin, which was observed to be active by Tritsch et al. (6), was as effective as 0.1 μmole of hemoglobin. Albumin had little activity in the antithyrotoxic assay in vivo presumably because it was digested in the intestine. The acid-insoluble precipitate obtained from the alkaline hydrolysate of hemoglobin was about as active as would be expected from its iron porphyrin content when tested at concentrations of less than 0.15 μmole of hemin; at higher concentrations this fraction tended to reverse the inhibitory effect. Suspensions of soy protein, cottonseed meal or the cottonseed meal concentrate (1 mg) also stimulated the peroxidase system in the absence

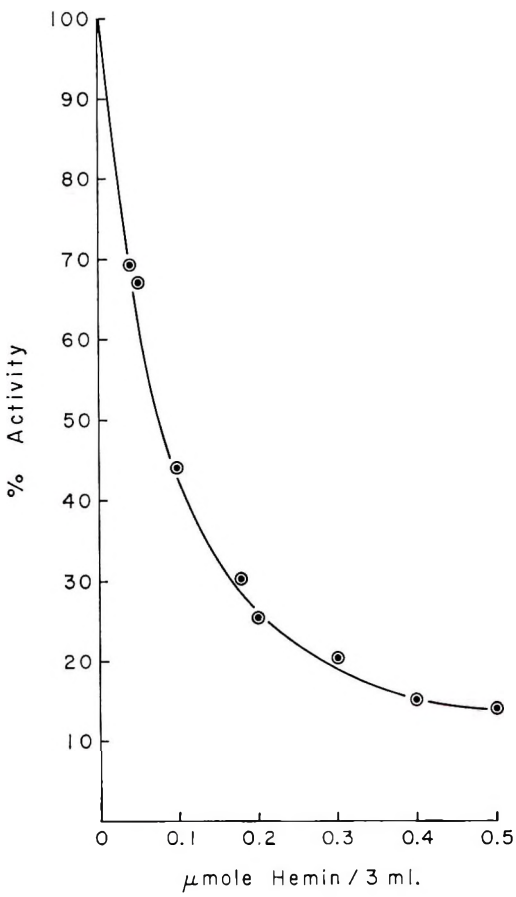

Fig. 2 Effects of varying the hemin concentration on the rate of oxidation of DPNH in the peroxidase system containing 0.02 μmole of thyroxine. The rate of oxygen consumed in the absence of hemin = 100% activity.

of thyroxine because they contained substances which could be substituted for thyroxine as the catalyst in this test. Liver residue concentrate had little or no effect but it was also relatively insoluble at pH 7.

Intestinal absorption of thyroxine. At least some of the antithyrotoxic effect of liver residue and hemoglobin can be attributed to a decreased absorption of dietary thyroxine (3). Table 3 shows similar effects from hemoglobin, hemin, and the acid-insoluble concentrates prepared from hemoglobin. Globin was relatively inactive in the usual antithyrotoxic assay, and was also inactive in these absorption tests. Qualitatively all of these substances had the same effect in the usual 11-day antithyrotoxic assay as in this short 10-hour absorption test, but the quantitative relationships were different. A diet containing 10% hemoglobin had 100 units of antithyrotoxic activity and reduced the 10-hour blood radioactivity in the thyroxine absorption test to 55% of the control value. A diet containing 0.1% hemin had less than 10 units of antithyrotoxic activity but decreased the blood radioactivity to 35% of the control value. The 1.5% acid-insoluble fraction had 148 units of antithyrotoxic activity; it contributed approximately 0.13% hemin-like material to the diet and was about as active as the hemin in the absorption test. Hemin was particularly effective in the thyroxine absorption test, and as little as 0.01% in the diet decreased the 10-hour blood radioactivity to 60% of that obtained with the basal diet.

A variety of other materials were also tested by this procedure in order to study the correlation between their known (1, 8) antithyrotoxic activities and the degree to which they interfered with the absorption

TABLE 2

Relative binding of thyroxine (T_4) by hemin when increasing amounts of the latter were added to 0.02 μmoles of T_4 and tested for activity in the peroxidase assay system (all values are in μmoles/3 ml)

Hemin added	0.050	0.100	0.200	0.300	0.400	0.500
Total T_4 added	0.0200	0.0200	0.0200	0.0200	0.0200	0.0200
Free T_4 remaining	0.0126	0.0085	0.0052	0.0038	0.0030	0.0028
Bound T_4 (by difference)	0.0074	0.0115	0.0148	0.0162	0.0170	0.0172
Free hemin [1]	0.0426	0.0885	0.1852	0.2838	0.3830	0.4828
Dissociation constant (K) [1]	2.42×10^{-5}	2.17×10^{-5}	2.16×10^{-5}	2.22×10^{-5}	2.25×10^{-5}	2.63×10^{-5}

[1] Assuming a 1:1 molar reaction between hemin and T_4, the bound T_4 equaled the bound hemin (or the hemin:T_4 complex), and the free hemin was obtained by difference. After converting all values to molar concentrations, the dissociation constant for the hemin:T_4complex was calculated from:

$$\frac{(\text{Free hemin}) \times (\text{Free } T_4)}{(\text{Hemin:}T_4 \text{ complex})} = K.$$

TABLE 3

Effect of hemoglobin products on the absorption of thyroxine from the gastrointestinal tract

Substance tested	% of diet	No. of exps. [1]	Blood radioactivity relative to that in blood of rats fed basal diet
			%
None (basal diet)	—	–	100
Hemoglobin	5	1	62
Hemoglobin	10	5	55
Hemoglobin	15	1	45
Globin	10	5	94
Hemin	0.1	3	35
Acid-insoluble ppt of hydrolyzed Hb	1.5	2	33

[1] Four rats per experiment.

TABLE 4

Effect of various dietary constituents on the absorption of thyroxine from the gastrointestinal tract

Substances	% in diet	No. of exps. [1]	Anti-thyrotoxic activity	Blood radioactivity relative to that in blood of rats fed basal diet
			Hb units [2]	%
Gelatin	10	2	8	120
Thymus	10	1	8	89
Cholesterol	1	2	0	92
Chitin	4	2	16	80
Deoxycholic acid	0.4	2	30	88
Glycocholic acid	0.4	2	40	115
Soy protein	20	2	20	101
Soy protein conc [3]	5	1	55	101
Fibrin	20	2	21	106
Lactalbumin	20	2	25	120
Lactalbumin conc [3]	5	1	82	90
Deacylated chitin	4	2	51	40
Liver residue fat	10	1	47	56
Cottonseed meal	10	2	47	73
Cottonseed meal conc [3]	3	2	> 42	73
Liver residue	15	1	48	74
Liver residue conc [3]	5	2	85	63
Keratin	10	2	> 50	70

[1] Four rats per experiment.

[2] Where necessary, the antithyrotoxic activities of the compounds listed were recalculated from previously reported (1, 8) liver residue (LR) units (3 LR units = 1 Hb unit), or adjusted to reflect the dietary concentrations used in these tests.

[3] The preparation of the concentrate has been described elsewhere (1).

of thyroxine. The first 4 substances listed in table 4 had little or no activity in either test, and the last 7 substances were active in both the 11-day GPD assay and the 10-hour T_4 absorption test. However, the bile salts, fibrin, soy protein or lactalbumin (and their concentrates) were active in the antithyrotoxic assay but had no effect on the intestinal absorption of T_4. This suggests a mechanism of antithyrotoxic action for these substances which differs from any interference with intestinal absorption. If all the antithyrotoxic effect of hemoglobin can be attributed to an intestinal absorption phenomenon, then liver residue, cottonseed meal, and their concentrates also appeared to have somewhat more antithyrotoyic activity than could be accounted for on this basis. Removal of the acetyl groups from chitin increased its antithyrotoxic activity threefold, and the deacylated chitin was as active as hemoglobin in both the GPD assay and T_4 absorption tests.

DISCUSSION

Ruegamer and Wallace (3) have reported that dietary antithyrotoxic materials such as liver residue and hemoglobin interfere with the absorption and enterohepatic recycling of thyroxine given either orally or subcutaneously. An oral dose of thyroxine moved through the lower ileum, cecum and large intestine more rapidly when the diet contained such antithyrotoxic substances; approximately twice as much thyroxine was excreted into the feces, and half as much thyroxine was present in the plasma. Schenkman (3) observed that the thyroxine excreted in the feces, with a basal diet, was biologically active when refed, but was relatively inert when the original diet contained hemoglobin. Certainly a portion (and possibly all) of the antithyrotoxic effect of hemoglobin can be attributed to an interference with the intestinal absorption of thyroxine.

The present experiments were designed to determine which part of the hemoglobin

molecule was responsible for binding the thyroxine, and whether all of the antithyrotoxic effect of hemoglobin could be explained on this basis. Thyroxine was bound by free iron porphyrin, as shown in the peroxidase test system, and dietary hemin interfered with the intestinal absorption of thyroxine. However, free iron porphyrin exhibited less antithyrotoxic activity in the GPD assay than an equivalent amount of hemoglobin, and the acid-insoluble precipitate derived from an alkaline hydrolysate of hemoglobin was more active in the GPD assay than could be attributed to its porphyrin content. Five-tenths per cent of the acid-insoluble fraction in the diet assayed at 55 units and contributed no more than 0.042% of iron porphyrin to the diet (42 mg/100 g diet). At the 1% level it gave essentially complete protection (> 60 Hb units), whereas a dietary concentration of 0.1 to 0.2% free hemin exhibited only 20 to 30 units.

If the antithyrotoxic effect of hemoglobin and related compounds is due entirely to a binding of T_4 by the iron porphyrin moiety, then this binding is more effective in vitro with free hemin and more effective in vivo when the iron porphyrin is bound to globin or its hydrolytic products. Free hemin produced diarrhea, whereas the bound form or alkali-treated hemin did not, and this may have complicated the 11-day GPD assay; it did not prevent the hemin from being very effective in the 10-hour T_4 absorption test. The best evidence that a "bound porphyrin" was the potent antithyrotoxic moiety in hemoglobin, was the creation of additional activity when hemoglobin or a mixture of hemin plus globin was hydrolyzed in alkali. The nature of this degradation product is unknown, but some combination of the porphyrin with an amino acid (or degradation products of each) is indicated, since the alkaline hydrolysis of each one alone did not enhance their respective AT activities. It can be postulated that during the intestinal digestion of hemoglobin, a "bound porphyrin" remains in the intestinal tract which does not produce diarrhea but still binds thyroxine effectively.

The difficulty with attributing all of the antithyrotoxic activity of hemoglobin to an intestinal binding of T_4 lies in the quantitative relationships of the parameters measured so far. A 10% hemoglobin diet reduced the blood level of administered T_4 by about 50%, but it reduced the effectiveness of the T_4 on the tissues by about 90% in the BMR and GPD assays. Either the blood level of labeled T_4 (as measured in these tests) does not reflect the quantitative effect of T_4 on the tissues, or the hemoglobin (and especially some other antithyrotoxic substances) exert an effect which is different from (and is additive to, or is additive to) the intestinal binding phemonenon.

Some substances which were reasonably antithyrotoxic in the 11-day GPD assay had little or no activity in the 10-hour T_4 absorption test. Although this suggests a mechanism of action other than an interference with T_4 absorption, it is also possible that a substance which did not interfere with T_4 absorption in a 10-hour test might still do so in an 11-day assay. Deoxycholate, for example, had no effect in the 10-hour test but was reasonably active in the 11-day assay, and is known to produce histological changes in the intestine and gallbladder on prolonged feeding (9).

It was suggested previously that the antithyrotoxic activity of each concentrate obtained from different starting materials was not due to the same chemical substance on the basis of differences in stability and solubility properties (1). Here again the antithyrotoxic effect does not appear to be anything which is specific for hemoglobin since deacylated chitin, which bears no structural similarities to hemoglobin, is active as well.

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Growth of Rats Fed Chlortetracycline or an Exchange Resin [1]

JORDAN L. HOLTZMAN [2] AND WILLARD J. VISEK [3]
Department of Pharmacology, University of Chicago, Chicago, Illinois

ABSTRACT Weanling male Holtzman rats were fed a basal liquid diet containing a suboptimal concentration of protein. Addition of chlortetracycline (250 mg/kg) or a cationic exchange resin (3.85%) increased the rate of body weight gain. The hydrolysis of urea in the gastrointestinal tract was reduced by chlortetracycline but not by the resin. Calculations of energy required for detoxification of ammonia from urea hydrolysis showed that growth differences due to the antibiotic or to the resin greatly exceeded energy sparing attributable to reductions in ammonia produced by bacteria. Regression analysis of weight gain vs. feed intake of the animals receiving supplements showed a reduction in dietary maintenance requirements as compared with control, but no significant change in nutrient absorption. Values are presented showing that the present results are compatible with the hypothesis that the sparing effect on protein or other nutrients by antibiotics is secondary to reduced requirements for maintaining the intestinal mucosa.

Numerous investigators have attempted to elucidate the mechanisms whereby dietary antimicrobial agents at concentrations below the therapeutic range stimulate growth in birds and mammals. It is generally agreed that this response is secondary to the presence of bacteria in the gastrointestinal tract (1–3). Among the mechanisms which have been postulated is a net improvement in the absorption of nutrients secondary to the reduction in the thickness of the wall of the gastrointestinal tract (1, 2). Previous evidence has been presented suggesting that the action of antibiotics is related to a reduction in ammonia produced in the gastrointestinal tract from hydrolysis of endogenous urea by bacterial urease(s) (1, 4).

In the present paper gastrointestinal hydrolysis of ^{14}C urea was determined in rats receiving measured amounts of a liquid basal diet supplemented with chlortetracycline or a polystyrene cation exchange resin known to reduce blood ammonia concentrations. The quantity of ammonia released by urea hydrolysis was calculated from ^{14}C urea turnover. The effects on growth of the basal diet supplemented with chlortetracycline and the resin were studied in rats fed ad libitum. The possibility that the growth differences observed were the result of reduced energy and maintenance requirements for processes of cellular renewal in the wall of the gastrointestinal tract is considered. Consideration is also given to the possible influence that the additives exerted on nutrient absorption. A preliminary report of these investigations has been presented.[4]

EXPERIMENTAL METHODS

Growth experiments. Seven experiments were conducted with a total of 101 male weanling rats of the Sprague-Dawley strain [5] having initial weights of 45 to 55 g. The animals were housed individually in hanging wire cages and maintained in clean air conditioned quarters. Assignment to treatment and cages was by random numbers. A liquid basal diet was fed which provided 8% of the calculated caloric content as total protein from evaporated milk and contained a final concentration of 42% glucose. Vitamins and minerals were included to meet recommended allowances (5). Microbial fermentation in this mixture was found to be minimal under animal room conditions for periods of at least 5 days. Feeding was ad libitum

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[2] Present address: Laboratory of Chemical Pharmacology, National Heart Institute, Bethesda, Maryland.
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[4] Holtzman, J. L., and W. J. Visek 1964 Growth and urea metabolism in rats fed chlortetracycline and a cation exchange resin. Federation Proc., 23: 499 (abstract).
[5] Sprague-Dawley Farms, Madison, Wisconsin.

from calibrated drinking bottles [6] which remained on the cages for 2 to 3 days and were then replaced with clean bottles containing fresh diet. Stock diet was stored at 4° for a maximum of 3 weeks. In experiments 1, 2 and 3, experimental groups received the basal diet supplemented with soluble chlortetracycline [7] at a concentration of 250 ppm. In experiments 4, 5, 6 and 7, control animals were fed the liquid basal diet described above except for the addition of carboxymethyl cellulose [8] to facilitate suspension of a polystyrene sulfonate cation exchange resin.[9] The experimental groups in this series of experiments received the modified basal diet supplemented with 3.85% of the resin which had been equilibrated with Krebs-Ringer solution for 24 hours at room temperature. Ten milliliters of equilibrating solution per gram of resin were used in experiments 4, 6 and 7, and 5 ml in experiment 5. After equilibration, the supernatant solution was decanted and the resin was dried to constant weight at 110° before incorporation into the diet. Both the carboxymethyl cellulose and the resin were incorporated at the expense of all other constituents. A feeding period of 28 days was used for experiment 7, whereas all other experiments lasted 21 days. Body weights were obtained at weekly intervals.

Studies of urea hydrolysis. Seventy-five Sprague-Dawley rats were fed ad libitum a commercially available diet until they weighed 150 to 225 g, whereupon they were changed to the liquid diet (table 1). This was given 4 times daily by stomach tube for 6 days. Animals weighing less than 200 g received 7 ml of solution/feeding, and those weighing over 200 g received 10 ml. Experimental animals received the same diet supplemented with chlortetracycline or with the resin as described above.[10] At the end of the fourth day of tube feeding, each rat was injected intraperitoneally with 1 to 2 μc of ^{14}C urea (33 μc/mmole). Urine separated from feces was collected at 24 and 48 hours thereafter. Assignment to treatment groups was random.

Determination of specific radioactivity of urinary urea. Urinary urea was determined spectrophotometrically by the acetyl monoxime method (6). Decolorized aliquots of urine (7) were used for assay of radioactivity in a liquid scintillation spectrometer.[11] The scintillator solution was the dioxane-cellulose preparation of Bruno and Christian (8). Hydrolysis of urea in the gastrointestinal tract was calculated using equation I:

$$U_H = U_u \frac{1 - P}{P} \qquad (I)$$

where U_H represents millimoles of urea hydrolyzed in the gastrointestinal tract per kilogram of body weight per day; U_u, the millimoles of urinary urea excreted per kilogram of body weight per day; and P, the fraction of injected ^{14}C urea which was excreted in the urine in 48 hours. Preliminary experiments and published data (9, 10) indicated the validity of this method as an estimate of urea hydrolysis in the gastrointestinal tract.

RESULTS AND DISCUSSION

A summary of the weight gain and feed consumption for experiments 1, 2 and 3 is shown in table 2. The data were analyzed by analysis of variance with the main effects of time and treatment. Addition of antibiotics to the diet increased growth and efficiency of feed utilization regardless of the statistical model of comparison. The gain in body weight and the efficiency of feed utilization for the 4 experiments with the polystyrene sulfonate cation exchange resin are listed in table 3. Efficiency of feed utilization was consistently higher in the animals given the resin supplement, and in 3 of the 4 experiments, the differences were statistically significant ($P < 0.02$). A statistically significant difference in overall growth was observed only in experiment 4. Feed intakes showed no statistically significant differences. Average values for the pooled results are listed in table 3, and are to be interpreted with the realization that 5 ml of Krebs-Ringer solution/g were used for equilibration of the

[6] Kimax no. 44875. Kimble Glass Company, Toledo, Ohio.

[7] Aureomycin; Lederle Laboratories Division, American Cyanamid Corporation, Pearl River, New York.

[8] No. 7HP; Hercules Powder Company, Oakbrook, Illinois.

[9] Kayexalate; Winthrop Laboratories, New York.

[10] Carboxymethyl cellulose was omitted in this experiment. The resin was maintained in suspension by stirring while aliquots were removed for administration.

[11] Packard Instrument Corporation, La Grange, Illinois.

TABLE 1

Composition of liquid diet [1]

Ingredients added in the order listed:	
Water	1028 g
Glucose, anhydrous	797 g
Calcium lactate, N.F.	36 g
Sodium glycerophosphate, N.F.	42 g
Choline chloride	700 mg
Calcium pantothenate	24 mg
Niacin	20 mg
Thiamine	4 mg
Add solution containing:	
$CuSO_4$	20 mg
Zn $(C_2H_3O_2)\cdot 2H_2O$	20 mg
Water	10 g
Add solution containing:	
$FeSO_4\cdot 7H_2O$	165 mg
$MnSO_4$	200 mg
Water	10 g
Add, after vigorous stirring, a mixture containing:	
Tween 80 [2]	4.5 ml
dl-α-Tocopherol [3]	85 mg
Vitamin A [4]	30,000 IU
Linoleic acid [5]	4.28 g
Water	50 g
Add: Evaporated milk	1642 g
Add water to make 3,614 g	

[1] All ingredients C.P. grade unless otherwise indicated. Water and utensils were sterilized. Gulcose and calcium lactate added with water temperature at 90° to 100°. Vitamins and evaporated milk were incorporated at temperatures under 60°. Calculated caloric and protein content per millimeter were 1.6 kcal and 28 mg respectively. Carboxymethyl cellulose for experiment 7, no. 7HP, Hercules Powder Corporation, Oakbrook, Illinois.

[2] Tween 80 (polyalkylene derivative of sorbitan monostearate); Nutritional Biochemicals Corporation, Cleveland.

[3] *dl-α*-tocopherol (250 IU vitamin E/g); Nutritional Biochemicals Corporation, Cleveland.

[4] Vitamin A in gelatin (500,000 IU/g). Nutritional Biochemicals Corporation, Cleveland.

[5] Linoleic acid (purified) A-165; Fisher Scientific Corporation, Fair Lawn, New Jersey.

resin in experiment 5, whereas 10 ml were used in the other experiments.

The average urinary urea excretion per kilogram of body weight per day and the average hydrolysis of urea in the gastrointestinal tract as calculated using equation I are shown in table 4. There was no apparent difference in the total urinary urea excretion between treatments, but the animals given the chlortetracycline supplement showed a marked reduction in urea hydrolysis as compared with either the control animals or those given the resin supplement ($P < 0.05$).

The quantity of protein and the calories of energy consumed per unit of weight gain were calculated for each treatment group from the data of tables 2 and 3 using the calculated caloric and protein contents of the diet (table 1). Corrections for decreases in energy and protein content were used when carboxymethyl cellulose and the resin were added. In all experiments the calories or protein required per gram of gain were greater for control groups than for those fed chlortetracycline or the resin. These differences were also observed in diet intake per unit of weight gain. Growth of the animals in the resin experiment was below that of comparable groups in the chlortetracycline experiments. The cause for this growth difference may have been the carboxymethyl cellulose.

According to present knowledge synthesis of glutamine is a prerequisite step in the formation of urea, and one mole of

TABLE 2

Weight gain and feed consumption of rats fed a liquid diet for 3 weeks and without 250 ppm of chlortetracycline

Treatment	Exp. no.	Diet consumed	Weight gain		Body wt [1]
		ml	*g*	*g/100 ml*	*g*
Basal	1 (6) [2]	360 ± 46 [3]	30 ± 5 [4]	8.1 ± 0.5	70 ± 7
	2 (6)	447 ± 59	39 ± 4	8.8 ± 0.7	72 ± 4
	3 (6)	564 ± 54	46 ± 5	8.3 ± 0.3	68 ± 5
	Avg	457 ± 51	38 ± 5	8.4 ± 0.5	70 ± 5
Chlortetracycline	1 (6)	388 ± 36	42 ± 4	10.8 ± 0.4	76 ± 5
	2 (5)	523 ± 53	48 ± 5	11.0 ± 0.9	80 ± 6
	3 (6)	634 ± 68	60 ± 8	9.3 ± 0.5	74 ± 6
	Avg	515 ± 51	50 ± 6 [5]	10.4 ± 0.6 [5]	77 ± 6

[1] (Final weight plus initial weight)/2.

[2] Figures in parentheses indicate number of animals.

[3] Average ± SD.

[4] Initial weights: control, range 45 to 55 g; chlortetracycline, range 45 to 55 g.

[5] The difference in weight gain between the 2 groups is consistent from experiment to experiment. The average difference in weight gain was 12 g. Statistical analysis verifies that this difference is significant at the 5% level. The same is true of the feed efficiency.

TABLE 3

Weight gain, feed consumption, and efficiency of feed utilization for rats fed for 3 weeks a liquid diet with and without supplementation with a polystyrene sulfonate cation exchange resin

Treatment	Exp. no.	Diet consumed	Weight gain		Body wt [1]
		ml	*g*	*g/100 ml*	*g*
Basal	4 (6) [2]	363 ± 32 [3]	18 ± 4	4.8 ± 0.8	67 ± 6
	5 (6)	381 ± 45	16 ± 5	4.2 ± 1.0	60 ± 5
	6 (6)	334 ± 42	20 ± 4	5.9 ± 0.7	55 ± 5
	7 [4] (15)	549 ± 49	30 ± 5	5.4 ± 0.5	72 ± 4
	Avg	407 ± 42	21 ± 5	5.1 ± 0.8	64 ± 5
Resin	4 (6)	356 ± 38	25 ± 5 [5]	7.0 ± 0.9 [6]	71 ± 5
	5 (6)	342 ± 45	18 ± 3	5.2 ± 0.6	62 ± 5
	6 (6)	329 ± 33	25 ± 7	7.6 ± 1.3 [7]	56 ± 6
	7 [4] (15)	523 ± 45	34 ± 8	6.4 ± 0.8 [6]	73 ± 4
	Avg	388 ± 43	26 ± 6	6.6 ± 0.7	66 ± 5

[1] (Final weight plus initial weight)/2.
[2] Figures in parentheses indicate number of animals.
[3] Average ± SD.
[4] Experiment 7 lasted 4 weeks.
[5] $P < 0.01$.
[6] $P < 0.001$.
[7] $P < 0.02$.

TABLE 4

Urinary urea excretion and hydrolysis of urea by rats tube-fed a liquid basal diet with or without chlortetracycline (250 ppm) or polystyrene sulfonate cation exchange resin (3.85%).

Treatment	Urinary urea	Hydrolysis of urea
	mmole/kg/day	*mmole/kg/day*
Basal (27) [1]	10.0 ± 1.66 [2]	3.60 ± 1.42
Chlortetracycline (23)	10.6 ± 1.59	2.67 ± 1.06 [3]
Polystyrene sulfonate cation exchange resin (25)	10.5 ± 1.05	3.58 ± 1.08

[1] Figures in parentheses indicate number of animals.
[2] Average ± SD.
[3] $P < 0.05$.

urea formed would utilize 6 moles of ATP (11, 12). With 38 moles of ATP formed following the complete oxidation of one mole of glucose (13), the formation of a mole of urea in mammals would demand about 115 kcal of dietary energy. From the turnover studies hydrolysis of urea in the gastrointestinal tract was 0.36 and 0.26 mmole/100 g of body weight/day for the control animals and those given the chlortetracycline supplement, respectively. The corresponding quantities of NH_3 released would have required 0.86 kcal and 0.62 kcal in 3 weeks for detoxification. These calculations and the regression analysis of the growth data described below, show that the energy required to detoxify ammonia arising from this source was less than 1% of the apparent energy spared the animals given the chlortetracycline-supplement and agree with direct determinations on rats fed urea (14).

Removal of ammonia by the resin in a rat consuming 400 ml of diet would have approximated 46 mEq if binding of ammonia equaled the capacity of the resin (3.1 mEq/g). Removal of this quantity of ammonia would spare 1.7 kcal of dietary energy for the 3-week period. From the known physiological actions of the resin it is reasonable to postulate that binding and removal of ammonia took place (15, 16) but that the quantity was insufficient to cause a significant difference in urinary urea excretion. Furthermore, the presence of resin in the diet did not alter urea hydrolysis in the gastrointestinal tract as did chlortetracycline. These data indicate that bacterial production of ammonia from urea and the resultant processes of detoxification play a minor role in depriving the animal of energy for growth. However, they do not invalidate previous data suggesting that ammonia released in the gastrointestinal tract may have a significant effect upon growth and other physiological processes in the host (1, 4).

Although the experiments were designed to study the influence of chlortetracycline and the resin upon urea hydrolysis and growth, the data were subjected to regres-

sion analysis for purposes of evaluating dietary requirements for growth and maintenance and for assessment of possible effects on nutrient absorption. The growth data were pooled by treatment from the experiments in which chlortetracycline was fed and regressed versus feed intake according to equation II.[12] For the purposes of these calculations it was assumed that the energy equivalents of respective gains were the same for the control and supplemented groups.

According to standard methods of comparison (20), the lines were parallel but not congruent ($P < 0.001$) (fig. 1). The correlation coefficients were 0.854 ($P < 0.001$) for the basal diet and 0.951 ($P < 0.001$) for the basal diet plus chlortetracycline. The estimated difference in dietary intake for equivalent growth when chlortetracycline was added was 64 ml with 95% confidence bounds of 36 and 93 ml. At a caloric content of 1.6 kcal/ml, this represents a sparing of 102 kcal/animal for the experimental period with 95% confidence bounds of 57 to 148 kcal. With a concentration of 28 mg/ml of diet, the 95% confidence bounds encompass a sparing of dietary protein due to chlortetracycline supplementation ranging from 1.0 to 2.6 g/three weeks or between 50 and 125 mg/day, with an average of 86 mg/day. Since the regression lines were parallel and not congruent, the regression analysis indicates that the main effect of antibiotic supplementation was to reduce the maintenance requirement of the animal for essential nutrients or energy and not to enhance absorption of nutrients. When growth versus feed consumption were regressed for experiment 7, in which the resin was fed (fig. 2), the lines obtained using all of the data for each of the 2 treatments have significantly different slopes (table 5). The basal group had a regression slope of 8.92 ml of diet/g gained, whereas the respective value for the animals given the resin supplement was 5.34. The respective correlation coefficients were 0.895 and 0.898. The difference between these 2 slopes was significantly different from zero ($0.05 > P < 0.025$). The regression constants varied inversely and not proportionately with the regression slopes.

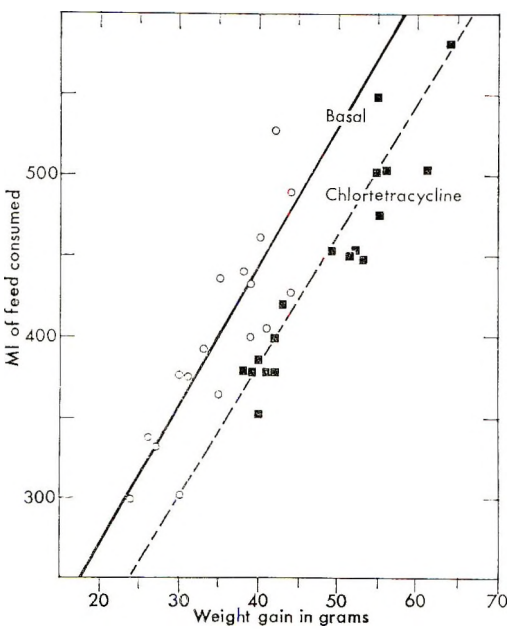

Fig. 1 Feed consumption vs. weight gain of male weanling rats fed a liquid diet deficient in protein with and without chlortetracycline supplementation (250 ppm) for 3 weeks.

[12] Equations for regression analysis:

The growth data vs. the feed intake were regressed according to equation I,

$$f = b\,g + k \qquad \text{(II)}$$

where f is the total feed intake for the experimental period, g is the total weight gain, and b and k the appropriate regression parameters. The term bg represents the energy of growth and k, the energy of maintenance. Variations in b give an estimate of the effect of absorption on growth. The equations of Wood and Capstick (17), Brody (18), and Winchester and Hendricks (19), were also examined to ascertain the effect of the variation in the average weight on the analysis of the data. However, since the animals were in a period of rapid growth, these terms did not significantly improve the analysis and therefore, equation II was used.

If the treatment does not alter the energy necessary for formation of body tissues and maintenance of the animal from absorbed nutrients, then the absorptive effect can be estimated by:

$$A_t f = b\,g + k \qquad \text{(III)}$$

where A_t is the constant of absorption for each treatment, t: and b and k are the universal constants of the regression equation for all treatments. Dividing by A_t gives

$$f = (b/A_t)\,g + k/A_t \qquad \text{(IV)}$$

The quantities b/A_t and k/A_t are represented by b_t and k_t, and are derived from the regression coefficients of the observed data. Two treatments, $t1$ and $t2$, give constants from the regression equation of

$$b_{t1} = b/A_{t1} \text{ and } k_{t1} = k/A_{t1} \qquad \text{(V)}$$

and

$$b_{t2} = b/A_{t2} \text{ and } k_{t2} = k/A_{t2}$$

Dividing b_{t1} by b_{t2} gives A_{t2}/A_{t1}, as does a similar division for k_{t1} by k_{t2}:

$$b_{t1}/b_{t2} = A_{t2}/A_{t1} = k_{t1}/k_{t2}. \qquad \text{(VI)}$$

Thus a test for the effect on absorption of a treatment is given by the simultaneous test of the null hypothesis:

$$b_{t1} - b_{t2} = 0 \text{ and } k_{t1} - k_{t2} = 0 \qquad \text{(VII)}$$

If the composite null hypothesis cannot be rejected, then absorption is not a significant factor in the observed differences. Further, the differences in the 2 regression coefficients must be in the same direction to satisfy equation VI.

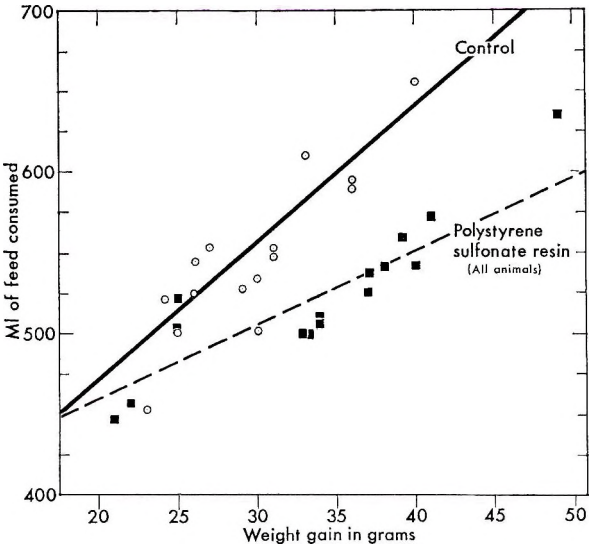

Fig. 2 Feed consumption vs. weight gain of male weanling rats fed a liquid diet deficient in protein with and without supplementation by a polystyrene sulfonate cation exchange resin (3.85%) for 4 weeks.

TABLE 5

Regression coefficients for the comparison of growth of animals fed a liquid diet with and without a polystyrene sulfonate cation exchange resin.[1]

Treatment	Correlation coefficient	Slope	Constant of regression equation
		ml diet/g gain	*ml diet*
Basal (15) [2]	0.895	8.920	283
Polystyrene sulfonate cation exchange resin (15)	0.898	5.342 [3]	355

[1] Data from experiment 7.
[2] Figures in parentheses indicate number of animals.
[3] $0.05 > P > 0.025$.

Thus, they do not satisfy the conditions of equation VI and indicate that absorption was not the primary factor in causing this difference. The quantity of dietary protein consumed per gram of increase in body weight for the animals of experiment 7 was calculated from the regression slopes derived as described above. For the control animals 8.92 ml of diet containing 28 mg of protein/ml would have provided about 250 mg of protein/g of increase in body weight. The regression slope of 5.34 ml of diet/g of body weight gain would have provided the 15 resin-supplemented animals with about 150 mg of protein/g of growth, which is considerably below the dietary requirement of 216 mg of protein/g of weight gain in rats of comparable size as determined by body composition (21). The value calculated for the resin-supplemented group suggested a marked difference from control in body composition which appeared unlikely. Plotting of the observed frequency of efficiencies of feed utilization showed that the animals given the resin supplement were not a homogenous population. Examination of the regression lines in figure 2 demonstrated a convergence of the control and resin animals at the end of the curve representing the lowest feed intakes. When regression lines (fig. 3) were calculated omitting all of the animals that gained less than 26 g, the regression slope for the control animals was found to be 8.11 ml of diet consumed /g of weight gained with a correlation coefficient of 0.836. Corresponding values for the resin-fed animals were 8.45 and 0.985. The residual mean squares of the lines were significantly different. Comparison of the slopes (20) gave a *t*-function value of 1.812 which is not statistically significant. The lines were then compared using the combined slope of 8.29 as was

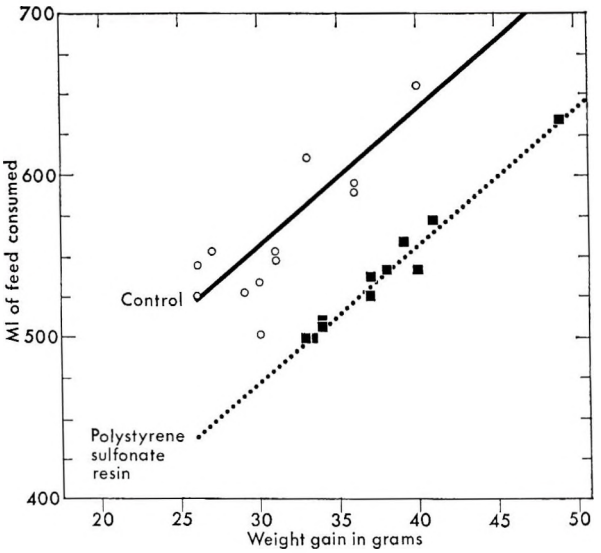

Fig. 3 Feed consumption vs. weight gain of male weanling rats gaining more than 26 g when fed a liquid diet deficient in protein with and without supplementation by a polystyrene sulfonate cation exchange resin (3.85%) for 4 weeks.

done in experiments 1, 2 and 3 with chlortetracycline. A difference of 77 ml was calculated with the *t*-function being 26.2 for 14 degrees of freedom ($P < 0.001$). The 95% confidence bounds were 55 and 100 ml of diet and encompass a sparing of dietary protein ranging from 55 to 100 mg/day or an average of 78 mg.

It is known that the presence of bacteria in the gastrointestinal tract leads to a thickening of the intestinal wall which is reduced by antibacterial substances and not observed in germfree animals (2, 22, 23). A number of experiments have been reported on the turnover and loss of cellular substances from the intestinal mucosa of conventional and germfree animals (23, 24). The mucosa represents about 25% of the weight of the small intestine (25, 26) and the small intestine can equal as much as 4% of the total body weight in young rats (27). Thus, the small intestinal mucosa may equal one gram of tissue per 100 g of body weight. If the mucosa of the small intestine contains 216 mg of protein/g (21) and the turnover rate is 2 days (23, 24), then 108 mg of protein would be required daily to maintain the integrity of the small intestinal mucosa. If antibiotics reduce both the turnover time and cellular area of the mucosa by 30% (2, 23, 24), the protein requirement for maintenance of this turnover rate would be reduced by 58 mg/day. This value approaches the 95% confidence area calculated for the sparing of protein in these studies. The value of 108 mg of protein required per day to maintain the small intestinal mucosa assumes a complete loss of mucosal cell protein through turnover which may not be true. However, this value does not give consideration to cellular losses in other segments of the alimentary tract and to losses in enteric secretions. The observations appear to be in agreement with the hypothesis which is presented for further consideration. Other nutrients required for maintenance of rapidly reproducing cell populations have been spared or their efficiency of utilization increased by antibacterial agents, thereby suggesting that the mechanism described above is common to other experimental conditions (2).

Recent evidence on the magnitude of cellular losses and turnover of gastrointestinal mucosal cells indicate that the above estimates are conservative.[13] Nasset (28) and associates have shown that a large

[13] Altmann, G. G. 1964 Cell numbers as a measure of growth and exfoliation of epithelium of the small intestine of rats. M. Sci. Thesis, McGill University.

amount of endogenous nitrogen is secreted into the gastrointestinal lumen and that the quantity may be profoundly influenced by diet. Others have shown that protein from cellular material is of substantial significance in this contribution. Although the regression analysis may suggest a chance relationship, the present results and those previously obtained suggest that ammonia (1, 2, 4) normally released by bacteria in the gastrointestinal tract may lead to a tissue reaction in the wall of the gastrointestinal tract with a resultant increase in nutrient requirements or a secondary loss of essential substances in the fecal stream.

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Effects of B-Vitamin Deficiencies and of Starvation on Liver Enzyme Activities of Growing Rats [1]

W. J. STIELAU,[2] R. A. FREEDLAND AND J. H. MEYER
Departments of Animal Husbandry and Physiological Sciences, University of California, Davis, California

ABSTRACT The feasibility of using enzyme assays to evaluate a specific vitamin deficiency in the presence of multiple vitamin deficiencies was investigated in a series of studies in which rats were fed complete, B-vitamin free, pyridoxine-deficient or thiamine-deficient diets. The activities of alanine and aspartate aminotransferase, serine dehydratase, and the pentose phosphate-metabolizing system were measured in liver homogenates. In a simple pyridoxine or thiamine deficiency, the activities of enzymes requiring these vitamins as co-factors were significantly lowered. The pentose phosphate-metabolizing system was also depressed in a multiple B-vitamin deficiency. The response of the pyridoxine-dependent enzymes to a thiamine deficiency and to a B-vitamin free diet was similar. The activities of serine dehydrates and alanine aminotransferase were increased, whereas aspartate amino transferase activity was somewhat decreased. With the exception of aspartate aminotransferase, the response of the liver enzymes to fasting and to the vitamin-free diet was also similar. It was concluded that measurements of the pentose phosphate-metabolizing system can possibly be used to evaluate a thiamine deficiency in malnutrition. However, the pyridoxine-dependent enzymes cannot reliably be used to predict a pyridoxine deficiency when other B-vitamins are limiting in the diet because these enzymes are sensitive to changes in food intake and to other B-vitamin deficiencies.

Several investigators have suggested that nutritional deficiencies of pyridoxine and thiamine can be evaluated by measuring the activities of enzyme systems requiring these vitamins as co-factors (1–4).

There is general agreement that aminotransferase activity is depressed in the liver and blood serum under conditions of pyridoxine deficiency (1, 5, 6) and that this depression can often be reversed by the administration of pyridoxine. Alanine aminotransferase activity (glutamic-pyruvic transaminase) appears to be more sensitive to a lack of pyridoxine than aspartate aminotransferase activity (glutamic-oxalacetic transaminase) (1).

A thiamine deficiency in rats has been shown to decrease the activity of the pentose phosphate-metabolizing enzyme system (measured as transketolase activity) of blood erythrocytes (3, 7) and of other tissues including liver (4). Deficiencies of pyridoxine, riboflavin and protein have no effect on erythrocyte transketolase activity in the rat (7).

Deficiencies of single vitamins were studied in the work mentioned above, but multiple vitamin deficiencies are far more common under natural conditions. An enzyme assay might be used as a parameter of vitamin insufficiency in malnutrition if the activity of that enzyme were sensitive to a specific vitamin deficiency and relatively insensitive to other vitamin deficiencies or dietary variables. The effects of multiple and single B-vitamin deficiencies on the activities of certain pyridoxine- and thiamine-dependent enzymes were investigated in the present studies. In addition, the behavior of the enzymes during short periods of starvation was assessed.

EXPERIMENTAL

The response of liver enzyme activities to B-vitamin free, pyridoxine-deficient or thiamine-deficient diets or to fasting was studied in a series of 3 experiments. Weanling rats of the Sprague-Dawley strain, obtained commercially,[3] were used in all studies. The rats were housed individually in wire-bottom cages in a room maintained at 26 ± 1°. Each rat was provided with water ad libitum.

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[2] Present address: University of Natal, Pietermaritzburg, Natal, South Africa.
[3] Simonsen Laboratories, Inc., Gilroy, California.

The basic vitamin-free diet fed to the rats was composed of the following parts by weight: vitamin-free casein, 25; mineral mixture, 4;[4] sucrose, 65; and cottonseed oil, 5. The vitaminized ration was prepared by adding crystalline B-vitamins to the vitamin-free diet (8). A solution of B-vitamins was made up in distilled water such that a known number of drops contained the same amounts of B-vitamins as present in 10 g of the vitaminized diet. This solution, made up frequently and stored under refrigeration, was used for the oral vitamin supplementation of the rats. Ample amounts of vitamins A, D, E and K in cottonseed oil were administered orally to each rat at the beginning of each trial and at weekly intervals.

The pyridoxine-free and thiamine-free rations were identical in composition to the fully vitaminized diet, except that thiamine and pyridoxine, respectively, were excluded.

The liver enzymes studied were alanine and aspartate aminotransferase, serine dehydratase and the pentose phosphate-metabolizing enzyme system.

After decapitation and exsanguination of each rat, the liver was rapidly and completely removed, weighed and placed in ice. A weighed portion of the chilled liver was analyzed for glycogen using a method based on the turbidity of aqueous-alcohol suspensions of glycogen (9). Another weighed portion was used to prepare a 10% homogenate in 0.14 M KCl. The homogenate was centrifuged at zero to 4° for 30 minutes at 35,000 × *g*, and the supernatant was analyzed for protein with a biuret reagent (10) and assayed for enzyme activity.

Enzyme activities were measured at 25° on a Beckman model DU spectrophotometer coupled to a Gilford model 2000 sample absorbance recorder. Alanine aminotransferase activity was measured in a system similar to that described by Bowers et al. (11). The activity of the aspartate aminotransferase system was assayed using a modification of the methods of Karmen (12) and of Steinberg et al. (13), and serine dehydratase activity was determined according to the method of Freedland and Avery (14). Aminotransferase and serine dehydratase activities were estimated by measuring the disappearance of NADH from the assay mixtures by decreases in absorption at 340 mμ. Determination of the activity of the pentose phosphate-metabolizing enzyme system by the method of Benevenga et al. (15) was accomplished by estimating the rate of formation of NADH.

Details of the experimental design follow.

Experiment 1. Two groups of weanling male rats were fed ad libitum a B-vitamin free and a B-vitamin supplemented diet, respectively. Three rats were killed on the first day of the trial and enzyme activities determined. Two rats fed each diet were killed at weekly intervals for assay of liver enzyme activities.

Another group of 10 weanling rats was fed a B-vitamin free diet for 10 days. Eight of these rats were then each given a single oral dose of B-vitamins, equivalent to the B-vitamin content of 10 g of vitaminized diet. Two rats receiving the supplement and two that did not receive any supplement were killed 12 hours later; the remaining rats were killed in pairs at daily intervals to determine the response of enzyme activities to a single dose of B-vitamins. This procedure was repeated with another group of 10 rats which were fed the B-vitamin free diet for 18 days before 8 rats were each given a single oral dose of B-vitamins.

Experiment 2. Twelve weanling male rats were fed the B-vitamin free diet for 3 days, after which 6 rats were changed to the vitaminized ration for 4 days. Food was then removed from 3 rats fed each diet, but water remained freely available to all animals. At intervals of 24, 48, and 72 hours after the beginning of starvation, a fed and a fasted rat on each treatment were killed in the usual manner and differences in the activities of their liver enzyme systems were measured.

Experiment 3. A group of 14 weanling male rats was fed the vitamin-free diet for a standardization period of 4 days after which 2 rats were killed and liver enzyme activities determined. The remaining 12 rats were allocated to 4 groups of 3 rats each and were placed on one of the fol-

[4] Phillips, P. H., and E. B. Hart, J. Biol. Chem., *109*: 657, 1935.

lowing regimens: (A) complete diet; (B) pyridoxine-free diet; (C) pyridoxine-free diet + 0.4 mg deoxypyridoxine/day, intraperitoneally; (D) B-vitamin free diet + 0.4 mg deoxypyridoxine/day, intraperitoneally. After 7, 14, and 17 days, one rat from each treatment was killed and liver enzyme activities were assayed.

In a similar manner groups of weanling rats were allocated to the following four treatments: (A) complete diet; (B) thiamine-free diet; (C) thiamine-free diet + 0.1 mg oxythiamine/day, intraperitoneally; (D) B-vitamin free diet + 0.1 mg oxythiamine/day, intraperitoneally. These rats were also killed, one rat from each treatment, at intervals of 7, 14, and 17 days and liver enzyme activities determined.

RESULTS

Multiple B-vitamin deficiency. Comparison of the chemical composition of the livers of rats fed vitamin-free or vitaminized diets (exp. 1) showed that the exclusion of B-vitamins from the diet tended to result in reduced glycogen levels (fig. 1). In vitamin-deficient rats, liver glycogen content increased markedly in response to a single dose of the B-vitamins. The glycogen content returned rapidly to a low level, however. The increased glycogen level might have been related to a marked stimulation of food intake after the vitamin dose rather than to any metabolic consequences of these vitamins. The liver glycogen levels in this and subsequent experiments confirmed that the vitamin-deficient rats were eating and were not, biochemically, in the same state as fasted rats which in experiment 2 had no measurable liver glycogen. No appreciable differences in liver protein percentages were recorded between different treatments and experiments.

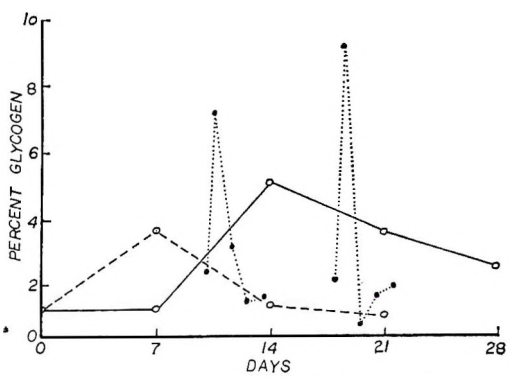

Fig. 1 Response of liver glycogen percentages to a vitamin-free diet and single oral doses of a B-vitamin mixture. Complete diet ○———○, vitamin-free diet ○– – – –○, vitamin-free diet for 10 and 18 days before a single B-vitamin dose ●. . . . .●.

The liver enzyme activities of rats fed complete and B-vitamin free diets (exp. 1) are shown in figure 2. The enzyme activities were expressed on a body weight basis to relate them to the functional need of the animal as suggested by Weber and Cantero (16). The rats fed the B-vitamin free diet had increased activities of liver alanine aminotransferase and of serine dehydratase, whereas the pentose phosphate-metabolizing system was depressed. The activity of aspartate aminotransferase also tended to be below the control level. When B-vitamin depleted rats were given a single dose of B-vitamins, liver enzyme activities generally approached control levels. This response was of short duration; activities tended to revert rapidly to pre-dosage levels.

Effects of fasting. The data shown in figure 3 (exp. 2) demonstrate that fasts lasting 48 and 72 hours caused appreciable increases in the aminotransferase and serine dehydratase activities of rats fed complete and B-vitamin free diets. The activity of the pentose phosphate-metabolizing system was somewhat reduced. With the exception of aspartate aminotransferase, the response of the liver enzymes to B-vitamin free diets and starvation was similar in nature. The enzyme activities observed during fasting tended to be lower when rats were fed the vitamin-free instead of the complete diet, beforehand. Apparently, enzyme activities obtained with fasting rats are influenced by the diet fed previously.

Pyridoxine deficiency. A simple pyridoxine deficiency caused aminotransferase and serine dehydratase activities to decrease within 7 days (fig. 4). In contrast with the observations of Brin et al. (1), aspartate aminotransferase appeared to be decreased more than alanine aminotransferase by the pyridoxine deficiency. The activity of the pentose phosphate-metabo-

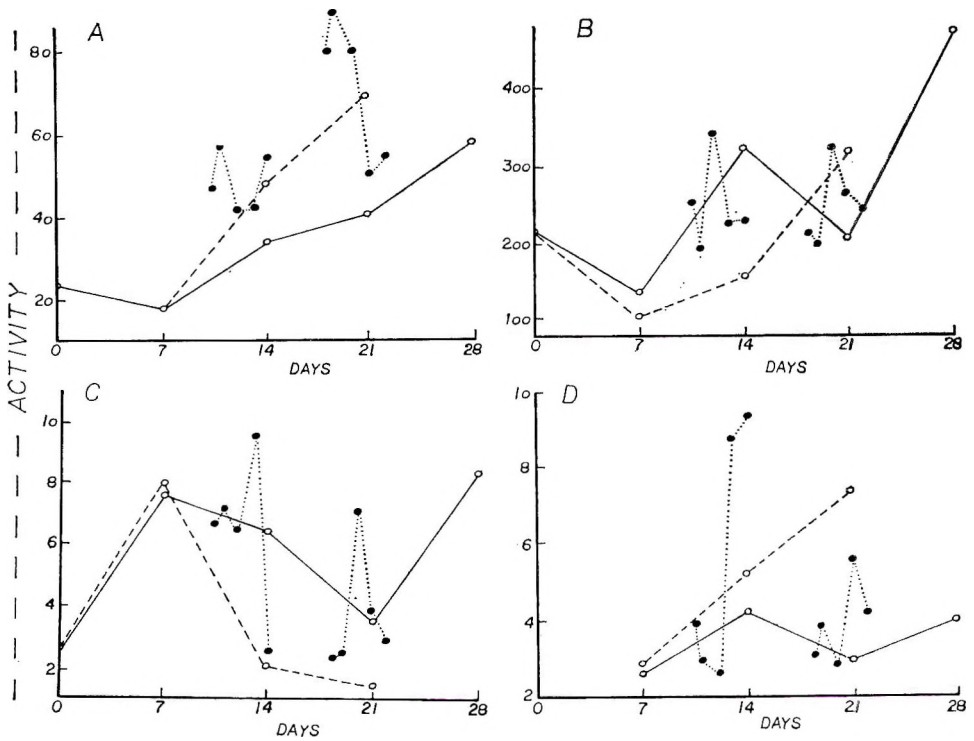

Fig. 2 Effect of vitamin-free diet on liver enzyme activities expressed as μmoles of NADH formed or oxidized/minute/100 g live weight. (A) alanine aminotransferase; (B) aspartate aminotransferase; (C) pentose phosphate-metabolizing system; (D) serine dehydratase. Complete diet ○——○, vitamin-free diet ○- - - - -○, vitamin-free diet for 10 and 18 days before a single B-vitamin dose ● . . . . . ●.

lizing system was also slightly reduced but did not appear to be particularly sensitive to a pyridoxine deficiency. The slight reduction might have been a function of decreased food intake. Concomitant treatment with deoxypyridoxine did not appear to accentuate the vitamin B_6 deficiency. The activities of the aminotransferases were slightly reduced below the levels observed with the pyridoxine-deficient diet alone. However, after 7 days of treatment with deoxypyridoxine the activities of all enzymes increased above this level although aminotransferase activities never exceeded the corresponding values observed in control rats. The antivitamin increased serine dehydratase activity throughout the experiment. Hartsook et al. (17) observed that the addition of deoxypridoxine to the diet reduced aspartate aminotransferase activity more markedly than a simple pyridoxine deficiency. However, Caldwell and McHenry (19) reported that deoxypyridoxine had no significant effect on aspartate aminotransferase and markedly increased alanine aminotransferase activity. Thus, a pyridoxine deficiency induced by the antagonist, deoxypyridoxine, might not duplicate in vivo a simple pyridoxine deficiency. However, the possibility of increased tissue breakdown due to a lower food intake after the administration of deoxypyridoxine may partially explain the increase in certain of these enzymes associated with protein catabolism.

Similar responses were observed for deoxypyridoxine treatments of rats fed a vitamin-free diet except that the pentose phosphate-metabolizing system was depressed.

Thiamine deficiency. The data in figure 5 demonstrate that the activity of the pentose phosphate-metabolizing system was consistently decreased by the exclu-

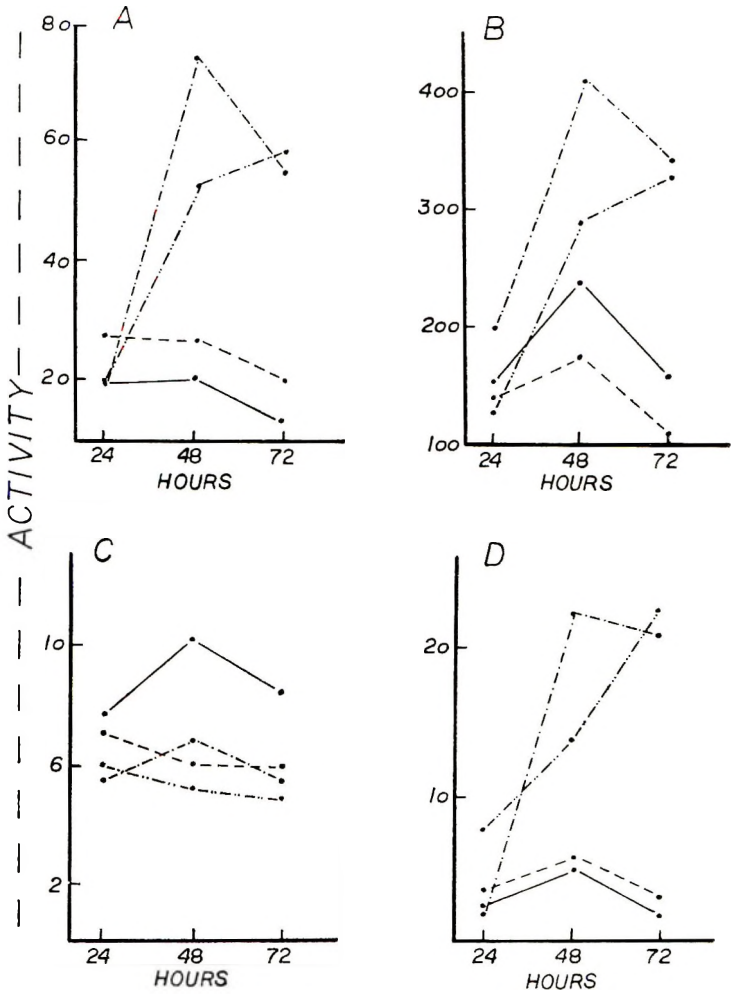

Fig. 3 Effects of fasting on liver enzyme activities expressed as μmoles of NADH formed or oxidized/minute/100 g live weight. (A) alanine aminotransferase; (B) aspartate aminotransferase; (C) pentose phosphate-metabolizing system; (D) serine dehydratase. Complete diet, not fasted ●——●, vitamin-free diet, not fasted ●– – – –●, complete diet, fasted ●–·–·–●, vitamin-free diet, fasted ●–··–··–●.

sion of thiamine from the diet for 17 days, whereas serine dehydratase activity was consistently increased. The effect of thiamine deficiency on aminotransferase activity was variable. Aspartate aminotransferase activity appeared to be depressed during the first 2 weeks of treatment.

The antivitamin treatment in conjunction with either the B-vitamin or thiamine-free diets resulted in a greater reduction of the pentose phosphate-metabolizing system. Whenever thiamine was excluded from the diet, the resultant depression of the pentose phosphate-metabolizing system was greater than during fasting.

DISCUSSION

To provide a useful index of a specific vitamin deficiency, enzyme activities in malnutrition must deviate reliably from a "norm" and must be relatively insensitive to other vitamin deficiencies or variations in diet. The summarized results of experiments 1 and 3 (table 1) demonstrate that the enzymes studied satisfy the first requirement. Aminotransferase and serine

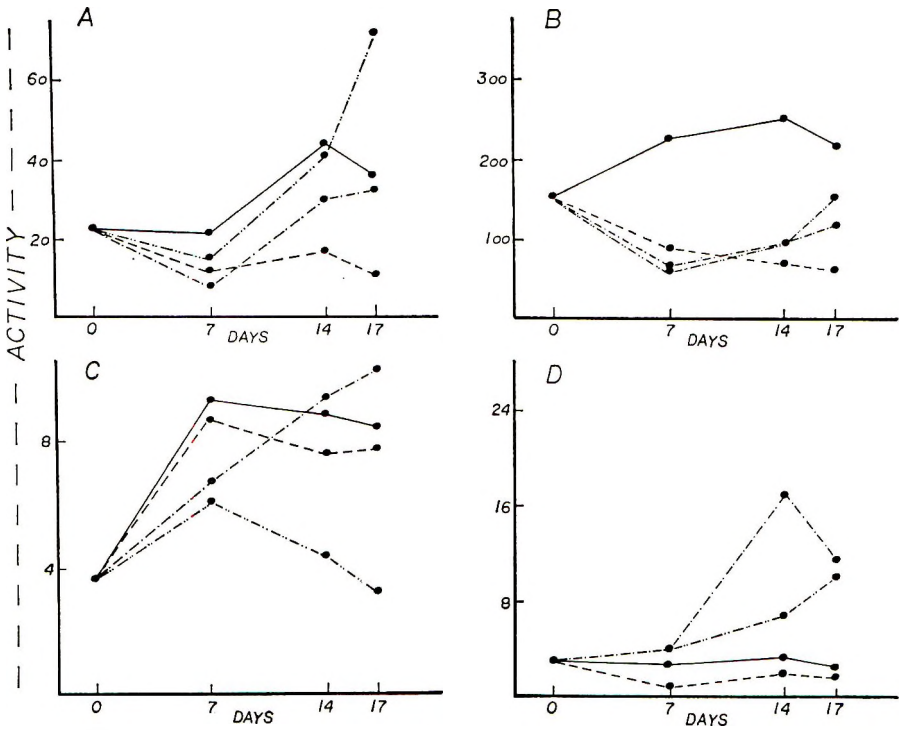

Fig. 4 Effects of a pyridoxine deficiency and of deoxypyridoxine on liver enzymes of rats, expressed as μmoles of NADH formed or oxidized/minute/100 g live weight. (A) alanine aminotransferase, (B) aspartate aminotransferase, (C) pentose phosphate-metabolizing system, (D) serine dehydratase. Complete diet ●——●, vitamin B_6-free diet ●– – – –●, vitamin B_6-free diet + deoxypyridoxine ● –·–·– ●, vitamin-free diet + deoxypyridoxine ● –··–··– ●.

dehydratase activities were significantly depressed by a pyridoxine deficiency as compared with a "norm" (rats fed a complete diet ad libitum). The activity of the pentose phosphate-metabolizing system was significantly reduced in a thiamine deficiency.

The overall effects of feeding a multiple B-vitamin deficient diet for 21 days on the 4 enzymes studied, however, were extremely variable. With respect to these enzymes, rats fed the B-vitamin free diet did not deviate significantly from rats fed the complete diet.

The effect of a multiple B-vitamin deficiency on enzyme activities tended to resemble that of a thiamine deficiency rather than a pyridoxine deficiency even though three of the four enzymes studied required pyridoxine as a co-factor. The influence of a thiamine deficiency on the activities of the pyridoxine-dependent enzymes may not be mediated solely through an effect on food intake. Babcock (5) and Caldwell and McHenry (6) have shown in studies of pyridoxine deficiency that serum and liver aminotransferase activities of pair-fed control rats exceeded those of control rats fed ad libitum. These results were in agreement with the observation that aminotransferase as well as serine dehydratase activities are elevated during fasting. Therefore, if reduced food intake were the only cause of alterations in the activities of pyridoxine-dependent enzymes, thiamine-deficient rats, which had food intakes similar to pyridoxine-deficient animals, would behave as pair-fed controls, whose liver enzyme activities should consistently be somewhat above the level of the controls fed ad libitium. This effect was demonstrated for serine dehydratase (the enzyme which appears to be most sensitive to reduced food intake) but not for the

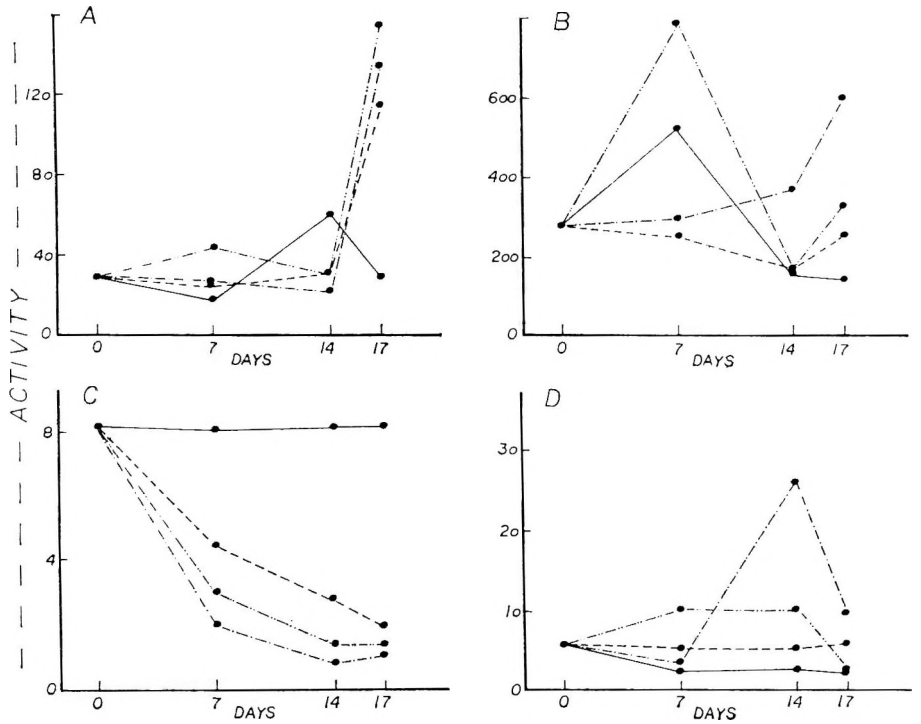

Fig. 5 Effects of a thiamine deficiency and of oxythiamine on the liver enzyme activities of rats expressed as μmoles of NADH formed or oxidized/minute/100 g live weight. (A) alanine aminotransferase, (B) aspartate aminotransferase, (C) pentose phosphate-metabolizing system, (D) serine dehydrate. Complete diet ●——●, thiamine-free diet ●– – – –●, thiamine-free diet + oxythiamine ●–·–·–●, vitamin-free diet + oxythiamine ●–··–··–●.

TABLE 1

Mean liver enzyme activity during 17 to 21 days of treatment with B-vitamin deficient diets

Deficiency	Alanine amino-transferase	Aspartate amino-transferase	Pentose phosphate metabolizing enzymes	Serine dehydratase
	% of control value		*% of control value*	
Multiple B-vitamin	145.4 ± 48.2	85.3 ± 27.6	66.3 ± 33.6	155.5 ± 37.4
Pyridoxine	39.1 ± 5.3 [1]	32.3 ± 3.0 [1,2]	89.7 ± 3.4	55.1 ± 10.3 [1,3]
Thiamine	159.0 ± 46.8	81.9 ± 11.0	37.8 ± 7.4 [1,2]	233.0 ± 8.3 [1,3]

[1] Mean liver enzyme activity differed significantly from control group ($P < 0.05$).
[2] Regression of enzyme activity on days of treatment differed significantly from control group $P < 0.05$).
[3] Deviations from control group throughout treatment were significant ($P < 0.05$).

aminotransferases. Thus, it is possible that thiamine deficiency can influence aminotransferase activities by mechanisms partially independent of food intake. For example, the accumulation of metabolites such as pyruvic acid in thiamine-deficient animals, may affect aminotransferase activity. Concomitant deficiencies of other B-vitamins and the metabolic interactions of the deficiency states could also produce enzymic responses which might obscure the response to a simple pyridoxine deficiency.

Present knowledge indicates, therefore, that aminotransferase and serine dehydratase cannot be used reliably to assess pyridoxine status in the presence of other B-vitamin deficiencies. In this respect, the

use of the pentose phosphate-metabolizing system to evaluate a thiamine deficiency shows more promise. More information concerning factors which exert an effect upon the ultimate activity of an enzyme system is required before enzyme activities can be interpreted with confidence.

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In Vitro Studies of ^{59}Fe Absorption by Everted Intestinal Sacs of the Rat [1]

W. N. PEARSON AND MARTHA REICH
Division of Nutrition, Department of Biochemistry, Vanderbilt University Medical School, Nashville, Tennessee

ABSTRACT Iron-59 transport was studied in vitro using everted gut sacs or intestinal segments. A definite concentration of iron in the mucosa was noted but there was little transfer into the inner pouch fluid. Saturation of the transport mechanism was not demonstrable over a wide range of iron concentrations. Mucosal uptake was significantly decreased by certain metabolic inhibitors. Ascorbic acid increased uptake at low, but not high, iron concentrations. Both ferrous and ferric iron were concentrated at about the same rate by mucosal cells. Under our experimental conditions muscosal and pouch fluid iron uptakes were not consistently altered in rats with anemia induced by phenylhydrazine injections or by dietary means.

Despite the large number of studies of iron absorption reported in the literature, the means by which iron is absorbed from the intestine is still not known. Recently, everted gut sac systems have been used in an attempt to define the mechanism of iron uptake but with contradictory results. Brown and Justus (1), for example, found no evidence for active transport of iron using everted sacs of rat intestine. Conversely, Dowdle et al. (2) and Manis and Schachter (3–5) reported that iron uptake was the result of an energy-requiring active transport process with flow against a concentration gradient. In an attempt to resolve these divergent views we have reexamined the use of this technique in studies of iron absorption.

METHODS

Since it was deemed desirable to carry out experiments under conditions permitting multiple sampling, the method of Crane and Wilson (6) was used initially. This technique proved to be susceptible to large errors at low iron concentrations, however, and the "multiple transfer" approach detailed below was devised. This permitted precise estimations of uptake to be made because the initial and final counts in the incubation medium could be determined easily without having to transfer from the incubation vessel to counting tube with its concomitant losses.

Albino female rats (125 to 175 g) of the Sprague-Dawley strain were fasted for 24 hours and decapitated. They were maintained with commercial rat chow [2] unless otherwise noted. The small intestine was cut at the pylorus and cecum and dissected free of the mesentery. It was then placed in an O_2-saturated ice-cold 0.146 M NaCl–0.004 M KCl solution and sacs or segments were made from various parts of the gut depending upon the specific experiment. The intestinal segments were cut, tied at one end, everted, and rinsed in the NaCl–KCl solution. The sacs were approximately 6.0 cm long and weighed an average of 550 to 650 mg by wet weight. In most cases they were filled with medium of the following composition: 0.45 M NaCl; 0.04 M D-mannose, 0.004 M tris [3] buffer at pH 7.4. This medium is a modification of that of Manis and Schachter (3), omitting $CaCl_2$ and sodium ascorbate. A bubble of O_2 was placed in each sac which was then tied, weighed, and placed in a plastic tube containing 3 ml of medium plus sufficient ^{59}Fe-labeled Cl_2 [4] and stable iron if necessary to give concentrations of 0.004 μg Fe/ml or higher. Prior to the experiment, oxygen was bubbled through the medium in each tube for 20 seconds. The tubes

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[2] The Ralston Purina Company, St. Louis.
[3] Hydroxymethylaminomethane. Sigma Chemical Company, St. Louis.
[4] Obtained from Abbott Laboratories, North Chicago, Illinois.

were then stoppered and an initial count obtained in a well-type scintillation counter [5] prior to the introduction of the sac for incubation.

Each tube containing a sac plus 5 additional pre-counted tubes containing oxygenated medium was agitated in a water-bath at 37°. The following procedure was used: a sac was placed in tube 1 for 30 minutes, then removed and placed in tube 2 for 30 minutes, then removed to tube 3 for 30 minutes, etc., to give a total incubation time of 3 hours. Transfer was rapidly effected with a pair of forceps. After the final incubation period the sac was removed from the tube and an incision was made over a clean plastic tube to obtain the inner pouch fluid. The incubation tubes were then recounted and the amount of iron absorbed by the sacs was calculated from the decrease in counts. The empty sac was reweighed, digested in 3 ml of 5 N HNO_3 in a glass test tube in an 80 to 90° water bath and counted. This gave a direct estimation of iron absorption which always approximated 85 to 90% of that determined by difference. In some early experiments, untied 1- to 2-cm intestinal segments were used without eversion and the uptake was measured for a one-hour period.

Plastic tubes were used for incubation because, in early experiments, radioiron was observed to be adsorbed to the Pyrex flasks used. In addition, the use of disposable plastic tubes eliminated the need for decontamination and permitted pre-counting of outside medium.

In some cases the amounts of iron uptake were compared by calculating indexes of mucosal and pouch fluid uptake in a manner similar to that of Brown and Justus (1). That is:

$$\text{Index of mucosal } {}^{59}\text{Fe uptake} = \frac{\text{mucosal [6] cpm}}{0.8 \text{ wt sac [7] in g} \times \text{initial cpm/ml incubation fluid}}$$

$$\text{Index of pouch fluid } {}^{59}\text{Fe uptake} = \frac{\text{cpm/ml pouch fluid}}{\text{Initial cpm/ml incubation fluid}}.$$

Any index > 1.0 indicates concentration of iron; an index of ≦1.0 is characteristic of a diffusive process.

RESULTS AND DISCUSSION

Since the method used in our gut sac studies involved replenishment of the incubation medium at fixed intervals during the experiment, comparisons were made with a conventional method. The periodic renewal of the iron concentration increased the amount of iron available for absorption and, as expected, more was absorbed. The observed difference in absorption between a sac incubated in a single tube for 3 hours and a sac transferred to new medium at 30-minute intervals during the 3 hours was exclusively a function of iron concentration as determined in experiments in which the energy source alone or the iron alone was periodically restored to its initial concentration level.

To determine whether a marked anatomic gradient occurred in the capacity of the gut to absorb iron, the entire intestine of each of 7 rats was cut into 2.0-cm segments (± 150 mg wet weight) and placed serially in 10-ml Erlenmeyer flasks with 3 ml of incubation medium containing 0.5 μg $^{59}Fe^{+++}$/ml. Odd numbered segments were incubated under oxygen and the even numbered segments were incubated under nitrogen. The iron uptake was measured over a one-hour period. The observed patterns of uptake are shown in figure 1. Under both oxygen and nitrogen an absorption peak occurred in the area 8- to 12-cm distal to the duodenum. This was followed by a 10-cm area of reduced

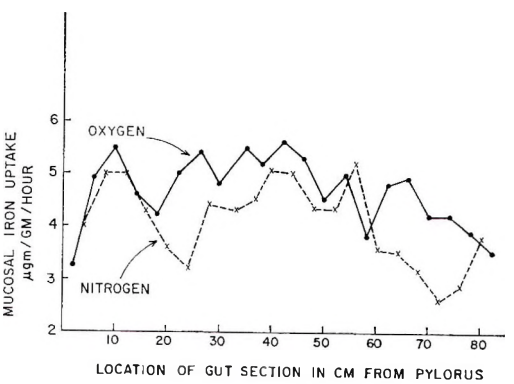

Figure 1

[5] Nuclear Chicago Model DS5-5 Scintillation Well Counter and a Model 132 Analyzer-Computer.

[6] This refers to the entire intestinal sac which consists largely of mucosa.

[7] Approximately 80% of the transferring mucosa is water.

absorption, a 30-cm area of greater uptake and a terminal 20-cm section of lower absorptive capacity. The first 2-cm gut segment distal to the pylorus showed the lowest iron absorption, being one-half that of the 2-cm segment located 10 cm distal to the duodenum. The absorption of ^{59}Fe under a nitrogen atmosphere was significantly less than that under oxygen ($P < 0.05$) when all segments were considered, but some areas were more affected than others. Iron uptake under nitrogen was particularly depressed in the section of the ileum just prior to the terminal 2 or 3 segments.

In subsequent experiments using the everted gut sac technique (as opposed to the gut segment technique described above), it was observed that the sac derived from the first 6.0 cm of gut distal to the pylorus always absorbed less iron than did a sac made from the next 6.0-cm segment. The observed difference in absorption usually approximated 20% (i.e., see fig. 2). This result is compatible with the pattern shown in figure 1 which was obtained with intestinal segments. The significant difference in uptake consistently observed in these 2 sacs not only made their use as experimental pairs impractical but led to erroneous conclusions in early experiments. On the other hand, consecutive 6.0-cm jejunal sacs prepared starting at a point 6.0 cm from the pylorus showed remarkably similar uptakes and were thus ideally suited for control-experimental pairs. Such pairs were usually used in this study.

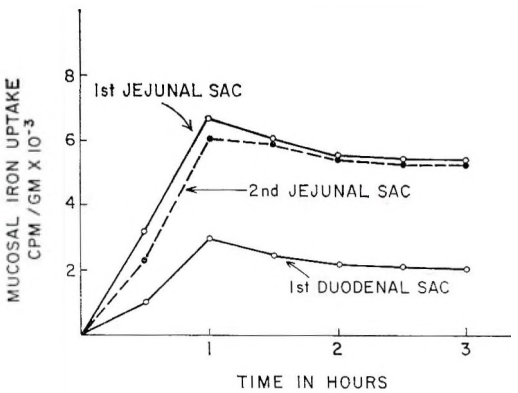

Figure 2

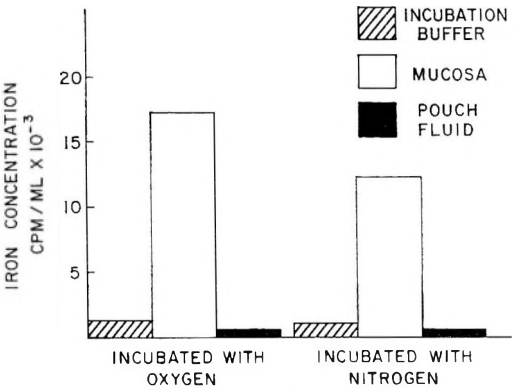

Figure 3

Mucosal uptake and serosal transfer. Using the multiple transfer technique with consecutive jejunal sacs, a rapid concentration of iron was observed in the mucosa, but little transfer of iron into the inner pouch medium could be demonstrated. Figure 3 summarizes an experiment with consecutive jejunal sacs which demonstrated concentration of iron in the mucosa (mucosal index > 1.0), the lack of concentration in the pouch fluid (pouch fluid index < 1.0) and the inhibition of mucosal uptake by nitrogen. The lack of concentration of iron in the pouch fluid was observed under a variety of experimental conditions in all sections of the gut using both multiple transfer technique and the single sac, 3-hour incubation method.

Effect of iron concentration. Six consecutive jejunal sacs were incubated using the multiple transfer technique at initial iron concentration levels of 0.004, 0.04, 0.4, 4.0, 40 and 400 μg/ml. The mucosal and pouch fluid uptakes under these conditions are plotted on a logarithmic basis in figure 4. The mucosal uptake of iron was linearly related to iron concentration even though sacs incubated at the 400 μg/ml level became "rigid" and appeared to have some iron deposited on the mucosal surface. The amount of transfer into the pouch fluid was also linearly related to the initial iron concentration. The more erratic relationship observed in the pouch fluid is partly a function of the low counting rates at the lower iron concentrations.

Iron efflux. Measurement of the magnitude of the diffusion of iron from the gut sac into the incubation mixture was con-

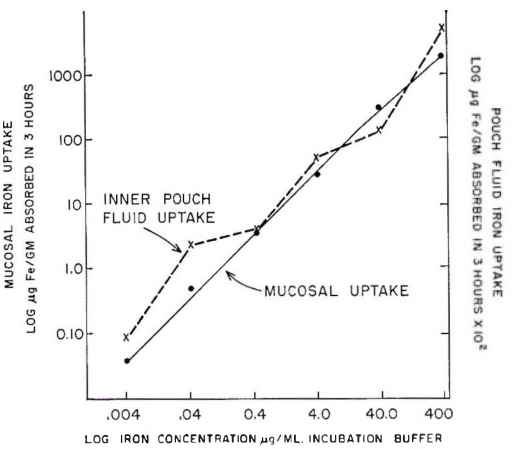

Figure 4

sidered to be of critical importance in these studies since tissue accumulation of radioisotope per se is not necessarily evidence for a net iron concentration. Accumulation of isotope might be the result of a unidirectional flux or an exchange diffusion. Accordingly, 2 types of efflux experiments were performed. In the first, the ability of the absorbed ^{59}Fe to diffuse out again into the incubation medium was measured in gut sacs that were carried through the usual transfer procedure for the first hour and were then transferred at 30-minute intervals through 4 tubes containing iron-free medium. The results shown in figure 2 reveal an iron loss of from 10 to 25% during the first hour of incubation in the iron-free medium but little loss thereafter. This suggests that the bulk of the absorbed iron has been converted to a non-diffusible form (i.e., by binding to protein). This is in agreement with the in vivo observations of Brown and Rother (7) who reported that the bulk of the non-particulate mucosal iron was protein-bound after one hour.

Because it was possible that the labeled iron was not uniformly distributed among all the iron compartments of the gut sac under the conditions of the experiment just described, another study was carried out in which the intestine was labeled in vivo with ^{59}Fe. This was accomplished by feeding 4 rats a diet containing ^{59}Fe so that their daily intake was 0.17 µg of ^{59}Fe for 3 consecutive days. At experiment, the animals were killed and jejunal gut sacs prepared. They were then placed in iron-free medium and the multiple transfer technique was carried out for 3 hours. The results (fig. 5) show a pattern of ^{59}Fe loss similar to that in the in vitro experiments. Most of the efflux (25%) occurred during the first hour of incubation with virtually no loss thereafter (± 5%).

In this in vivo study the magnitude of the total ^{59}Fe loss from each gut sac amounted to 3 to 5 µg during the 3-hour incubation period. Thus in our in vitro studies at iron concentrations of 0.004 and 0.04 µg/ml (fig. 4) it may be presumed that more iron actually diffused out of the sac than into the sac. Conversely, in the in vitro studies at iron concentrations greater than 0.4 µg/ml, there was a net accumulation of iron in the gut sac. Since no gross departure from linearity of uptake at the lower concentrations was apparent, it is probable that uptake and efflux were operating independently.

Effect of metabolic poisons. To determine whether iron uptake is dependent upon an energy-requiring process, the effects of 3 metabolic inhibitors were studied. The effect of nitrogen on mucosal iron uptake of gut segments when the iron concentration was 0.5 µg/ml has already been noted. The iron uptake of jejunal sacs was consistently depressed by nitrogen at an iron concentration of 0.004 µg/ml but not at concentrations of 4.0 or 400 µg/ml. This observation is similar to that of Brown and Justus (1) who reported that N_2 reduced mucosal uptake only if the iron concentration was low.

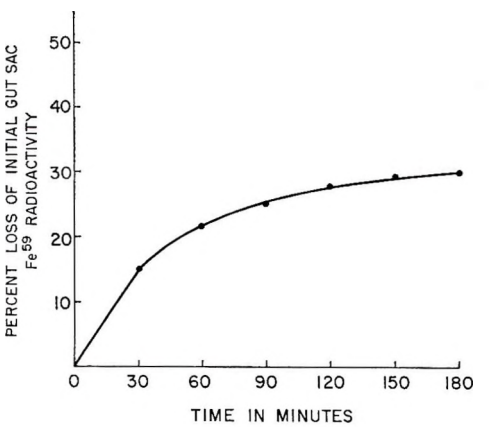

Figure 5

The effects of NaCN, 2-4-dinitrophenol (DNP) and N-ethyl maleimide on mucosal iron uptake are shown in table 1. Both cyanide and DNP markedly inhibited iron uptake at all 3 iron concentrations used but N-ethyl maleimide had little or no effect. A marked efflux of iron from the gut sacs during the last hour of the 3-hour experiment was observed when cyanide or DNP was present. It is possible that death of the tissues and autolysis may have had such a consequence.

Ascorbic acid and iron absorption. In these studies, the effects of ascorbic acid on mucosal iron uptake were determined. Paired jejunal sacs were first incubated for 90 minutes in the conventional medium and the experimental sac was then transferred to medium containing sufficient ascorbic acid to make the final concentration 8×10^{-4} M. The results expressed in table 2 indicate that ascorbic acid enhanced considerably the mucosal uptake of both ferrous and ferric iron when the iron concentration was very low (0.004 μg/ml). In general the amount of radioactivity noted in the inner pouch fluid of the intestinal sacs was somewhat less in the presence of ascorbic acid but no consistent effect was demonstrable.

Effect of anemia on iron absorption. Two studies were carried out using intestinal sacs obtained from rats made anemic by the injection of phenylhydrazine. In the first trial, female rats (150 to 170 g) were made anemic by intraperitoneal injections (8 mg/rat/day) on 3 consecutive days. The animals were killed on the fourth day and sacs were prepared from various parts of the gut. At this time, the animals usually had hemoglobin levels in the range of 5 to 7 g/100 ml. In the second trial, male rats (175 to 225 g) were made anemic in the same way but were then kept for periods of time up to one week to permit active hematopoiesis to be-

TABLE 1

Effects of metabolic inhibitors on mucosal uptake of iron by everted gut sacs from rats [1]

Exp. no.	Inhibitor	Index of mucosal uptake					
		Iron concentration, μg/ml					
		0.004		4.0		40.0	
		Inhibitor	No inhibitor	Inhibitor	No inhibitor	Inhibitor	No inhibitor
1	0.01 M NaCN	4.7	12.8	7.4	15.7	3.5	18.4
2	0.02 M 2-4-Dinitrophenol	5.3	13.0	7.7	24.4	2.6	19.5
3	0.01 M N-ethyl maleimide	12.1	13.5	15.6	17.7	18.3	19.7

[1] Six consecutive jejunal sacs from each of 2 rats were used for each experiment. Values are means of 2 sacs.

TABLE 2

Effect of ascorbic acid on mucosal uptake of ferrous and ferric iron by gut sacs from rats [1]

Conditions	Index of mucosal uptake	
	No ascorbic acid	Plus ascorbic acid [2]
Ferric chloride (ferric iron conc, 0.004 μg/ml)	13.2 ± 0.8 [3]	17.7 ± 1.1
Ferrous sulfate [4] (ferrous iron conc, 0.004 μg/ml)	13.8 ± 1.3	17.0 ± 1.0

[1] Paired jejunal sacs from 8 rats were used. The sacs were first incubated for 90 minutes in the usual medium and then the medium was made 8×10^{-4} M in ascorbic acid for the remaining 90 minutes of incubation. Indexes of uptake were calculated for the 3-hour period.
[2] No ascorbic acid vs. plus ascorbic acid, $P < 0.01$.
[3] All results are expressed as averages ± SE of mean.
[4] Ferric chloride vs. ferrous sulfate not significantly different.

TABLE 3

Effect of phenylhydrazine-induced anemia on mucosal iron uptake by everted gut sacs prepared from various sections of the intestine [1]

Location of sac	No. of sacs	Index of mucosal uptake		
		Controls	Phenylhydrazine-treated	Significance
		Experiment 1 [2]		
First duodenal	8	15.7 ± 1.2 [3]	16.3 ± 1.4	ns [4]
Second duodenal	8	19.4 ± 1.4	19.2 ± 2.3	ns
Jejunal	11	18.1 ± 1.0	19.1 ± 1.0	ns
		Experiment 2 [5]		
First duodenal	9	18.8 ± 1.3	14.4 ± 1.2	$P < 0.01$
Second duodenal	9	16.8 ± 0.7	15.7 ± 1.4	ns
Jejunal	18	19.2 ± 0.6	18.9 ± 0.8	ns

[1] In experiment 1, the treated rats were injected for 3 consecutive days (8 mg phenylhydrazine/day) and were studied on the fourth day (hemoglobin levels were 5–7 g/100 ml). In experiment 2, the rats were injected as in experiment 1 but were then retained for one week to permit regeneration of hemoglobin to levels of 9–12 g/100 ml. Control animals had hemoglobin levels of 13–16 g/100 ml. Iron concentration was 0.004 μg/ml incubation medium.
[2] Eight rats/group.
[3] All results are expressed as averages ± SE of mean.
[4] Not significant.
[5] Nine rats/group.

gin. The usual hemoglobin range for these animals at experiment was 9 to 12 g/100 ml. The data from these 2 studies are shown in table 3. Neither anemic animals nor anemic animals who were actively regenerating hemoglobin showed increased mucosal iron uptake. In fact, in the second experiment the first duodenal sacs from control rats absorbed significantly more iron than did those from rats treated with phenylhydrazine.

Effect of diet on mucosal iron uptake. To investigate the relationship between dietary iron and the iron uptake of gut sacs, 15 weanling rats were fed an "adequate" or "low" iron diet. The latter consisted of vitamin-free casein, 18%; hydrogenated cottonseed oil,[8] 5%; sucrose, 73%; adequate vitamins and an iron-free salt mixture. The following vitamins were added at the levels indicated per 100 g of basal diet: vitamin A, 400 IU; vitamin D, 40 IU; (and in milligrams) vitamin E, 7.0; thiamine, 0.5; riboflavin, 0.8; niacin, 4.0; Ca pantothenate, 4.0; biotin, 0.04; folic acid, 0.20; menadione, 0.50; inositol, 100.0; pyridoxine, 0.5; and choline chloride, 100.0. The mineral mixture was designed to meet the intakes recommended for the rat by the Committee on Animal Nutrition of the National Research Council (8) except for iron. It contained (in grams per kg of diet): $CaHPO_4$, 20.2; $NaHCO_3$, 2.7; $MgSO_4$, 1.9; KCl, 3.1; K_2CO_3, 0.5; $CuSO_4$, 0.05; $MnSO_4$, 0.11; KI, 0.07; NaF, 0.012; $ZnCl_2$, 0.025. Growth with this diet was superior to that obtained with the diet containing the iron-free salt mixture described by Gubler et al. (9) and weanling rats consistently became anemic with the low-iron diet in contrast to rats fed the Gubler diet.

The "adequate" iron diet was prepared by adding 100 mg of ferric citrate per 100 g of the "low" iron diet. The low iron diet contained 17 μg Fe/g diet (by analysis) and the adequate iron diet contained 211 μg/g (by calculation).

Three animals from each group were killed at weekly intervals from the third through the seventh week of the experiment. First duodenal, second duodenal, and jejunal sacs were studied using the multiple transfer technique and an iron concentration of 0.004 μg/ml.

Rats fed the low iron diet were anemic by the end of the second week. Since neither the hemoglobin levels nor the mucosal iron uptakes showed any particular trend during the experimental period, the data were assembled into 2 groups for the statistical analyses shown in table 4. First duodenal sacs absorbed significantly less iron than did second duodenal or jejunal sacs. Diet had no apparent effect on uptake by the duodenal sacs but the jejunal sacs from rats fed the low iron diet ab-

[8] Snowdrift, Hunt-Wesson Sales Company, Fullerton, California.

TABLE 4

Effect of dietary iron on mucosal iron uptake by everted gut sacs from various sections of the intestine [1]

Diet	Mean Hb	Index of mucosal uptake [2]		
		1st duodenal	2nd duodenal	Jejunal
	g/100 ml			
Adequate	15.6	15.5 ± 1.3 [a,3]	19.8 ± 1.8 [b]	17.5 ± 1.0 [c]
Low	8.3	13.9 ± 0.8 [a]	19.0 ± 1.0 [b]	20.3 ± 1.0 [c]

[1] Iron concentration was 0.004 μg/ml incubation medium; *a* vs. *a* and *b* vs. *b* not significantly different; c vs. c $P < 0.01$.
[2] Fifteen rats/group.
[3] All results are expressed as averages ± SE of mean.

sorbed slightly more iron than did those from rats fed the adequate iron diet.

Finally, in experiments not detailed here, no deviations from the normal in the uptake of ^{59}Fe were observed in intestinal sacs taken from rats suffering from overt deficiencies induced by dietary means of thiamine, riboflavin, pyridoxine or protein respectively.

DISCUSSION

Our observation that the first 6.0-cm gut sac distal to the pylorus absorbed less iron than did the next sac is of interest because Dowdle et al. (2) and Manis and Schachter (3) have reported in vitro transport to be maximal at the pylorus. These workers drew this conclusion largely on the basis of active serosal transfer. Their limited data on mucosal uptake suggest no marked differences. In general, our data agree with those of Brown and Justus (1) although these workers did not examine the entire gut, and the exact locations of the segments studied are not clear. There do not appear to be remarkable in vitro differences in the capacity of various areas of the gut to absorb iron — at least at the single iron concentration used in our studies.

The experiments described here demonstrate the ability of the mucosa actively to concentrate ^{59}Fe, thus confirming the reports of others (2, 5). Since iron was taken up against a concentration gradient and the uptake was markedly reduced by inhibitors of oxidative metabolism there is no doubt that iron passage into the mucosa is in part an active process. On the other hand, our data suggest that the overall process may also contain a passive component. The failure to observe saturation kinetics with increasing iron concentrations (fig. 4) suggests that iron enters the cell by diffusion. But it is also possible that we were observing an active process at the lower concentrations and a diffusion process at the higher concentrations with no sharp delineation between them. Had we extended our observations at the lower iron concentrations (i.e., 40 to 100 μg) a saturation effect might have been observed. Wheby et al. (10) have reported alteration of the kinetics of iron absorption from enzymatic to diffusive when an in vivo test dose exceeded 50 μg in normal rats. Similar results have been reported by Gitlin and Cruchaud in the mouse (11).

Retention within the cell appears to be facilitated by combination with protein since the bulk of the absorbed iron does not move easily into the inner pouch or the incubation fluid. Although Crosby (12) considers that ferritin is the protein involved, both Brown and Rother (7) and Manis and Schachter (5) present evidence to the contrary.

Our failure to observe concentration of iron in the pouch fluid is at variance with the results of Dowdle et al. (2) even though we used their incubation mixture and a similar procedure. Although most of our experiments employed jejunal sacs, we were also unable to demonstrate active concentration of iron in the pouch fluid of sacs taken from the duodenum or the ileum. We cannot account for this discrepancy but it should be added that neither did Brown and Justus (1) demonstrate active transfer of iron into the inner pouch fluid.

The failure to observe a consistently enhanced absorption in rats made anemic by phenylhydrazine injection or by dietary

means suggests that the principle mechanism controlling iron absorption does not reside in the intestinal mucosa or becomes non-operative under in vitro conditions. Again these results differ from those of Manis and Schachter (4) who demonstrated an uptake-depressing effect of dietary iron on both mucosal uptake and serosal transfer. However, these investigators made this observation only under conditions of relatively high dietary iron intakes, i.e., when comparing groups of rats whose daily intakes we calculate to be 0.6, 5.6, and 56.0 mg of iron, respectively. When groups consuming about 0.01 and 0.6 mg of iron daily were compared, no differences in absorption were observed even though the first group was markedly anemic.

The relative insensitivity of an in vitro gut sac system to physiological changes known to alter iron uptake dramatically in vivo, suggests that mucosal participation in iron transport may be under extramucosal control. If this is true, then in vivo approaches to the iron transport mechanism should prove to be more profitable.

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Vitamin B_6 Requirement of the Male Albino Rat [1]

G. H. BEATON AND M. C. CHENEY
Department of Nutrition, School of Hygiene, University of Toronto, Toronto, Canada

ABSTRACT The vitamin B_6 requirement of the male Wistar strain rat fed a 20% casein, 20% corn oil diet, was investigated in experiments designed to provide dose response curves over the range of 5 to 80 μg/day. Pyridoxine hydrochloride was administered by subcutaneous injection, by daily oral dosing and by incorporation in the diet. All routes gave comparable results. A linear relationship was noted between the logarithm of the dose administered and the body weight gain and the erythrocyte transaminase activities over considerable portions of the dose response curves. For maximal weight gain, the requirement was between 40 and 80 μg pyridoxine hydrochloride/day. For maximal erythrocyte glutamic-oxalacetic transaminase activity, the requirement was also in the range of 40 to 80 μg/day; for the glutamic-pyruvic enzyme, the requirement was 80 μg or more/day. The 3 parameters provide comparable estimates of vitamin B_6 requirement. It is suggested that rat diets should provide about 100 μg pyridoxine hydrochloride/day, or 60 to 70 μg/10 g diet, to ensure the adequacy of the intake. This is considerably higher than the commonly accepted vitamin B_6 requirement of the rat.

After reviewing the available evidence, the Committee on Animal Nutrition of the National Research Council (1) concluded that 12 μg of pyridoxine hydrochloride/day were adequate to meet the requirements of growing, pregnant or lactating rats. The Committee noted that the individual authors cited had suggested requirements varying between 8 and 25 μg/day.

After a similar review of the literature and studies made by Cuthbertson (2) in his laboratory, he concluded that the vitamin B_6 requirement of the rat was about 20 μg/10 g diet. He observed that increasing the level above 80 μg/10 g diet did not improve the growth rate even when the animals were subjected to a variety of stresses.

Cheney and Beaton (3), as a part of a study of specificity of biochemical alterations in B vitamin deficiencies, undertook an experiment, having a factorial design in which thiamine and pyridoxine were administered at 5 levels of dosage ranging from 5 to 80 μg/day. This experiment suggested that in the presence of adequate thiamine (approximately 20 μg/day), the pyridoxine hydrochloride requirement of the rat for maximal growth was at least 40 μg/day. The present studies were undertaken to confirm and extend this observation to include a consideration of the possible effect of the route of administration and source of the vitamin. Since Williams and Briggs (4) had commented upon the inadequacy of many mineral mixtures in common use, and since it was felt that this might affect the growth response, the effect of a second salts mixture was also examined.

MATERIALS AND METHODS

In all of the studies to be reported, male rats of the Wistar strain [2] were housed individually in suspended screen-bottom cages in a room maintained at approximately 24°. Food and tap water were offered ad libitum.

The basal diet used contained the following materials per 100 g diet: vitamin-free casein, [3] 20 g; sucrose, 54 g; corn oil, 20 g; cellulose, [4] 2 g; salts mixture (Steenbock-Nelson no. 40(5)), 4 g; choline, 200 mg; and inositol, 200 mg. Vitamins were added to provide the following amounts per 10 g diet: thiamine · HCl, 50 μg; riboflavin, 50 μg; Ca pantothenate, 200 μg; nicotinic acid, 200 μg; *p*-aminobenzoic acid, 200 μg; biotin, 10 μg; folic acid, 10 μg; menadione, 10 μg; vitamin B_{12}, 0.1

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[1] This research was supported by Public Health Research Grant 165-7-147, and Medical Research Council Grant MT725 from the Government of Canada.
[2] CFN strain, Carworth Farms colony, New City, Rockland County, New York.
[3] Nutritional Biochemicals Corporation, Cleveland; the casein contained approximately 0.6 μg vitamin B_6/g (providing about 1.2 μg/10 g diet).
[4] Alphacel, Nutritional Biochemicals Corporation.

μg; vitamin A, 300 IU, vitamin D, 75 IU; and α-tocopheryl acetate, 600 μg.

In experiment 2, one half of the animals were provided with a diet containing a salts mixture based upon that of Briggs and Williams.[5] The mixture was modified by the omission of the copper salt since the inclusion of any of a number of copper salts had been observed to produce rancidity in the 10% fat diets. Recently Costa et al.[6] have commented upon the production of rancidity with their salts mixture. The amount of copper contributed to the diet as a contaminant of other ingredients or in the tap water used for drinking is not known.

Pyridoxine hydrochloride was administered by 3 different routes: subcutaneous injection, oral dosing, and incorporation in the diet. To ensure that the results obtained were not attributable to the source of the vitamins, 2 sources[7] of pyridoxine hydrochloride were compared in one study.

At the end of the feeding period, animals were fasted for 18 hours and anesthetized with ether. Blood was removed by cardiac puncture. The red cells were separated, washed once with saline, then hemolyzed by dilution with 2 volumes of distilled water and frozen. Erythrocyte glutamic-oxalacetic (GOT) and glutamic-pyruvic (GPT) transaminase activities were determined by the methods previously described for blood hemolysates (6).

The results were examined statistically by analysis of variance.[8]

RESULTS

Response to injected and orally administered pyridoxine. Rats having an initial average body weight of 157 g were divided into 10 groups of 5 rats each. Five of the groups were given pyridoxine hydrochloride by daily subcutaneous injections. The remaining 5 groups received comparable doses by daily oral dosing. The vitamins were administered at 5 levels (5, 10, 20, 40 or 80 μg/day). The data on body weight gain, food intake and erythrocyte transaminase activities are shown in table 1. Since statistical analysis revealed that the route of administration did not affect

[5] Briggs, G. M., and M. A. Williams 1963 A new mineral mixture for experimental rat diets and evaluation of other mineral mixtures. Federation Proc., 22: 261 (abstract).

[6] Costa, M. A., M. A. Williams and G. M. Briggs 1965 Mineral mixtures and vitamin stability in purified rat diets. Federation Proc., 24: 691 (abstract).

[7] Pyridoxine hydrochloride was obtained from British Drug Houses, London, and from Sigma Cemical Company, St. Louis.

[8] Processing of data was conducted at the Institute of Computer Science, University of Toronto.

TABLE 1

Response to injected and oral pyridoxine hydrochloride

Route of administration	Dosage [1]	No. of rats	Body wt gain	Avg food intake (0–53 days)	Erythrocyte transaminase activity [2]	
					Glutamic-oxalacetic	Glutamic-pyruvic
	μg/day		*g/53 days*	*g/day*		
Subcutaneous injection	5	5	98 ± 3.96 [3]	12.1	943 ± 42.3	47.0 ± 4.25
	10	5	129 ± 7.80	12.8	1290 ± 39.8	44.2 ± 5.30
	20	5	181 ± 13.9	13.2	1910 ± 265	94.0 ± 14.4
	40	5	198 ± 20.0	14.1	2400 ± 114	135 ± 16.5
	80	5	210 ± 24.2	14.3	2530 ± 110	216 ± 18.0
Oral	5	5	104 ± 10.3	11.5	838 ± 78.0	35.0 ± 5.35
	10	5	149 ± 13.5	12.7	1170 ± 99.0	46.8 ± 6.62
	20	5	160 ± 22.1	13.3	1690 ± 158	80.2 ± 14.1
	40	5	193 ± 13.1	13.8	2510 ± 198	170 ± 12.9
	80	5	219 ± 14.2	14.3	2550 ± 179	183 ± 16.9
Combined data	5	10	101 ± 5.30	11.8	891 ± 60.2	41.0 ± 3.76
	10	10	139 ± 8.10	12.8	1230 ± 54.5	45.5 ± 4.04
	20	10	171 ± 12.8	13.2	1800 ± 149	87.1 ± 9.65
	40	10	196 ± 11.3	14.0	2450 ± 96.0	150 ± 11.9
	80	10	215 ± 13.2	14.3	2540 ± 97.3	200 ± 12.9

[1] As pyridoxine hydrochloride.
[2] After 84 days' feeding; expressed as μg pyruvate formed per ml hemolysate per hour.
[3] Mean ± SE.

the body weight or transaminase activities either with respect to general difference or trends with dose, the data for the 2 routes of administration were pooled by vitamin B_6 dosage and are included in figures 1, 2, and 3.

Response to dietary pyridoxine. Rats having an initial body weight of 114 g were divided into 4 sets of 4 groups. Each set was offered a diet containing 6, 12, 24, or 48 μg pyridoxine hydrochloride/10 g diet. The groups within each set received salts I and pyridoxine I, salts I and pyridoxine II, salts II and pyridoxine I, or salts II and pyridoxine II as described in table 2.

The data on food intake (and calculated vitamin intake), body weight gain and erythrocyte transaminase activity are shown in table 2. Statistical analyses of the data revealed that neither the salts mixture nor the source of vitamin B_6 had a significant effect upon body weight gain or transaminase activity or upon the trend of the response to dosage. Therefore, the data were pooled by level of vitamin administration and are incorporated in figures 1, 2, and 3; they are plotted by calculated intake so that comparison with experiment 1 is possible.

DISCUSSION

Figures 1, 2, and 3 present the data from experiments 1 and 2. For purposes of comparison, data previously reported (3) in a study of concurrent thiamine and pyridoxine administration are included. The animals selected from this study received 20, 40, or 80 μg thiamine hydrochloride/day; in other respects, these ani-

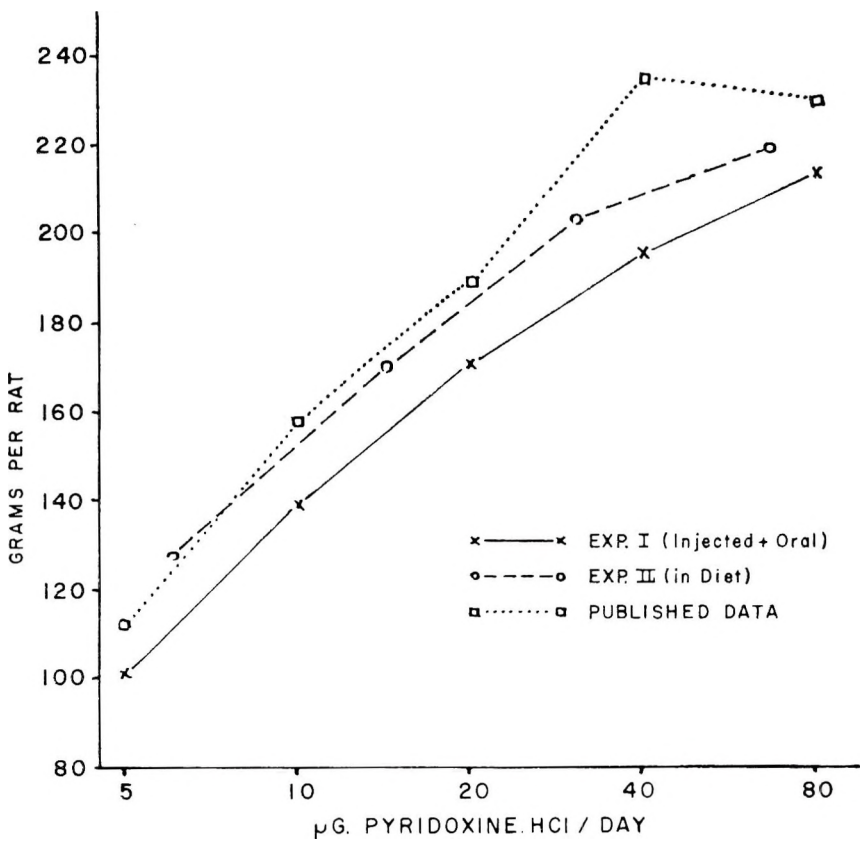

Fig. 1 Effect of vitamin B_6 dosage on body weight gain. Data have been pooled in experiments 1 and 2. Previously reported data (3) are included for comparison. Dosage is plotted on a logarithmic scale.

TABLE 2

Response to dietary pyridoxine hydrochloride after 50 days

Level of vitamin B_6 [1]	Salts [2]	Vitamin [3]	Avg food intake	Avg vitamin intake [1]	Body wt gain	Erythrocyte transaminase activity [4]	
						Glutamic-oxalacetic	Glutamic-pyruvic
μg/10 g diet			*g/day*	*μg/day*	*g/50 days*		
6	I	I	9.5	5.7	(4) [5] 130 ± 18.5 [6]	(4) 969 ± 58.1	(4) 64.0 ± 11.4
	I	II	10.1	6.1	(4) 120 ± 11.0	(4) 1060 ± 43.8	(4) 75.4 ± 15.1
	II	I	10.8	6.5	(4) 125 ± 10.5	(4) 939 ± 76.5	(4) 72.4 ± 9.78
	II	II	10.6	6.4	(5) 136 ± 16.0	(5) 1060 ± 41.6	(4) 88.9 ± 10.0
	Pooled data		10.4	6.2	(17) 128 ± 8.81	(17) 1010 ± 27.6	(16) 70.8 ± 5.57
12	I	I	11.6	13.9	(5) 167 ± 11.0	(5) 1400 ± 72.8	(5) 70.9 ± 10.5
	I	II	12.3	14.8	(4) 189 ± 14.5	(4) 1400 ± 63.9	(4) 74.1 ± 11.5
	II	I	12.1	14.5	(5) 147 ± 10.1	(5) 1500 ± 71.0	(5) 67.1 ± 14.5
	II	II	12.4	14.9	(5) 180 ± 15.8	(5) 1480 ± 125	(4) 64.9 ± 6.27
	Pooled data		12.1	14.5	(19) 170 ± 6.99	(19) 1450 ± 42.3	(18) 69.7 ± 5.32
24	I	I	13.5	32.0	(5) 200 ± 12.7	(5) 2400 ± 91.7	(5) 164 ± 15.0
	I	II	12.3	29.5	(5) 180 ± 6.11	(5) 2280 ± 81.6	(5) 128 ± 22.7
	II	I	14.5	34.8	(5) 220 ± 14.0	(4) 2090 ± 96.7	(4) 183 ± 25.8
	II	II	13.8	33.1	(5) 217 ± 3.80	(5) 2190 ± 109	(4) 191 ± 24.9
	Pooled data		13.5	32.3	(20) 204 ± 5.89	(19) 2250 ± 51.0	(18) 164 ± 11.3
48	I	I	13.2	63.4	(5) 220 ± 13.6	(5) 2460 ± 106	(5) 290 ± 23.5
	I	II	14.0	67.2	(5) 225 ± 21.3	(5) 2630 ± 103	(5) 286 ± 22.7
	II	I	13.4	64.3	(5) 215 ± 13.8	(5) 2350 ± 87.4	(5) 307 ± 30.6
	II	II	14.5	69.6	(5) 216 ± 12.7	(5) 2240 ± 100	(5) 252 ± 21.4
	Pooled data		13.8	66.2	(20) 219 ± 7.30	(20) 2420 ± 56.0	(20) 284 ± 12.3

[1] As pyridoxine hydrochloride.
[2] Salts mixtures: I, modified Briggs mixture (see footnote 4 in text); II, Steenbock-Nelson mixture (5).
[3] Vitamin sources: I, British Drug Houses, London; II, Sigma Chemical Company, St. Louis.
[4] Expressed as μg pyruvate formed/ml hemolysate/ hour.
[5] Numbers in parentheses refer to the number of animals.
[6] Mean ± SE.

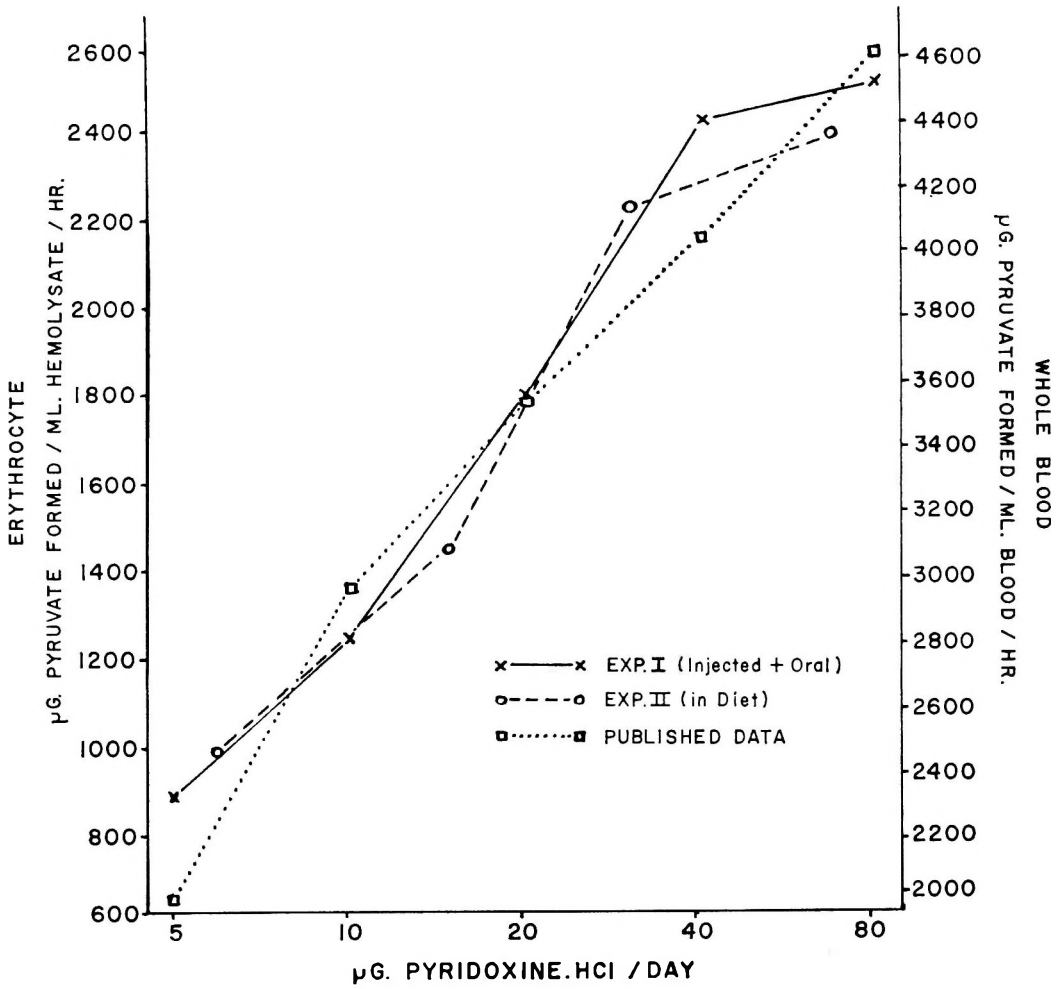

Fig. 2 Effect of vitamin B_6 dosage on erythrocyte glutamic-oxalacetic transaminase activity. Data have been pooled in experiments 1 and 2. Data on blood transaminase activity from a previous study (3) are included for comparison. Dosage is plotted on a logarithmic scale.

mals were treated in a manner similar to those in experiment 1. There were 15 rats for each level of pyridoxine hydrochloride administered. The initial body weight was 154 g; feeding was continued for 84 days. In this study, whole blood rather than erythrocyte transaminase activities were measured.

Visual inspection of these graphs indicates that a logarithmic relationship existed between the dose of vitamin B_6 administered and body weight increase (fig. 1), and erythrocyte GOT activity (fig. 2), and erythrocyte GPT activity (fig. 3), over a considerable range of dosage. The plots also reveal parallel responses in each of the 3 experiments; the results are not attributable to the method of administration.

Statistical examination revealed no significant departure from a linear (log scale) relationship between body weight gain and pyridoxine dosage in experiment 1. However, in experiment 2, a significant ($P < 0.05$) departure from linearity was noted. Graphically, this departure from the linear relationship is apparent (fig. 1) in the decreased weight response above

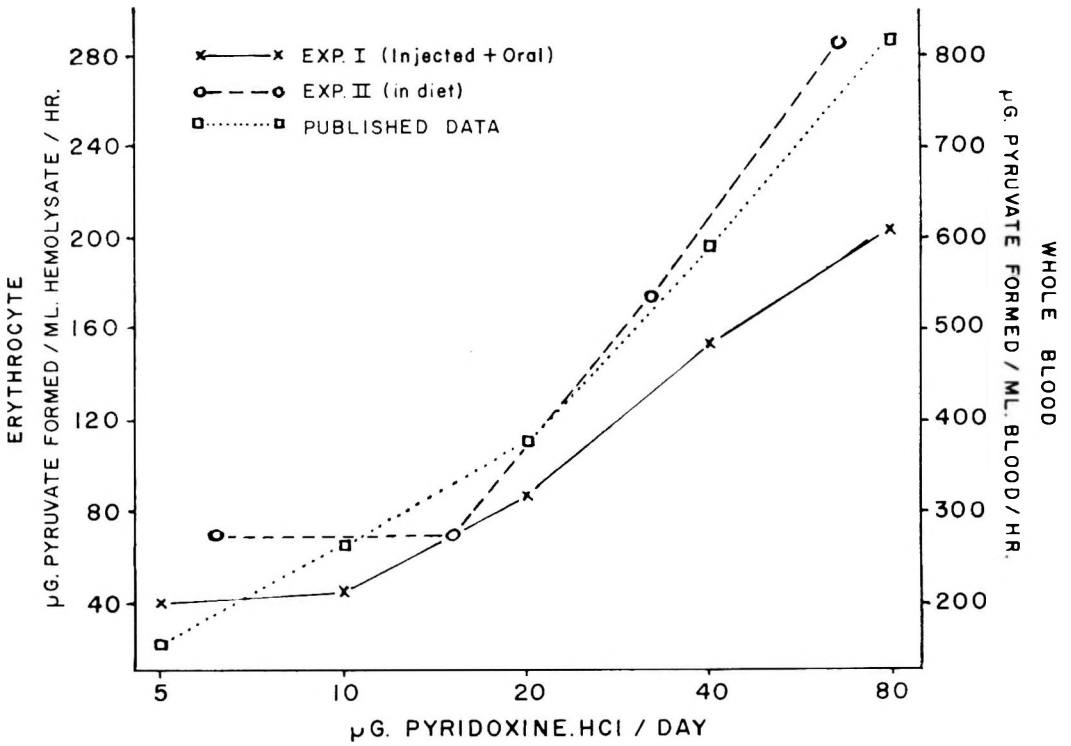

Fig. 3 Effect of vitamin B_6 dosage on erythrocyte glutamic-pyruvic transaminase activity. Data have been pooled in experiments 1 and 2. Data on blood transaminase activity from a previous study (3) are included for comparison. Dosage is plotted on a logarithmic scale.

the 40-μg dose level. A similar change in the dose response curve is evident in the published data (3). These data suggest that the pyridoxine hydrochloride requirement for maximal weight gain is at least 40 μg/day and may approximate 80 μg/day. Williams[9] has reported that body weight gain increased with increasing pyridoxine hydrochloride in the diet up to 40 to 50 μg/10 g diet; increasing the level to 96 μg did not produce a further increase in body weight. These results are in agreement with those of Heddle et al. (6) who observed little difference in body weight gain between rats given 50 μg pyridoxine hydrochloride/10 g diet (approximately 75 μg/day) and those given 2000 μg/10 g diet (approximately 2800 μg/day). Morrison and Sarett showed a slightly (but not significantly) greater body weight response when the intake of vitamin B_6 was increased from 15 to 750 μg/10 g diet (7) and from 20 to 1000 μg/day (8). The data of Clarke and Lechycka (9) also show that male rats had not achieved a maximal weight response at a dosage of 32 μg/day although the slope of the dose response curve was much lower than with levels of 1 to 8 μg/day. This study also suggested that the vitamin B_6 requirement of the male rat was higher than that of the female. Beaton et al. (10) observed that during vitamin B_6 deprivation the appearance of acrodynia was more frequent and more severe in male rats than in females, thus suggesting that the male has a higher requirement than the female.

The data for erythrocyte GOT activity (fig. 2) also show a logarithmic relationship to vitamin B_6 dosage. Significant departures from linearity were noted in experiment 1 (cubic trend, $P < 0.05$) and experiment 2 (quadratic and cubic trends, $P < 0.01$). The plotted data reveal that a

[9] Williams, M. A. 1965 Evaluation of the pyridoxine requirement of the male rat. Federation Proc., 24: 624 (abstract). These studies were carried out with weanling Long-Evans rats offered a 20% casein, 6% fat diet.

pleateau of transaminase response exists above the 40-μg dose level. Therefore, the data suggest that maximal erythrocyte GOT activity is produced with 40 to 80 μg pyridoxine hydrochloride/day.

The response of erythrocyte GPT activity to pyridoxine dosage shows an initial "lag" phase (fig. 3) at low dosage levels. This phenomenon has been noted previously (3). Above 10 μg/day, the response is linear (log scale) to the 80-μg dosage, suggesting that the vitamin B_6 requirement for maximal activity of this enzyme is at least 80 μg/day. Statistical examination of this data confirmed the existence of significant departures from linearity in both experiments ($P < 0.01$).

In the studies of Heddle et al. (6), the blood GOT activity did not increase with the increase in vitamin intake to 2000 μg/10 g diet, whereas the blood GPT activity increased slightly. Again these results are consistent with the present conclusions about the requirements for maximal erythrocyte transaminase activities. Furthermore, the results of Heddle et al. demonstrated that a 40-fold increase in pyridoxine hydrochloride administration did not cause an in vivo "induction" of enzyme activity which supports the contention that erythrocyte transaminase activity is a sensitive indicator of vitamin B_6 nutriture (3, 11). Beaton (12) has already commented upon the implications of these results in the definition of requirements for vitamin B_6. Beaton and McHenry (13) reported that the liver vitamin B_6 level increased with vitamin B_6 dosage (as free base) up to about 15 μg/day, but did not increase further as the dosage was increased to 25 μg/day. However, the body weight response was still increasing at the 25-μg dose level. Cheney and Beaton (3) demonstrated that liver GPT and GOT activities attained maximal levels with a daily intake of 20 μg/day. In contrast with these results, the present studies suggest that maximal body weight gain and erythrocyte GOT activity require a dose of 40 to 80 μg/day and maximal erythrocyte GPT activity requires a dose of 80 μg or more of pyridoxine hydrochloride per day. The need to clearly state the criterion used in describing vitamin requirements is apparent. These observations should also raise questions about the suitability of criteria used in the investigation of human vitamin requirements.

In the investigations of vitamin B_6 requirements of the rat cited by the Committee on Animal Nutrition (1), the criterion used was body weight gain. Many of the early studies of nutrient requirements were concerned with the development of biological assays for the vitamins (9, 14) and the authors were primarily interested in searching for a linear response of body weight to dose at low levels of intake; no real attempt was made to determine the maximal weight gain or to define requirements. The early studies were also conducted before supplies of purified growth factors were available (14, 15); the crude diets used make interpretation difficult. At least one study (16) provided only 10 μg of thiamine/day. Other reports provide insufficient information to judge the importance of factors which might affect the interpretation of the results (2, 17).

In conclusion, in the present studies, using relatively long feeding periods and designed to determine the maximal response obtainable, the requirement for body weight gain may be set at a value between 40 and 80 μg pyridoxine hydrochloride/day; the requirement may approximate the upper limit of this range. This is 3 to 4 times as high as the dosage required to produce maximal liver vitamin B_6 levels or liver transaminase activities. The requirement for maximal erythrocyte GOT activity lies in the 40- to 80-μg range; the erythrocyte GPT activity responds to dosages as high as 80 μg/day but this level produces near-maximal activity. Thus the body weight response and erythrocyte transaminase activities all support comparable requirement figures; this is of importance in considering the possible use of the erythrocyte transaminase activities as criteria of vitamin B_6 nutriture.

On the basis of the present results, it is suggested that experimental diets for rats should supply about 100 μg of pyridoxine hydrochloride, or its equivalent in other forms of the vitamin, per rat per day to ensure the adequacy of the diet. This would be equivalent to about 60 to 70 μg pyridoxine hydrochloride/10 g diet. It should also be recognized that increasing

the protein content of the diet above the 20% level may increase the vitamin B_6 requirement above the 100 μg intake suggested above.

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Invitation for Nominations for 1966 American Institute of Nutrition Awards

Nominations are requested for the 1966 annual awards administered by the American Institute of Nutrition to be presented at the next annual meeting. Nominations may be made by anyone, including members of the Nominating Committees and non-members of the Institute.

The following information must be submitted: (1) Name of the award for which the candidate is proposed. (2) A brief convincing statement setting forth the basis for the nomination. A bibliography and supporting letters are not to be submitted. (3) Five copies of the nominating letter must be sent to the chairman of the appropriate nominating committee *before October 1, 1965*, to be considered for the 1966 awards.

General regulations for A.I.N. awards. Membership in the American Institute of Nutrition is not a requirement for eligibility for an award and there is no limitation as to age except as specified for the Mead Johnson Award. An individual who has received one Institute award is ineligible to receive another Institute award unless it is for outstanding research subsequent to or not covered by the first award. A Jury of Award composed of A.I.N. members, which makes final selection and remains anonymous, may recommend that an award be omitted in any given year if in its opinion the work of the candidates nominated does not warrant the award. An award is usually given to one person, but, if circumstances and justice so dictate, a Jury of Award may recommend that any particular award be divided between two or more collaborators in a given research.

Presentation of awards will be made at the annual dinner at the annual meeting.

1966 Borden Award in Nutrition

The Borden Award in Nutrition, consisting of $1000 and a gold medal, is made available by the Borden Company Foundation, Inc. The award is given in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of milk or its components. The award will be made primarily for the publication of specific papers during the previous calendar year, but the Jury of Award may recommend that it be given for important contributions made over a more extended period of time not necessarily including the previous calendar year. Employees of the Borden Company are not eligible for this award nor are individuals who have received a Borden Award from another administering association unless the new award be for outstanding research on a different subject or for specific accomplishment subsequent to the first award.

Former recipients of this award are:

1944 – E. V. McCollum
1945 – H. H. Mitchell
1946 – P. C. Jeans and Genevieve Stearns
1947 – L. A. Maynard
1948 – C. A. Cary
1949 – H. J. Deuel, Jr.
1950 – H. C. Sherman
1951 – P. György
1952 – M. Kleiber
1953 – H. H. Williams
1954 – A. F. Morgan and A. H. Smith
1955 – A. G. Hogan
1956 – F. M. Strong
1957 – no award
1958 – L. D. Wright
1959 – H. Steenbock
1960 – R. G. Hansen
1961 – K. Schwarz
1962 – H. A. Barker
1963 – Arthur L. Black
1964 – G. K. Davis
1965 – A. E. Harper

NOMINATING COMMITTEE:

L. D. WRIGHT, *Chairman*
E. L. HOVE
P. H. WESWIG

Send nominations to:

DR. L. D. WRIGHT
Graduate School of Nutrition
Savage Hall, Cornell University
Ithaca, New York

1966 Osborne and Mendel Award

The Osborne and Mendel Award of $1000 and an inscribed scroll has been established by the Nutrition Foundation, Inc., for the recognition of outstanding recent basic research accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the

most significant published contribution in approximately the calendar year preceding the annual meeting of the Institute, or who has published recently a series of papers of outstanding significance. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration.

Former recipients of this award are:

1949 – W. C. Rose
1950 – C. A. Elvehjem
1951 – E. E. Snell
1952 – Icie Macy Hoobler
1953 – V. du Vigneaud
1954 – L. A. Maynard
1955 – E. V. McCollum
1956 – A. G. Hogan
1957 – G. R. Cowgill
1958 – P. György
1959 – Grace A. Goldsmith
1960 – N. S. Scrimshaw
1961 – Max K. Horwitt
1962 – William J. Darby
1963 – James B. Allison
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M. O. SCHULTZE
ALEX BLACK

Send nominations to:

DR. NEVIN SCRIMSHAW
Department of Nutrition and Food Science
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

1966 Mead Johnson Award for Research in Nutrition

The Mead Johnson Award of $1000 and an inscribed scroll is made available by Mead Johnson and Company to an investigator who has not reached his 46th birthday during the calendar year in which the Award is given. Selection by the Jury of Award will be based primarily on a single outstanding piece of research in nutrition published in the year preceding the annual meeting or on a series of papers on the same subject published within not more than the three years preceding the annual meeting.

Former recipients of this award are:

1939 – C. A. Elvehjem
1940 – W. H. Sebrell, Jr.
J. C. Keresztesy
J. R. Stevens
S. A. Harris
E. T. Stiller
K. Folkers
1941 – R. J. Williams
1942 – G. R. Cowgill
1943 – V. du Vigneaud
1944 – A. G. Hogan
1945 – D. W. Woolley
1946 – E. E. Snell
1947 – W. J. Darby
P. L. Day
E. L. R. Stokstad
1948 – F. Lipmann
1949 – Mary S. Shorb
K. Folkers
1950 – W. B. Castle
1951 – no award
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1964 – J. S. Dinning
1965 – J. G. Bieri

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Send nominations to:

DR. B. CONNOR JOHNSON
University of Oklahoma
School of Medicine
Oklahoma City, Oklahoma 73104

1966 Conrad A. Elvehjem Award for Public Service in Nutrition

The American Institute of Nutrition is pleased to announce the establishment of the Conrad A. Elvehjem Award for Public Service in Nutrition. It is to consist of $1000 and an inscribed scroll and will be made available annually by the Wisconsin Alumni Research Foundation. The award is to be bestowed in recognition of distinguished service to the public through the science of nutrition. Such service, primarily, would be through distinctive activities in the public interest in governmental, industrial, private, or international institutions but would not exclude, necessarily, contributions of an investigative character.

NOMINATING COMMITTEE:

B. S. SCHWEIGERT, *Chairman*
E. E. HOWE
F. W. QUACKENBUSH

Send nominations to:

DR. B. S. SCHWEIGERT, *Chairman*
Food Science Department
Michigan State University
East Lansing, Michigan 48823

Invitation for Nominations for 1966 American Institute of Nutrition Fellows

The Fellows Committee of the American Institute of Nutrition invites nominations for Fellows in the Society. Eligible candidates are active or retired members of the Society who have passed their sixty-fifth birthday (by the time of the annual meeting) and who have had distinguished careers in nutrition. Up to three Fellows will be chosen each year.

Nominations may be made to the Chairman of the Fellows Committee by any member of the Society, including members of the Committee.

Nominations (in 5 copies) are due by October 1. A supporting statement giving the reason for the nomination is desirable.

Final selection will be made by the Fellows Committee and a suitable citation will be presented at the Annual Dinner in April.

Fellows Committee:

CHARLOTTE M. YOUNG, *Chairman*
W. D. SALMON
S. L. HANSARD
PAUL E. JOHNSON
W. H. GRIFFITH

Send nominations to:

DR. CHARLOTTE M. YOUNG
Cornell University
Savage Hall
Ithaca, New York 14850

The following persons have been elected previously as Fellows of the Society:

J. B. Brown (1964)
Thorne M. Carpenter (1958)
George R. Cowgill (1958)
Henrik Dam (1964)
Eugene F. DuBois (1958)
R. Adams Dutcher (1961)
Ernest B. Forbes (1958)
Casimir Funk (1958)
Wendell H. Griffith (1963)
Paul György (1965)
Albert G. Hogan (1959)
Icie Macy Hoobler (1960)
Paul E. Howe (1960)
J. S. Hughes (1962)
C. Glen King (1963)
Leonard A. Maynard (1960)
Elmer V. McCollum (1958)
Harold H. Mitchell (1958)
Agnes Fay Morgan (1959)
John R. Murlin (1958)
Leo C. Norris (1963)
Helen T. Parsons (1961)
Lydia J. Roberts (1962)
William C. Rose (1959)
W. D. Salmon (1962)
Arthur H. Smith (1961)
Genevieve Stearns (1965)
Harry Steenbock (1958)
Hazel K. Stiebeling (1964)
Raymond W. Swift (1965)
Robert R. Williams (1958)

Invitation for Nominations for Honorary Membership in the American Institute of Nutrition

The Committee on Honorary Memberships of the American Institute of Nutrition invites nominations for Honorary Members.

Distinguished individuals of any country who are not members of the American Institute of Nutrition and who have contributed to the advance of the science of nutrition shall be eligible for proposal as Honorary Members of the Society.

Nominations may be made to the Chairman of the Committee on Honorary Memberships by two members of the Society.

Nominations (in three copies) are due by October 1. A supporting statement giving the reason for the nomination is desirable but not necessary.

Final selection of nominees will be made by the Council of the American Institute of Nutrition and such nominations submitted to the Society at the spring meeting. Election requires a two-thirds majority of the ballots cast.

Honorary members pay no membership fees but are eligible to subscribe to the official journal(s) at member's rates.

Committee on Honorary Memberships:

PAUL GYÖRGY, *Chairman*
PAUL DAY
GRACE GOLDSMITH

Send nominations to:

DR. PAUL GYÖRGY
University of Pennsylvania
Pennsylvania General Hospital
Philadelphia, Pennsylvania 19104

The following persons have been elected previously as Honorary Members of the Society:

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W. R. Aykroyd
Frank B. Berry
Edward Jean Bigwood
Frank G. Boudreau
Robert C. Burgess
Harriette Chick
F. W. A. Clements
David P. Cuthbertson
Herbert M. Evans
Joachim Kühnau
Toshio Oiso
Lord John Boyd Orr
Conrado R. Pascual
V. N. Patwardhan
Emile F. Terroine
Eric John Underwood
Artturi I. Virtanen

ERRATUM

Srivastava, U., A. Devi and N. K. Sarkar 1965 Biochemical changes in progressive muscular dystrophy. III. Nucleic acid, phosphorus and creatine metabolism in the muscle, liver and brain of rabbits maintained with a choline-deficient diet. J. Nutrition, *86:* 298. The description of the diet should have included the information that all rabbits, whether maintained with a choline-supplemented diet or a choline-deficient diet, received 10 mg of α-tocopheryl acetate daily by oral administration, as described by Hove and Copeland (J. Nutrition, *53:* 391, 1954).

To correct the description of the diet composition in your copy of volume 86, number 3, please cut along lines of reprinted section below and paste at the bottom of page 299.

Column 1, lines 2 and 3 should read: L-cystine, 0.1; vitamin premix, 5; and 10 mg α-tocopheryl (administered orally); as recommended by Hove and Copeland (1).
