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ANDRE MAYER

(1875–1956)

André Mayer

André Mayer

— A Biographical Sketch

(1875 – 1956)

André Mayer was born in Paris on November 9, 1875. His father, Myrtil, was a remarkable man. The son of a Vosges mountain woodcutter, he had been sent away by his father at the age of 12 with a silver five-franc piece to seek his fortune in the Alsatian Valley lest he, too, end up an illiterate lumberjack if he stayed in the hills. A resourceful boy, he decided not to become an apprentice but instead used his small capital to start in business buying and selling feathers for eiderdowns and pillows, while educating himself through books he bought or borrowed. Already prosperous at the age of twenty he switched to the textile industry, setting himself up as a draper. A gifted inventor, he successfully experimented with more and more complex machinery which could bind feathers to cloth to make "fur." The feather boa, so popular in the second half of the nineteenth century, was his brainchild. He moved to Sedan, then to Paris, and married a young lady from a well-established family in Lille. He became active in liberal causes (he was one of the heads of the Masons, the main organized opposition to Napoléon III) and in social reform (he pioneered in medico-social services and better employment conditions). He founded a number of hospitals, clinics, and sanatoriums the largest of which, situated in the Vosges, bears his name.

André, an only child, was a brilliant student in the sciences as well as in the classics. He entered medical school at the age of 16 and toward the end of his medical studies started concurrently a science degree at the Sorbonne in the laboratory of Albert Dastre, Claude Bernard's favorite student. Dastre's laboratory was an extraordinarily live place intellectually and in the span of a few years André Mayer met there many of the most active physiologists of his time. He also spent a short period in Ostwald's laboratory in Leipzig. At twenty-two he was, to all intents and purposes, an independent investigator. Within a year or two he had become the head of an active laboratory supported, first, largely by his father and after 1905 by his inheritance. His collaborators were attracted as much by his kindness, his generosity, his gaiety and his unfailing good manners as by the clarity of his intelligence, his disciplined imagination, his vigorous inductive powers and his encyclopedic scholarship. Until World War I he started his day by spending two hours operating a free clinic in a poor district of Paris.

When he was twenty-three he demonstrated the constancy of the osmotic pressure of the internal environment and showed that sudation, pulmonary evaporation and even fluid deprivation altered osmotic concentration only very slowly. The concept of osmotic constancy was so new that the Société de Biologie appointed a special commission to examine the implications of this finding. André Mayer went on to study the means by which osmotic homeostasis is maintained: reflex cardiovascular changes, use of subcutaneous and intramuscular water reserves. He examined the physical and chemical correlants of thirst, especially as related to changes in osmotic concentrations. His book "On Thirst," which appeared in 1900, created a sensation among psychologists and philosophers as well as among physiologists; for the first time a basic psychological drive was related to measurable parameters in the body; and a physiological regulation entailing a specific behavior was clearly related to physico-chemical variations.

The study of changes in the viscosity of plasma led André Mayer to study the

colloidal state of protoplasm. He went on to discover the existence of complexes: glucoproteins and lipoproteins in particular. He studied the conditions in which such complexes were reversibly or irreversibly dissociated or were precipitated. He became deeply involved in the physical chemistry of colloids and founded the French Society of Physical Chemistry of which, at a very early age, he became President. One of the fellow charter members with whom he became friendly was Pierre Curie. This friendship led him to make the first observation of the effect of radiation on living organisms. Curie said, in the course of one of their conversations: "You are a physician, I wish you would look at a sore I have which does not heal." Mayer looked at the sore which resembled a burn and was situated just under Curie's right vest pocket. Mayer asked whether Curie carried in his pocket anything which might rub against his skin. Curie said no, all he carried in this pocket was a small tube with a little radium in it. Mayer tested the tube on a colloid preparation which collapsed, then taped it onto the skin of a mouse which developed a burn similar to that of Curie.

The demonstration with Victor Henri that cytoplasm is a colloidal gel, the physical properties of which could be modified by very small chemical changes, led Mayer, together with a small group of friends whom he attracted to his laboratory —among them George Schaeffer, Emile Terroine, Emmanuel Fauré-Fremiet and F. Rathery—to investigate the difference in composition between tissues. This work led them to the concept of "cellular constants" (such as the lipocytic ratio, cholesterol:fatty acids, which they correlated with the degree of hydration of cells, and the cholesterol:phospholipids ratio). It also led to Mayer's fundamental contribution establishing the existence of lipoproteins of various types. The demonstration that, contrary to the theories of Overton, lipoprotein complexes were present inside as well as at the periphery of cells led André Mayer and his collaborators to the discovery that lipoproteins were present in large amounts in mitochondria. They showed that mitochondria were also rich in unsaturated fatty acids and that they took an active part in the metabolism of cells. In 1913, Mayer undertook with Terroine a study of the mechanism of development of fatty livers. He also developed with Armand-Delile the first "synthetic" medium for the culture of bacteria, an achievement of major importance for both bacteriology and nutrition.

Then came World War I. André Mayer volunteered on the first day and became battalion surgeon at the First Marne, then at Verdun. When the German army attacked the Canadians with poison gas, he was called back to organize the biological component of the Allied Chemical Warfare Service. Given overriding authority to call back from the Army the personnel he required and to requisition laboratories, he showed himself a superb administrator of large-scale scientific, military and industrial programs, and within the span of a few months had been given enormous executive powers and become a trusted major advisor to the Allied Commander-in-Chief and the Allied Governments.

The first reaction of many Allied scientists had been that it was folly to believe that France and the United Kingdom could win a "chemical" war against Germany and that the only course of action was immediate negotiation—in effect a capitulation. André Mayer and his British associate, Joseph Barcroft, convinced the doubters that the physiology, pharmacology, and biochemistry they could deploy were a match for "German" science and were the key to victory in "chemical" warfare. They conceived, manufactured, and distributed in record time millions of the first military gas masks so that the second German chemical attack—which occurred only a few weeks after the pilot experiment on the Canadian front—failed. Through the development of new compounds and techniques they went on to put the enemy on the defensive in this field. They were helped from 1917 on by their American colleagues, Walter Cannon and L. J. Henderson in particular. André Mayer's crucial contributions to the Allied victory were recognized by high decorations from almost all Allied armies: French, British, Rumanian, Greek, Yugoslav, Japanese, etc.

They also gave him a position of international leadership among physiologists which made it possible for him to exert a decisive influence in the creation of the first international organizations devoted to nutrition and to health (particularly, as we shall see, the United Nations Relief and Rehabilitation Administration and the Food and Agriculture Organization).

In spite of the formidable amount of activity which he had to expend in directing his laboratories, his pilot plants, and his front-line observations and experiments, André Mayer continued to have the serenity of mind necessary to permit the serendipity of the first-rate scientist. One example among many others will serve: when he was told in 1916 that a number of workers manufacturing picric acid died in extreme hyperthermia, he refused to accept the diagnosis that there must be an epidemic among them, because he was struck by the peculiar epidemiologic characteristics of these deaths: they occurred only among workers involved in the second nitration of phenol. Some experiments he conducted himself, during what little spare time he had, led him to discover the enormous increase in heat production due to dinitrophenol, a discovery which had immediate practical as well as theoretical implications.

In 1919 André Mayer married a physiologist who had been one of his prewar students and wartime assistants, Jeanne Eugénie, and he was named Professor of Physiology in the Medical School of the University of Strasbourg. He took a leading part in reorganizing the University and built an Institute which has been ever since extremely active. In 1922 he was elected Professor at the Collège de France in Paris and once again immediately attracted to his laboratory a number of the best French (and other European) scientists. In 1929 he became co-director of the Institute for Biophysics and Biochemistry with Jean Perrin, a physicist and Nobel laureate, and with the great chemist, George Urbain, the discoverer of eleven new (rare earth) elements and the theoretician of inorganic complexes. He continued concurrently to direct his laboratory at the Collège de France, of which he became Vice President for Sciences in 1930. He was also to be elected in that period a member of the National Education Council, the National Research Council, and the National Defense Research Board, as well as of the French and Belgian Academies of Medicine and later of the French Academy of Sciences. He served at various times as President of the Biochemical, Physiological, and Psychological societies as well as of the French Society for the Advancement of Science and the Federated Biological Societies. While these appear to be and were formidable administrative tasks, he continued through this period to spend at least one-half of every day in the laboratory, and he repeatedly declined positions which would have taken him away permanently from the research he loved: he turned down the Presidency of the Collège de France, of the University of Paris, and ministerial positions.

It was in his laboratory at the Collège de France that pioneer work on the influence of oxygen and CO_2 pressure, of hydration and of toxicity, and ionic concentration on the respiration of animal and vegetable tissues—basic to the subsequent development of biochemistry—was undertaken in the early twenties in collaboration with L. Plantefol. André Mayer went back to the study of 1,2,4-dinitrophenol, the action of which he had discovered in 1916 in his famous toxicological investigation. He showed that its hyperthermic effect in the whole animal was due to a hypermetabolic effect in tissues. He concluded that there can be a purely chemical thermogenesis: heat production can be increased by methods other than shivering and exercise. Mayer's analysis of the mode of action of dinitrophenol through the use of methylene blue and other hydrogen acceptors, culminating in his demonstration in 1932 that the energy released by oxidative reactions is released in the presence of dinitrophenol as heat instead of being stored as high energy compounds, was a technical and intellectual tour de force considering the state of biochemistry at that period.

Mayer and his collaborators, Plantefol, Chevillard, and Gompel, among others,

studied the effect of decreasing oxygen pressure on body temperature, and of simultaneous decreases of environmental temperature and of oxygen pressure on oxidative reactions. He demonstrated for the first time that it was possible under certain conditions to bring down the body temperature of rats, dogs, and cats to 15 to 17° and to bring them back to normal temperature without any lasting damage, a discovery which was to have many important surgical and medical applications.

In the thirties, André Mayer went back to the study of regulatory mechanisms. Having studied both hyperthermia and hypothermia, it is not unexpected that he started with the examination of the regulation of body temperature, and in particular the role of evaporation in this regulation. Of more immediate interest to nutritionists was his series of basic papers on the regulation of food intake, at a time when this basic area was almost completely neglected. He and Gasnier demonstrated that food intake was a regulated phenomenon in mammalians, that the effectiveness of the regulatory mechanism could be quantitatively evaluated by defining its reliability, its accuracy, and its sensitivity, that these parameters varied independently under various conditions and that the results obtained supported an hypothesis of two related regulatory mechanisms, a short-term (daily) mechanism corrected by a long-term mechanism. André Mayer examined the effects of heat loss and of environmental temperature on the functioning of the regulation. His fundamental work was once again interrupted by war when, in September 1939, at the age of 64, he reported to Allied headquarters as a general officer.

To fully understand André Mayer's major contribution to modern thought on human nutrition and his decisive contribution to the creation of the international institutions concerned with nutrition, we must look at his own history and at the history of ideas during his lifetime. As an ardent and resourceful mountain climber he became familiar early in life with people who are forced to extract a meager and precarious livelihood from a difficult environment, and developed profound sympathy and, indeed, great affection for them. As a very young man, in the year 1898 when he was 23, he took a long trip to Morocco, including long forays into areas generally forbidden to outsiders. He looked at "underdeveloped" areas with what would now be considered a "modern" view, but was then considered a revolutionary perspective. Through the exotic facade he saw millions of men, women and children, not as picturesque natives inhabiting a romantic land, but as fellow human beings who were sick and malnourished in a world where the means existed to bring scarcity and sickness to an end. Throughout his life, he never lost the ability to combine a clear appraisal of the technical aspects of a health or nutritional situation with a feeling of outrage that a preventable problem had lasted so long, and he maintained the fortitude, the patience and the organizational ability to bring about the necessary correction.

In the 1920's he served as chairman of the Expert Committee of the International Red Cross on the Protection of the Civilian Populations. Both at the 1921 Washington Disarmament Conference and at numerous Geneva meetings, along with a few farsighted statesmen like young Anthony Eden, he fought for the banning of chemical and bacteriological warfare. In the early 1930's he became active in the health and nutrition activities of the League of Nations, working with a group which included Rajckman, Burnet, Aykroyd, Bigwood, Mellanby, Hazel Stiebeling, McDougall and John Boyd Orr. It is usually forgotten now (and, once again, those who forget history may repeat it) that economists of that period considered agricultural "overproduction" the main cause of the depression. In a world ravaged by hunger, and threatened even in its most industrialized and heretofore most prosperous areas, economists advocated further restriction in agricultural production. The small group of nutritionists, eventually helped by labor leaders, battled the economists to a standstill. At the International Labor Conference in 1935, they appealed for "the indispensable marriage of health and agriculture." The pioneer French surveys, organized by André Mayer

and carried out in the thirties by his student and friend Lucie Randoin, and linking minimum salary, employment, nutrition and health, paralleled Orr's surveys in Britain and Stiebeling's surveys in the United States. These led to a recognition that maldistribution and the collapse of buying power had resulted in agricultural surpluses, rather than vice versa.

During World War II André Mayer had the opportunity to translate his ideas on nutrition into major institutions. After having, once again, served as the chief scientist for defense against chemical warfare for the Allies in 1939–1940, he had become the head of the Free French medical and scientific mission to the United States and from 1941 to 1944 commuted between Cambridge, Massachusetts, and Washington, D.C. In 1942, André Mayer and Frank McDougall approached Mrs. Roosevelt and, through her, President Roosevelt, with a view to implementing the "Freedom from Want" through the creation of an international organization devoted to food and agriculture. President Roosevelt was impressed by the arguments of the two Geneva veterans and agreed to call a preliminary conference—the first United Nations Meeting—in Hot Springs, Virginia, in May 1943. A decision was taken to appoint a commission to elaborate a constitution for food and agriculture; André Mayer took the leading part in this work and at the first meeting of the FAO Conference—in Quebec City in October 1945—was elected chairman of the Executive Committee of FAO. He declined to succeed Orr as Director General, but was twice President of the General FAO Conference, Chairman of the Council, and represented FAO at the United Nations on the Coordination Committee of the United Nations Agencies (the "seven wise men"), and at the Councils of UNRRA, UNESCO, and UNICEF. He was universally known as "Mr. FAO" within the organization and within the United Nations generally. The Food and Agriculture Organization has perpetuated his memory within the organization in a number of ways, the most meaningful being the establishment of the André Mayer Fellowships. Beneficiaries of these fellowships, who number many distinguished agricultural scientists and economists among them, have made important contributions toward the realization of his ideal of a world free from hunger.

In 1945 at the time of the Quebec Conference, André Mayer was 70. His work for FAO would have sufficed to fill the time of a younger man, but he resumed the direction of his laboratory and his teaching and administrative duties at the Collège de France. He was appointed chairman of the French Interministerial Committee on Food and Agriculture and served as chairman of the Social Affairs Committee of UNRRA (he had participated as the French representative in the creation of this international institution in 1943). He took an active part in establishing the International Council of Scientific Unions and was chairman of the Board of a number of institutes and foundations (some of the latter had been created by his father). He spent a great deal of his time helping to modernize the Cancer Institute in Lille, an excellent institute founded by Herbert Hoover, of which he had been since 1938 chairman of the Board and of the Executive Committee. His work was not confined to meetings and board rooms. He re-equipped his laboratory, staffed it and resumed his lectures. In the summer he continued to go to his beloved Alps and initiated his grandchildren, as he had his two children,[1] in the beauties of nature and the joy of physical effort.

He wrote a great deal, not only about physiology but about science and its relation to society, about the history of ideas, about the state of underdeveloped countries and their future and about the role of international institutions. His style was simple but noble, often even poetic. Many of his articles, such as his introduction to the volume on Life of the French Encyclopedia, have become literary classics and American, British, and other European universities awarded him honorary degrees in literature and in law as well as in science.

[1] A son, born in 1920, this writer; and a daughter, born in 1924, Geneviève Sylvie (Massé), a graduate of Radcliffe College and, after service in World War II, the University of Paris Medical School and the Harvard School of Public Health. Well known for her studies of the growth pattern of West African children and now Professor at the French School of Public Health, she is the wife of a distinguished epidemiologist who is also Professor there.

He traveled extensively, often to distant areas in which he organized and supervised nutritional surveys. After his wife died in early 1956, he departed for Senegal and Mali where, at the age of 81, he inspected survey teams in isolated villages hundreds of miles from the coast. He returned with jaundice, first diagnosed in Africa as due to infectious hepatitis, but which turned out to be due to a carcinoma of the head of the pancreas. After less than ten days of enforced inactivity, following an unsuccessful operation, he died on May 27, 1956, interested in everything but his own suffering. To the very end, when he was in great physical pain, he showed to his visitors and to the hospital staff, including its humblest members, the same innate courtesy and concern for their welfare that he had shown to every man, woman or child his life had touched.

All who saw him and heard him at international meetings remember him with the same mixture of affection and awe. He would wait while everyone else spoke at great length, defending a viewpoint, arguing for this or that advantage for his country, his institution or himself, straying from the point, forgetting the main issue, bargaining for quid pro quos. Finally, he would ask for the floor and stand, his neat, trim figure very erect. He would make kind reference to any well-intentioned or practical statement others had made. And then he would speak of people.

In rooms filled with diplomats representing governments, scientists emerging from their laboratories, scholars just out of their libraries, the miserable villages of Africa, the crowded cities of India, the suffering, uneducated, helpless children of the world would take shape. The whole tone of the meeting would change as the comfortable delegates were reminded of what really was at stake. André Mayer would recall to them their tradition of knowledge and of compassion. A few sentences from the great prophets of mankind or from the Greek philosophers or the great encyclopedists of the eighteenth century, a simple and masterly summary of the technical aspects of the problems and, finally, a lucid, precise and generous solution would usually win the day. And all would leave proud of themselves, pleased and somewhat astonished at all the good there was in them, friendly toward one another and delighted at the achievements of a conference which, a few hours before, had seemed doomed to hopeless deadlock.

André Mayer firmly believed that greater knowledge carries greater responsibility, and that physiologists have a special duty to defend Man as an organism, physicians to defend Man as a patient, and nutritionists to defend Man as a nutritional entity. Like Pericles and the ancient Athenians, he believed that men silent in the presence of injustice are useless; he would have little use for nutritionists silent as crops are being destroyed from the air, as the children of the poor remain underfed in their own rich countries or while a whole people, as we witness now in Biafra, is being exterminated by famine.

At a time when the young are avoiding the natural sciences because they are repelled by the moral neutrality of scientists we can turn back to the memory of André Mayer as that of a scholar who spoke and fought for Man, a great pioneer in science who was a great pioneer in the new humanism.

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Pyridoxine Deficiency and Iron Metabolism in the Pregnant Rat: Fetal responses [1,2]

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ABSTRACT Iron intake of rats fed pyridoxine and pyridoxine-deficient rats was approximately doubled by oral administration of $FeSO_4$ supplements containing 2 mg elemental iron daily during gestation. Effects of the treatments on the iron content of fetal plasma and tissue storage fractions were investigated. Fetuses of deficient mothers had low levels of plasma iron and transferrin and increased transferrin saturation. These changes were associated with increased concentrations of total iron, non-heme and hemosiderin components in the fetus. The overall effect of the deficiency appeared to be an increase in iron transfer from placental to fetal tissues, with some mitigation by the low levels of transferrin in fetal plasma and of ferritin in placenta. The inability of iron supplements, administered to mothers fed the vitamin, to increase the iron content of fetal plasma or tissues indicated that iron passage from the placenta to the fetus was regulated. This control mechanism appeared to be operative, at least in part, in pyridoxine deficiency since iron supplements administered to deficient mothers whose tissues were replete with iron did not result in increased fetal iron content. The decrease in placental total ferritin content in deficient and in iron-supplemented mothers is discussed in relation to the regulation.

The possibility that pyridoxine is a factor in the regulation of iron absorption is controversial. The observations of Yeh et al.[3] and Neal and Pearson (1) have led to questions concerning earlier reports that pyridoxine-deficient rats (2) and swine (3) absorb large amounts of iron despite replete body stores. Recent data from this laboratory (4) suggested no major impairment in iron absorption in pyridoxine-deficient pregnant rats administered iron supplements orally during gestation, even though this is a period during which iron absorption is markedly increased (5, 6). Elevated levels of iron in liver and spleen of deficient animals were related largely to decreased utilization of iron in hematopoiesis and to reduced fetal demands. Alterations in iron storage components including increased hemosiderin-to-ferritin ratios, however, were observed in liver, spleen and duodenal tissues of deficient animals. The extent to which the deficiency may produce similar changes in placental tissue was of interest in view of Nylander's (7) suggestion that placental ferritin in the rat may play an important part in the transfer of iron from mother to fetus. Wöhler (8) and Bothwell et al. (9) have made similar postulations for maternal to fetal iron transfer in the rabbit. The present experiment was undertaken to determine in the rat whether pyridoxine deficiency per se, or in conjunction with oral iron supplements administered to the mother during gestation, leads to changes in placental and fetal iron components.

EXPERIMENTAL

Female rats of the Sprague-Dawley strain, 80 days of age, were used. Care of the animals and composition of the diets have been described previously (4). The basal diet contained 4 g Jones-Foster salt mixture (10) per 100 g, providing 0.2 mg elemental iron/g diet. Two groups of 10 animals each received pyridoxine-deficient diets for 3 weeks prior to mating and during the gestation period. Two additional groups received 8 μg pyridoxine/g diet for a comparable period of time. Mating was confirmed by the presence of sperm in a vaginal smear. Throughout the gestation period one deficient group and one group fed pyridoxine were given daily, by stomach tube, 1 ml of a solution of

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[1] Journal Paper no. 3430 of the Purdue University Agricultural Experiment Station.

[2] Supported in part by The Nutrition Foundation, Inc.

[3] Yeh, S. O. J., B. Padella and B. F. Chow 1962 Iron absorption by vitamin B_6-deficient rats. Federation Proc., *21:* 468 (abstract).

$FeSO_4 \cdot 7H_2O$ in 1% HCl, equivalent to 2 mg elemental iron.

On day 21 of gestation, animals were anesthetized by chloroform and the uterus removed intact. The uterine wall was split and the intact placenta and sac of each fetus was removed. Amniotic fluid was withdrawn by micropipette from each sac and pooled. Fetuses were removed and implantation sites, number and weights of live young were recorded. The placenta was separated from the sac and umbilical cord, blotted and weighed.

Blood was withdrawn from each fetus by cardiac puncture. The technique described by Grazer (11) was modified to eliminate a transfer of blood from the syringe by the insertion of a heparinized capillary tube in the system between the needle and syringe.

Hemoglobin was determined as oxyhemoglobin using 0.02 ml blood and measuring the absorbance at 545 mμ.

Plasma and amniotic fluid iron concentrations were determined by an ultramicro adaptation of the method of Trinder (12) using 20-μl samples. Total iron binding capacity of plasma and amniotic fluid was determined according to procedures reported previously for maternal plasma (4). Percentage saturation of iron binding capacity was obtained by dividing plasma iron concentration by total iron binding capacity.

Fifty-microliter samples of amniotic fluid were analyzed for total protein according to a modification of the method of Kingsley (13).

Placental and fetal tissue samples were dried at 110° and wet-washed according to the method of Reitz et al. (14). Total iron content was determined spectrophotometrically by the method of Sandell (15) using ortho-phenanthroline reagent.

Placental and fetal tissue samples were homogenized, diluted with deionized–distilled water and centrifuged at 2000 × *g*. Water-soluble ferritin and water-insoluble hemosiderin were separated by the procedures described by Kaldor (16), and the iron content of each fraction was determined by the method of Sandell (15).

The data were analyzed by analysis of variance techniques (17).

RESULTS AND DISCUSSION

Average numbers and weights of live young were presented in a previous report (4). The vitamin deficiency resulted in significant increases in fetal resorptions, decreases in both numbers and weights of live young and high placental-fetal ratios. Although the fetus is believed to draw pyridoxine from maternal blood (18) the observations of this study indicated that inadequate amounts of the vitamin were withdrawn from deficient mothers to provide for normal fetal growth. Iron supplementation per se did not significantly affect reproductive performance in deficient animals or in animals fed the vitamin.

Levels of hemoglobin in maternal or fetal blood were not influenced by pyridoxine deficiency (table 1). This is in contrast to the adverse effects of the deficiency on fetal growth and suggests priorities in the utilization of the vitamin. Pyridoxine deficiency in the mother resulted in polycythemia, low mean corpuscular hemoglobin (MCH) and low mean corpuscular volume (MCV) without changes in hemoglobin concentration (4). It is possible that hematological measures not assessed in the fetus were also altered by the deficiency. Hemoglobin levels of maternal blood in all groups were consistently higher than those of fetal blood. Iron supplementation did not change the levels in maternal or fetal blood in any group. Furthermore, the supplement did not prevent the decrease in hemoglobin concentration sometimes attributed to hemodilution of pregnancy (4). Values for pregnant animals, regardless of the level of mineral fed, were significantly less than for nonpregnant animals fed the same diet.

Concentrations of iron, total iron binding capacity (transferrin) and percentage saturation of iron binding capacity (transferrin saturation) of maternal and fetal plasma and amniotic fluid are presented in table 1. A concentration gradient between maternal and fetal plasma iron levels was evident for all groups with fetal values exceeding maternal values. Values for both fetal and maternal plasma exceeded those for amniotic fluid. The transferrin saturation data in this study sug-

TABLE 1

Concentrations of maternal and fetal hemoglobin and of iron, total iron binding capacity (transferrin) and percentage of saturation of iron binding capacity (transferrin saturation) in maternal and fetal plasma and amniotic fluid

	Pyridoxine fed Fe supplemented		Pyridoxine deficient Fe supplemented		Treatment significant ($P < 0.01$)
	0 mg	2 mg	0 mg	2 mg	
Hemoglobin, g/100 ml					
Maternal	14.7 [1] ± 0.6	14.8 ± 0.9	14.2 ± 0.7	14.6 ± 0.9	
Fetal	11.2 ± 0.3	11.0 ± 0.5	11.0 ± 0.4	11.3 ± 0.5	
Iron, μg/100 ml					
Maternal plasma	88 ± 4	105 ± 5	135 ± 4	149 ± 3	Vitamin
Fetal plasma	250 ± 5	233 ± 3	207 ± 4	215 ± 5	Vitamin
Amniotic fluid	60 ± 10	62 ± 7	45 ± 8	47 ± 9	Vitamin
Transferrin, μg/100 ml					
Maternal plasma	319 ± 10	329 ± 8	345 ± 9	352 ± 11	Vitamin
Fetal plasma	374 ± 12	324 ± 10	277 ± 4	293 ± 5	Vitamin
Amniotic fluid	130 ± 12	133 ± 10	100 ± 4	98 ± 5	Vitamin
Transferrin saturation, % [2]					
Maternal plasma	27.6 ± 1.2	31.9 ± 1.5	39.0 ± 1.1	42.3 ± 1.0	Vitamin, Fe
Fetal plasma	66.8 ± 2.0	71.9 ± 1.8	74.7 ± 1.2	73.4 ± 1.4	Vitamin
Amniotic fluid	46.1 ± 6.8	46.6 ± 6.2	45.0 ± 8.2	47.9 ± 9.0	

[1] Averages for 10 rats ± SEM.
[2] Iron (micrograms per 100 ml)/transferrin (micrograms per 100 ml), multiplied by 100.

gest a passive exchange across a semipermeable membrane could be via the pathway fetal to amniotic to maternal. A clear gradient between maternal and fetal transferrin saturation was apparent, thus emphasizing the active transport role of the placenta in exchanges of iron between the maternal and fetal systems. Similar relationships were observed in all groups including those deprived of vitamin and those receiving iron supplements.

The elevated levels of plasma iron and transferrin in deficient mothers may be related, in part, to less expansion of blood volume in the deficiency (19). In support of this suggestion the concentrations of these constituents in control and deficient nonpregnant groups were not significantly different (4). The possibility of increased absorption, however, is not ruled out. Increased transferrin saturation was observed in both maternal and fetal plasma of deficient groups, and in maternal plasma of iron-supplemented groups. These marked increases in transferrin saturation in maternal circulation are consistent with increased iron absorption. The increased saturation of maternal blood could conceivably lead to increased placental iron transfer to the fetus. The data on fetal iron concentration (table 2) support, in part, this suggestion since tissue concentrations were elevated in deficient groups. Further elevation, however, was not evident in fetuses of deficient mothers receiving iron supplements.

The low concentrations of iron and transferrin in plasma of fetuses of deficient mothers were paralleled by decreases in those values in amniotic fluid. Changes in transferrin levels are generally believed to be unrelated to iron absorption but the mobilization and transport of iron are protein dependent. Increases in transferrin and serum iron have been correlated with decreases in liver iron (20). Morgan (21) suggested that the level of transferrin reflected changes in its rate of cellular synthesis or destruction, or its withdrawal or destruction by the placenta. It is possible that pyridoxine, which is a necessary cofactor in the metabolism of many amino acids, was not adequate in the fetus for the synthesis of the protein moiety which binds iron.

Iron supplementation did not alter any of the parameters of plasma iron that were assessed in either maternal or fetal blood

TABLE 2

Concentration of iron and iron storage components in placenta and fetus, and of iron in maternal liver, spleen and duodenum

	Pyridoxine fed Fe supplemented		Pyridoxine deficient Fe supplemented		Treatment significant ($P < 0.01$)
	0 mg	2 mg	0 mg	2 mg	
Placenta					
Weight, g	0.502 [1] ± 0.038	0.482 ± 0.031	0.424 ± 0.070	0.434 ± 0.088	Vitamin
Total iron, μg	31.6 ± 2.0	26.8 ± 2.5	22.4 ± 1.8	23.0 ± 1.5	Vitamin
Total iron, μg/g wet tissue	63.3 ± 3.1	56.2 ± 2.1	53.2 ± 4.1	53.4 ± 4.0	
Heme iron, μg	23.0 ± 1.5	19.7 ± 1.3	13.1 ± 0.9	15.3 ± 1.0	Vitamin
Heme iron, μg/g wet tissue	46.2 ± 3.0	41.3 ± 3.2	31.2 ± 2.5	35.7 ± 3.0	Vitamin
Non-heme iron, μg	8.5 ± 0.8	7.1 ± 0.4	9.3 ± 0.7	7.7 ± 0.5	Fe
Non-heme iron, μg/g wet tissue	17.1 ± 1.5	14.9 ± 1.2	22.0 ± 2.0	17.7 ± 1.6	Vitamin, Fe
Ferritin, μg	6.5 ± 0.5	5.3 ± 0.4	5.5 ± 0.4	4.6 ± 0.3	Vitamin, Fe
Ferritin, μg/g wet tissue	13.0 ± 1.0	11.1 ± 1.1	13.0 ± 1.1	10.6 ± 1.0	Fe
Hemosiderin, μg	2.0 ± 0.1	1.8 ± 0.1	3.8 ± 0.2	3.1 ± 0.2	Vitamin
Hemosiderin, μg/g wet tissue	4.1 ± 0.3	3.8 ± 0.2	9.0 ± 0.4	7.0 ± 0.5	Vitamin
Hemosiderin/ferritin ratio	0.316 ± 0.012	0.342 ± 0.018	0.692 ± 0.022	0.670 ± 0.023	Vitamin
Fetus					
Weight, g	4.5 ± 0.4	4.4 ± 0.7	3.3 ± 0.7	3.2 ± 0.7	Vitamin
Total iron, μg	166.4 ± 10.1	154.5 ± 12.4	165.5 ± 16.0	157.5 ± 15.0	
Total iron, μg/g wet tissue	37.2 ± 3.2	35.6 ± 3.1	50.1 ± 5.0	49.1 ± 4.2	Vitamin
Heme iron, μg	22.3 ± 2.1	22.4 ± 2.0	17.3 ± 1.4	22.1 ± 2.0	
Heme iron, μg/g wet tissue	5.2 ± 0.3	5.2 ± 0.2	5.0 ± 0.3	7.1 ± 0.4	
Non-heme iron, μg	144.1 ± 14.0	132.1 ± 10.1	148.3 ± 7.1	135.4 ± 8.0	
Non-heme iron, μg/g wet tissue	32.0 ± 2.8	30.4 ± 1.9	45.1 ± 2.5	42.0 ± 3.0	Vitamin
Ferritin, μg	108.1 ± 7.1	101.0 ± 8.0	89.2 ± 6.1	87.2 ± 6.0	Vitamin
Ferritin, μg/g wet tissue	24.0 ± 1.8	23.1 ± 2.0	27.1 ± 2.1	27.0 ± 2.2	
Hemosiderin, μg	36.0 ± 2.5	31.1 ± 3.0	59.1 ± 4.0	48.2 ± 3.2	Vitamin
Hemosiderin, μg/g wet tissue	8.0 ± 0.5	7.3 ± 0.4	18.0 ± 1.2	15.0 ± 1.0	Vitamin
Hemosiderin/ferritin ratio	0.333 ± 0.014	0.304 ± 0.010	0.663 ± 0.020	0.552 ± 0.022	Vitamin
Maternal tissues					
Liver, total iron, μg	2112 ± 144	2946 ± 162	2665 ± 158	3276 ± 143	Vitamin, Fe
Spleen, total iron, μg	843 ± 92	955 ± 81	1117 ± 113	1151 ± 169	Vitamin
Duodenum, total iron, μg	19.7 ± 3.6	36.0 ± 4.1	41.7 ± 4.3	60.6 ± 4.8	Vitamin, Fe

[1] Averages for 10 rats ± SEM.

of any group. Iron supplements did not, therefore, prevent the drop in serum iron concentration observed in fetuses of deficient mothers. Perhaps the transport of iron in the deficiency had reached a maximum as indicated by the high percentage of transferrin saturation. In addition, the low transferrin levels observed in the deficiency possibly increased the difficulty of transporting the supplemental iron from the placenta to the fetal circulation. During the 3-week experimental period 42 mg elemental iron were administered orally to the mothers in addition to a comparable level fed in the diet. Wöhler (8) found that 30 mg iron sorbitol or saccharate injected into mothers resulted in only slight increases in serum iron, and that chronic medication with three 30-mg doses for 2 weeks did not lead to inundation of the fetus.

The concentrations of iron storage components in placenta are shown in table 2. Total iron per placenta was less for deficient animals than for animals fed the vitamin, but the concentration of iron per gram of tissue was similar. The concentration of heme and total heme was less and concentration of non-heme iron was slightly elevated in placentas of deficient mothers compared with groups fed the vitamin. The former is in contrast to the observation that the concentration of hemoglobin in maternal or fetal blood was not influenced by the deficiency. It is well established, however, that pyridoxal phosphate participates in the formation of an activated glycine derivative and also acts as a coenzyme in decarboxylation of α-amino-β-ketoadipic acid in the pathway of porphyrin synthesis.

Concentration of ferritin in the placenta was not influenced by the deficiency, but hemosiderin was increased resulting in an increased hemosiderin/ferritin (H/F) ratio. Although the concentration of ferritin was not affected by the deficiency, the quantity per placenta was less. If placental ferritin, or hemosiderin, or both, are involved in the transport of iron from the mother to the fetus as suggested by Wöhler (8) and Bothwell et al. (9), the total amount of ferritin may be of more importance than the concentration. If this assumption is true, then the decrease in ferritin could be a partial explanation for the decreased iron levels in fetal plasma and amniotic fluid, and the failure to transport large excesses of iron following iron supplementation of deficient mothers.

Neither total iron content nor concentration in placental tissue was affected when iron intake was about doubled by oral iron supplements administered during gestation. Wöhler (8) observed that even in the presence of maximal maternal serum iron values and elevated iron in all organs of the mother following short-term and long-term administration of large doses of injectable iron to rabbits, only slight increases occurred in the total iron content of the placenta. Morgan (21) found no difference in placental iron content in rats that were iron loaded or depleted prior to gestation. The decrease in nonheme iron in placenta of supplemented animals in this study is associated primarily with a decrease in ferritin. The decrease in this fraction may be related to the control of iron transport to the fetus.

Concentrations of iron and iron storage components in the fetus are shown in table 2. Total iron content per fetus was not affected by the vitamin deficiency but concentration per gram of tissue was markedly increased. The high concentration of iron in fetal tissues in the deficiency and the low concentration in fetal plasma are consistent with the increased transferrin saturation and the low transferrin levels, respectively, observed in the fetus. Increased transferrin saturation has been associated with increased iron transport from the placenta to the fetus. Iron transport, however, was not further increased by doubling the iron intake of the mother during gestation. Iron supplementation of mothers fed the vitamin did not increase fetal iron content or affect the storage forms.

In pyridoxine deficiency the pattern of change in fetal iron components was similar to that found in placental tissue. Total ferritin was less in the deficiency and hemosiderin was elevated resulting in a significantly higher H/F ratio. The significance of these changes is not known. Both

iron fractions are normally used in physiological functions and the total level of hemosiderin did not appear to be excessive enough to be considered toxic. No significant difference in total non-heme iron content in fetuses of deficient mothers was observed but concentration of non-heme iron was higher. This may serve some advantage in low birth weight young of deficient mothers since non-heme iron is a storage form which can be used for the formation of essential heme compounds during early life when rapid growth increases the need for these compounds. Pyridoxine is known to be essential for heme production and may have been used preferentially for this function because fetal growth was depressed by the deficiency without any alteration in heme concentration or total quantity.

Total iron content in maternal liver, spleen and duodenum are shown in table 2. Neither the deficiency nor the level of iron supplied to the mother influenced total fetal iron, whereas iron was considerably elevated in maternal liver and duodenum by both the deficiency and iron supplementation. Similar findings also have been observed in response to injectable iron compounds in the rabbit (8). Chronic medication of the mother failed to lead to inundation of the fetus although liver, kidney, heart, lung, muscle and spleen values were considerably increased in the mother. The investigator demonstrated by histochemical techniques that the administered iron was in the maternal vessels and the intervillous spaces but the descending fetal vessels were almost devoid of iron. The observations of the present study also indicated that when maternal tissues were replete with iron following iron supplementation the passage of iron from the placenta to the fetus was not increased. Furthermore, the control of absorption was operative, at least partially, in pyridoxine deficiency. Iron supplementation in the deficiency did not result in increased fetal iron. The decreased ferritin observed in placentas of both vitamin-deprived and iron-supplemented mothers may have served some regulatory function in preventing an excessive transfer of iron to the fetus.

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Effect of Urease Immunity on Urease and Antiurease Activities in Ruminants [1,2]

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ABSTRACT Jackbean urease was extracted and recrystallized three times. Subcutaneous injection of urease in sheep produced urease antibodies in blood and certain secretions. Concurrently urease activity in rumen fluid was reduced. Antiserum gave a positive precipitin ring reaction with ruminal urease. A surgical technique for the cannulation of the parotid duct of sheep is described.

Urease immunity has been shown to increase rate and efficiency of growth in lambs fed urea-rich diets (1, 2). The results of Sidhu et al. (2) indicated that this improved performance of urease-immunized lambs is related to a reduced rate of urea hydrolysis in the gastrointestinal tract.

The purpose of the present investigation was to determine effects of urease immunity in sheep on antiurease activity in blood serum, parotid saliva and gallbladder bile; on urease activity in rumen fluid; and on the reaction between antiserum and ruminal urease.

MATERIALS AND METHODS

Urease preparation and assay. Thrice crystalline urease (urea amidohydrolase, EC. 3.5.1.5) from jackbean (*Canavalia ensiformis* L.) was prepared by the procedure of Mamiya and Gorin (3) and assayed for urease at 25° by the alkalimetric method of Gorin and Chin (4). Enzymic activity was expressed as an International Union of Biochemistry (IUB) unit which is defined as the amount of enzyme which catalyzes the hydrolysis of 0.5 μmole of urea and liberates 1 μmole of ammonia in 1 minute. Eleven IUB units of urease assayed at 25° by the alkalimetric method are approximately equivalent to 1 Sumner unit (4).

Urease antibody assays. Procedures used to assay urease antibody were enzyme inhibition, hemagglutination, precipitin ring test and gel immunodiffusion. A brief description of each method is given as follows: The enzyme-inhibition technique is based on the principle of inhibition of urease by antiurease (2). In this study, the unit represents urease recovered after incubation of the sample with standard amount of the enzyme; smaller values represent more activity.

Sheep red blood cells (RBC) were prepared according to Stoffer [6] for the hemagglutination technique. The cells were tanned, sensitized and suspended in normal rabbit serum (NRS) as described by Kabat and Mayer (5). Each sample tested for antiurease was incubated at 56° for 30 minutes to destroy any complement present. The heat-inactivated sample was thoroughly mixed with an equal volume of washed packed sheep RBC and incubated at room temperature for 10 minutes to remove the heterophile antibodies. Controls contained NRS plus tanned RBC, NRS plus sensitized RBC, undiluted antiserum plus tanned RBC, and undiluted antiserum plus sensitized RBC. Hemagglutination titers were determined after incubation at room temperature for 12 hours.

The precipitin ring test was performed in 5-mm id serological tubes by using the procedure of Burrell and Mascoli (6). The

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two controls consisted of undiluted serum and undiluted antiserum; urease was incorporated in each. The concentration of urease found suitable for detecting antiurease was 90 IUB units/ml and this level was used to determine antiurease titer of the doubly diluted samples.

The gel immunodiffusion was adopted from Gelman procedures.[7] Urease at a level of 90 IUB units/ml was used to detect antiurease in undiluted samples.

Trial 1. The purpose of this trial was to determine the effect of urease injections on urease activity of rumen fluid. Eight yearling sheep, equipped with permanent rumen fistulas, were randomly assigned, four each, to two treatments, zero and 6800 IUB units of urease. The urease for each injection was dissolved in 5 ml physiological saline and injected subcutaneously by the schedule shown in table 1. The controls received 5 ml physiological saline. Animals were housed in individual wooden pens with wooden-slatted floors and were given diet 1 (table 2) and water ad libitum. Blood samples were obtained by jugular puncture on day 31 of the trial, and serum samples were tested for antiurease by enzyme-inhibition and gel-immunodiffusion techniques. Rumen fluid samples were collected, on days 33 and 40 of the trial, through rumen fistulas and were strained through four layers of cheesecloth. In preexperimental assays for jackbean urease it was found that chloramphenicol [8] had no effect on the activity of the enzyme. Therefore, 1 ml chloramphenicol, having a concentration of 1000 μg/ml, was added per 20 ml strained rumen fluid. Urease assays were made using the procedure of Gorin and Chin (4). Rumen fluid volumes were determined according to the procedure of Smith (7) by using polyethylene glycol,[9] thereby allowing the calculation of total ruminal fluid urease activity. Statistical analysis of the data was carried out using the analysis of variance method (8).

Trial 2. Two control and two urease-injected sheep from trial 1 were selected at random for the purpose of determining the effect of urease injections on the pos-

TABLE 1

Schedule of urease injections [1]

Injection no.	Trial 1		Trial 4	
	Day	Urease [2] injected	Day	Urease [2] injected
		IUB units		*IUB units*
1	0	900 [3]	0	900 [3]
2	4	1400	7	1400
3	10	2000	14	1900
4	17	2500	21	2400
Total		6800		6600
5 [4]	—	—	56	2700 [4]
6 [5]	—	—	96	3000 [5]

[1] Subcutaneous injections.
[2] Urease was added to physiological saline and assayed before each injection. Each animal received 5 ml.
[3] Each control animal was injected with 5 ml saline.
[4,5] Booster injections.

TABLE 2

Percentage composition of diets

Ingredient	Diet 1	Diet 2
	Trial no. 1, 2, 3	Trial no. 4
	%	%
Starch (corn)	32.00	25.84
Cellulose [1]	30.00	15.00
Glucose [2]	22.65	25.84
Alfalfa meal	—	10.00
Grain sorghum (ground)	—	10.00
Isolated soy protein [3]	6.32	—
Urea [4] (46% N)	2.00	4.20
Corn oil [5]	2.00	3.00
Polyethylene resin	—	2.00
K_2CO_3	2.23	1.81
$CaHPO_4$	1.33	1.08
$MgSO_4$	0.12	0.10
$MgCO_3 \cdot Mg(OH)_2 \cdot 3H_2O$	0.27	0.22
Na_2SO_4	0.25	0.20
NaCl	0.62	0.51
Trace mineral mix [6]	0.10	0.08
Choline chloride	0.10	0.10
Vitamin A and D mix [7]	0.01	0.02
Total	100.00	100.00

[1] Solka Floc (BW20) Brown Company, Berlin, N. H.
[2] Cerelose, Corn Products Company, Argo, Ill.
[3] Purina Assay Protein, RP-100, Ralston Purina Company, St. Louis, Mo.
[4] Crystalline urea, courtesy John Deere Chemical Company, Pryor, Okla.
[5] Mazola. Santoquin added to give 0.0125% in total diet.
[6] Composition of trace mineral mix: (in milligrams per gram) $FeSO_4$, 425.1; $MnSO_4 \cdot H_2O$, 153.7; $Na_2B_4O_7$, 125.6; $ZnSO_4 \cdot 7H_2O$, 263.5; $CuCO_3 \cdot Cu(OH)_2$, 19.7; KI, 0.7; $CoCl_2 \cdot 6H_2O$, 0.5; CaF_2, 2.0; $Na_2MoO_4 \cdot 2H_2O$, 5.0; $Cr_2(SO_4)_3$, 0.4; and Na_2SeO_4, 0.2.
[7] Diets contributed: (in IU per kilogram) vitamin A, 4000; and vitamin D, 500.

[7] Gelman procedures, techniques, and apparatus for electrophoresis. Gelman Instrument Company, Ann Arbor, Mich., p. 55, 1968.
[8] Chloromycetin (chloramphenicol) sodium succinate obtained from Parke, Davis & Company, Detroit, Mich.
[9] Polyethylene glycol obtained from Matheson Coleman and Bell Manufacturing Chemists, Norwood, Ohio.

sible presence of antiurease in saliva. Two weeks before the collection of saliva, the urease-injected sheep were given a "booster" injection of 3000 IUB units of urease dissolved in 5 ml physiological saline solution while the controls received only physiological saline. Saliva samples were collected via parotid cannulas fitted by the following surgical procedure. Twelve centimeters of polyethylene tubing (A), suitable to fit snugly into the sheep parotid duct, were used. It was cut at an angle of slightly greater than 60° at one end and square at the other (fig. 1A). A second polyethylene tubing (B) with a slightly larger internal diameter than tubing A, and of sufficient length to facilitate collection of saliva was selected. One end of tubing B was flared with a flame as is done for connecting polyethylene adapters. Using a hot 22-gauge needle, four holes were punched, equidistant, around the circumference of the flare (fig. 1B). The square end of tubing A was inserted into the flared end of tubing B (fig. 1C). A drop of plastic glue [10] was applied to the square end of tubing A prior to insertion. The connection was allowed to dry for 20 minutes. Sterilization with chlorhexidine [11] solution did not affect this glued junction.

Feed and water were withheld for 16 hours prior to the operation. Anesthesia was induced by intravenous injection of sodium thiamylal [12] and maintained by halothane.[13] A skin incision of 8 cm was made; the parotid duct was exposed by blunt dissection and subsequently incised. The angular end of tubing A was inserted into the duct which was ligated to the

[10] Dab obtained from R. M. Hollingshead Corporation, Sunnyvale, Calif.
[11] Nolvasan solution; 1,1'-hexamethylenebis [5-(*p*-chlorophenyl) biguanide] diacetate obtained from Fort Dodge Laboratories, Inc., Fort Dodge, Iowa.
[12] Surital obtained from Parke, Davis & Company.
[13] Fluothane (2-bromo-2-chloro-1,1,1-trifluoroethane) made by Ayerst Laboratories, Inc., New York, N. Y.

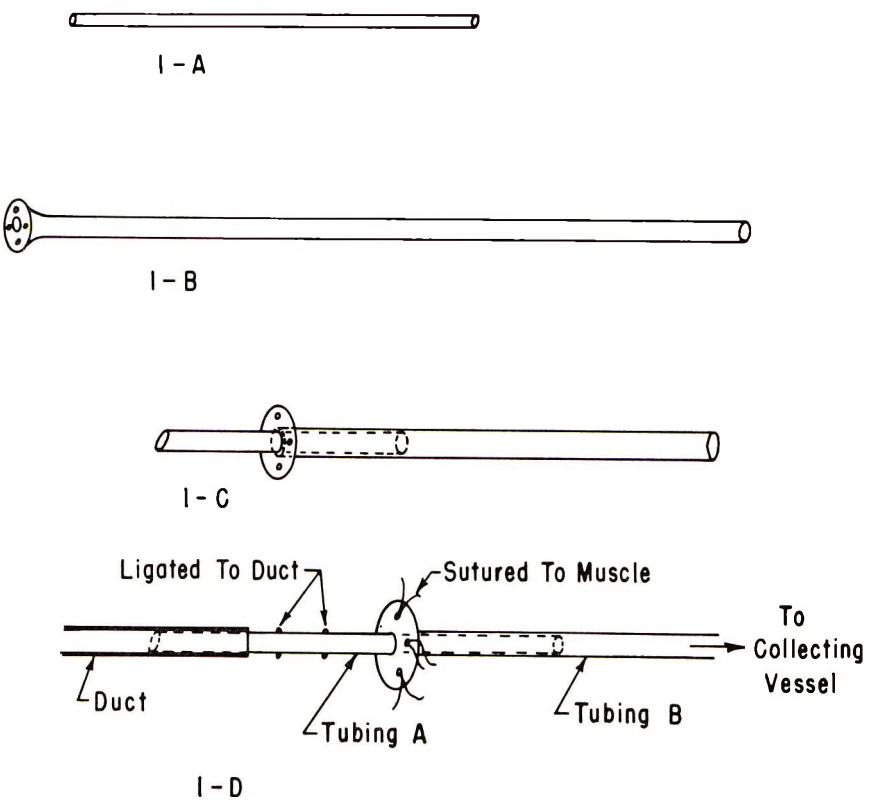

Fig. 1 Diagrammatic sketch of cannula used for collection of parotid saliva.

tubing. The flare of tubing B was sutured to surrounding muscles (fig. 1D) and the skin incision was repaired. The parotid cannula, thus fitted, allowed a free flow of saliva, prevented accidental removal, and did not restrict the jaw movements.

Each sample of saliva was concentrated 10-fold by dialyzing against polyethylene glycol. Blood samples were obtained 1 day prior to the collection of saliva samples. Antiurease activity of serum and concentrated saliva samples was determined by enzyme inhibition and hemagglutination techniques. Statistical analysis of the data was carried out by Student's t test (8). All other details were as described in trial 1.

Trial 3. Ruminal urease was prepared from mixed microorganisms from the rumen of a control-fistulated sheep fed diet 1 (table 2) to test for possible reaction between ruminal urease and antiserum. Approximately 400 ml of rumen fluid were withdrawn through the rumen fistula. It was strained twice through four layers of cheesecloth and, after straining, centrifuged at 15,000 × g at room temperature for 10 minutes. The sediment was suspended in 200 ml 0.02 M phosphate buffer (pH 7.0) and the mixture centrifuged as before. The sediment was resuspended in 100 ml 0.02 M phosphate buffer and the microbial cells were disintegrated by ultrasonic disintegration [14] for 3 minutes. The cell-free extract, which was obtained by centrifuging at 15,000 × g at 4° for 30 minutes, was found to have urease activity. The possible reaction between ruminal urease and antiserum was determined by the precipitin ring test. All other details were as described in trial 1.

Trial 4. The purpose of this trial was to determine antiurease activity in the bile of urease-immunized lambs. The animals used in this study had been immunized for another study (2) by the schedule shown in table 1; they had high antiurease activities. They were fed ad libitum diet 2 shown in table 2. The animals were killed 2 weeks after the last urease injection when gallbladder bile samples were collected. Prior to killing, blood samples were collected from each lamb by a jugular puncture. Bile samples were centrifuged at 7500 rpm at 4° for 10 minutes to remove the particulate matter. Antiurease assay on each supernate of bile samples was made by the enzyme-inhibition technique. The effect of bile on serum urease antibody titer was determined by the precipitin ring test.

A gamma globulin fraction was prepared from the supernatant of the bile collected from a urease-immunized lamb and assayed for antiurease by the hemagglutination technique. The fraction was prepared as follows: one-half volume of saturated ammonium sulfate was added to the supernate of bile. The precipitated fraction was obtained by centrifuging at 7500 rpm at 4° for 10 minutes. It was suspended in 1% NaCl solution, the volume of which was one-half the initial bile sample. The suspension was dialyzed against several changes of 0.85% NaCl solution. The suspension was poured into a graduated cylinder and dialyzed against moist polyethylene glycol until the concentration was 20 times that of the initial sample.

RESULTS AND DISCUSSION

The results of trial 1 are presented in table 3. Serum antiurease activity, determined by the enzyme-inhibition technique, was greater ($P < 0.05$) in the urease-injected sheep than in the controls. Presence of antiurease in the serum of urease-in-

TABLE 3

Effect of urease injections on the urease activity of rumen fluid and the antiurease activity of serum

Treatment [1]	Total rumen fluid urease	Serum antiurease activity
	IUB units × 10^4	*as antiurease* [2]
Control	32.4 [a]	33.6 [c]
Immunized	21.6 [b]	27.4 [d]
	2.99 [3]	0.72 [3]

[1] Four sheep per treatment and two observations per sheep.

[2] Antiurease is expressed as urease recovered after incubation of 33.0 IUB units of the enzyme with 0.2 ml serum.

[3] SE.

[a,b] Vertical values in same column are different ($P < 0.05$).

[c,d] Vertical values in same column are different ($P < 0.01$).

[14] Branson sonifier, model LS-75, manufactured by Ultrasonic Power Division, Branson Instruments Company, Stamford, Conn.

jected animals was confirmed by the gel-immunodiffusion technique (fig. 2) and the precipitin ring test. Urease-immunized sheep had lower ($P < 0.05$) total rumen fluid urease activity than the controls and it confirmed the earlier observations (2). Other workers have found low urease activity in gastrointestinal tract of urease-immunized rats, mice and guinea pigs (9, 10) and in intestines of urease-immunized swine (11).

Serum antiurease activity in trial 2 also was greater ($P < 0.05$) in the urease-injected animals (table 4). Concentrated saliva from urease-immunized lambs apparently ($P < 0.10$) inhibited jackbean urease. Mean values for hemagglutination titers of blood serum and concentrated saliva from urease-injected sheep were 1:1280 and 1:20, respectively. These results indicate that antiurease was present in the saliva of urease-immunized sheep.

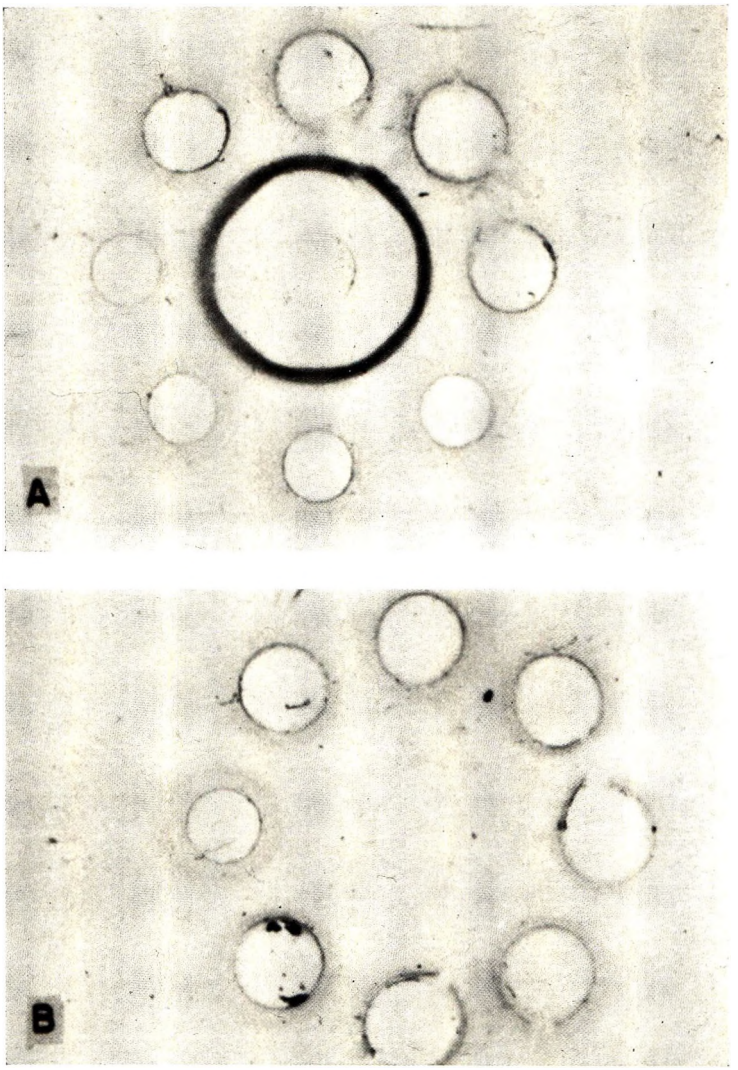

Fig. 2 Detection of serum antiurease activity by gel immunodiffusion. A = immunodiffusion of serum from a urease-injected lamb. B = immunodiffusion of serum from a control lamb. (The central well on each slide contained urease, 90 IUB units/ml, and all peripheral wells contained the serum sample tested.)

Salivary secretion of antibodies against bacterial antigens in human beings (12, 13) and against bovine plasma albumin in rats [15] has been reported.

The reaction between ruminal urease and antiserum, studied in trial 3, gave a precipitin titer of 1:160. These results obtained with sheep support the observation of Visek (14) that the serum of urease-immunized rats inhibited the gastrointestinal urease activity. It now appears that the low urease activity found in the rumen of the urease-immunized sheep could be due to a reaction between ruminal urease and salivary antiurease; antiurease was found in urease-immunized sheep saliva and the antiserum showed a positive precipitin ring reaction with ruminal urease.

Serum antiurease activity in trial 4 was greater ($P < 0.05$) in the immunized animals; however, no antiurease activity was found in the bile of urease-immunized lambs (table 5). Since the supply of bile was limited, the samples were pooled. Bile from urease-immunized lambs apparently raised antiserum precipitin titer, whereas bile from the control lambs had no effect (table 6). The gamma globulin fraction prepared from a sample of bile from a urease-immunized lamb had a hemagglutination titer of 1:160.

Sidhu et al. (2) observed low urease activity in fluids from the ileum and colon of urease-immunized lambs. It is possible that inhibition of intestinal urease results from antiurease activity of bile and possibly other intestinal secretions. Visek et al. (15) have detected urease copro antibodies in urease-immunized chicks.

Ruminal urease can penetrate to some unknown distance into rumen epithelial layers (16). The location of the urease-antiurease reaction has yet to be determined.

TABLE 4

Effect of urease injections on the antiurease activity of saliva and serum

Treatment [1]	Antiurease activity	
	Saliva [2]	Serum
	as antiurease [3]	
Control	35.5 [a]	35.8 [c]
Immunized [4]	33.1 [b]	28.0 [d]
	1.0 [5]	2.2 [5]
	as hemagglutination titer	
Control	—	—
Immunized [4]	1:20	1:1280

[1] Two sheep per treatment.
[2] Saliva concentrated 10 times.
[3] Antiurease is expressed as urease recovered after incubation of 35.8 IUB units of the enzyme with 0.2 ml serum or saliva.
[4] A booster injection of 3000 IUB was given 2 weeks before collection of samples.
[5] SE.
[a,b] Vertical values in same column are apparently different ($P < 0.10$).
[c,d] Vertical values in same column are different ($P < 0.05$).

TABLE 5

Effect of urease injections on the antiurease activity in bile and serum

Treatment [1]	Antiurease activity	
	Bile	Serum
	as antiurease [2]	
Control	35.8	35.8 [a]
Immunized [3]	33.2	25.4 [b]
	2.6 [4]	3.4 [4]

[1] Four lambs per treatment.
[2] Antiurease is expressed as urease recovered after incubation of 36.6 IUB units of the enzyme with 0.2 ml bile or serum.
[3] Two booster injections consisting of 2700 and 3000 IUB units of urease were given as shown in table 1.
[4] SE.
[a,b] Vertical values in same column are different ($P < 0.05$).

TABLE 6

Effects of bile on serum urease antibody titer

Diluent for antiserum	Serum precipitin titer
Saline	1:40
Pooled [1] bile from control lambs	1:40
Pooled [1] bile from urease-immunized lambs	1:160

[1] Four lambs per treatment.

ACKNOWLEDGMENTS

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Body Composition in Experimental Intrauterine Growth Retardation in the Rat [1]

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ABSTRACT The body compositions of a normally growing rat fetus were measured in the fetuses of eight pregnant rats during the last 5 days of intrauterine life. As the fetus grew in utero, there was a progressive contraction of body water and an increase in body protein (measured as nitrogen), fat, calcium and phosphorus contents. These changes were similar to those observed during postnatal growth. Intrauterine growth retardation was experimentally induced in five pregnant rats by ligating the uterine artery supplying one of the two uterine horns at 17 days of gestation. Intrauterine growth retardation (IUGR) was evident in 21 rat fetuses when delivered at 21 days of gestation, since their body weights were significantly lower than those of 21 control fetuses located in the unligated uterine horns. Chemical compositions were measured by tissue analysis. In IUGR fetuses, the total body water content was significantly higher, whereas the fat and protein contents were significantly lower than the control. The calcium and phosphorus contents were similar. The Ca/P ratio was lower in IUGR fetuses. The placental chemical compositions were similar between IUGR and control fetuses. It is suggested that the difference in chemical composition is probably a result of decreased placental transport of essential nutrients for fetal assimilation in utero.

The chemical growth of a newborn rat during the postnatal period has been well documented (1). It is known that after birth, a continuous contraction of body water, a progressive increase in protein, fat and mineral content occur with increasing age (1, 2). During intrauterine life, the pattern of chemical growth is not well defined, although Spray and Widdowson (1) have suggested that the changes in the prenatal and postnatal body composition follow an uninterrupted sequence. Hence, knowing the postnatal body composition, one may extrapolate and define the fetal chemical growth characteristic in utero.

In human intrauterine growth retardation (IUGR) the clinicians have observed evidence of decreased subcutaneous tissue and signs of recent wasting (3). The exact body composition of these subjects, however, has not been investigated. It is conceivable that with the disturbances in cell metabolism and growth, the body composition in IUGR is also affected. In this study, we induced IUGR in pregnant rats by uterine artery ligation. The animal model, orginally described by Wigglesworth (4), was used for tissue analysis of various chemical components to determine the changes involved.

MATERIALS AND METHODS

Thirteen female rats of the Holtzman strain [4] were mated overnight. Pregnancy was confirmed by a positive urine sperm test. In eight pregnant rats, the fetuses were delivered by cesarean section at 17, 19 and 21 days of gestation for determination of the normal chemical composition of placenta and carcass at these ages. To induce fetal growth retardation in utero, uterine artery ligation was performed in five other pregnant rats. At 17 days of gestation, a laparotomy was performed under ether anesthesia and under sterile conditions. The uterus was exposed and the uterine artery of one uterine horn was ligated. The artery of the opposite uterine horn was left untouched. The uteri were returned to the abdominal cavity and the

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[4] Holtzman Company Animal Breeders, Madison, Wis.

laparotomy wound was closed; the pregnancy was allowed to continue. For the duration of the pregnancy, the mother rat was fed commercial ration [5] and water ad libitum. At 21 days of gestation, the pregnancy was terminated by cesarean section under ether anesthesia. The rat fetuses were weighed on a torsion balance. Stunted growth, in utero, was evidenced by the discrepancy of birth weight of the fetuses found in the uterine horn whose uterine artery was ligated, compared with fetuses located in the opposite nonligated side (fig. 1). The fetuses were decapitated and total body carcass and placenta were immediately placed in separate preweighed glass containers. The tissues were weighed and minced with a scissors and subsequently dried at 100 to 105° for 72 hours. Dry weights were taken until they were constant. The difference between the wet and dry weights represented the total body water. The tissue fat was extracted five times (or until the supernate was clear) with a petroleum ethyl–ether mixture (1:1). The fat contents were equivalent to the difference between the dry weight and the fat-extracted tissue weight.

The dry fat-free solids (DFFS) were hydrolyzed with 6 N HCl for 48 hours at 100°, transferred to a volumetric flask, and made up to volume with redistilled water. Aliquots were used to measure calcium by an atomic absorption method (5); phosphorus was measured by a modified Fiske-Subbarow technique, and total nitrogen by use of Nessler's reagent after further nitric acid digestion (6).

RESULTS

Table 1 shows the changes in body weight, DFFS, total body water, fat, nitrogen, calcium (Ca), phosphorus (P), and Ca/P ratio of a normally growing rat fetus during the last 5 days of intrauterine life. During this period, body weight increased sixfold. Body water decreased from 90.6 to 87.1% of body weight. Total DFFS increased sevenfold. Fat was not detectable at 17 days of gestation, but increased to 6.2 mg/100 g DFFS at term. Nitrogen also increased from 9.4 to 12.3 g/100 g DFFS. Calcium showed the most rapid changes (0.37 to 1.89 g/100 g DFFS). Hence, despite an increasing phosphorus content (1.17 to 1.75 g/100 g DFFS), the Ca/P ratio increased from 0.31 to 1.09.

The difference in body composition of the IUGR rat fetuses and the controls, at 21 days of gestation, is evident in table 2.

[5] Purina Laboratory Chow, Ralston Purina Company, St. Louis, Mo.

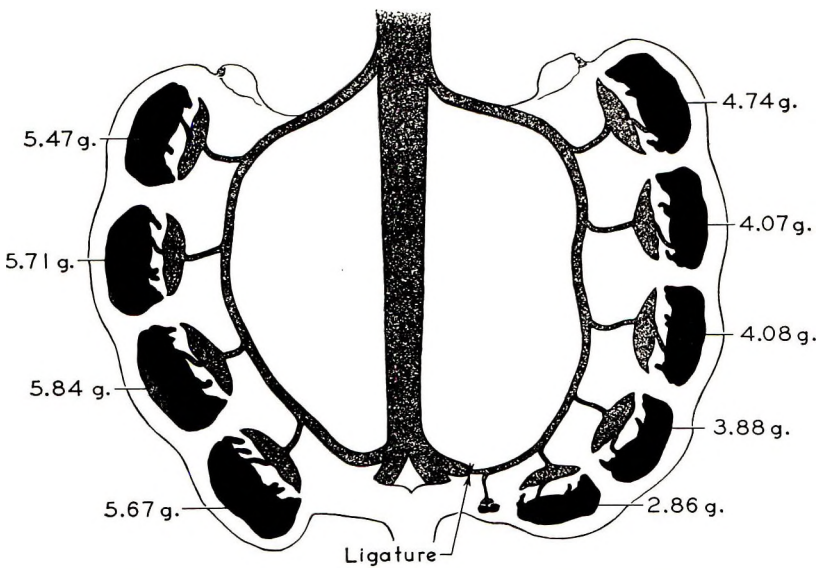

Fig. 1 Schematic presentation of animal model for experimental intrauterine growth retardation. (Modified from Wigglesworth, J. C. 1964 J. Pathol. Bacteriol., 88: 1).

TABLE 1

Chemical composition of rat fetus during the last 5 days of gestation

Gestation, day	17	19	21
Body wt, g	0.98 ± 0.02 [1]	2.4 ± 0.04	5.7 ± 0.06
Total DFFS,[2] mg	95 ± 3.0	310 ± 8.0	620 ± 20.0
Body water, % body wt	90.6 ± 0.2	87.5 ± 0.3	87.1 ± 0.1
Fat, mg/100 g DFFS	not detectable	2.9 ± 0.1	6.2 ± 0.6
Nitrogen, g/100 g DFFS	9.4 ± 0.2	11.5 ± 2.6	12.3 ± 0.7
Calcium, g/100 g DFFS	0.37 ± 0.006	1.27 ± 0.17	1.89 ± 0.06
Phosphorus, g/100 g DFFS	1.17 ± 0.03	1.30 ± 0.15	1.75 ± 0.06
Ca/P ratio	0.31 ± 0.01	0.95 ± 0.09	1.09 ± 0.4
No. of fetuses	11	19	21
No. of mothers	2	3	3

[1] Mean ± SEM.
[2] DFFS = dry fat-free solids.

TABLE 2

Body composition of intrauterine growth-retarded (IUGR) rat fetuses

	IUGR (21) [1]	Controls (21) [1]	$P <$
Body wt, g	4.6 ± 0.12 [2]	5.7 ± 0.06	0.001
DFFS,[3] g	0.49 ± 0.02	0.62 ± 0.02	0.001
DFFS, % body wt	11.76 ± 0.18	12.08 ± 0.08	ns [4]
Body water, % body wt	87.6 ± 0.12	87.1 ± 0.09	0.005
Fat, mg/100 g DFFS	4.5 ± 0.3	6.2 ± 0.6	0.005
Nitrogen, g/100 g DFFS	10.1 ± 0.5	12.3 ± 0.7	0.025
Calcium, g/100 g DFFS	1.78 ± 0.15	1.89 ± 0.06	ns
Phosphorus, g/100 g DFFS	1.93 ± 0.09	1.75 ± 0.06	ns
Ca/P ratio	0.93 ± 0.04	1.09 ± 0.04	0.01

[1] Number in parentheses indicates number of fetuses.
[2] Mean ± SEM.
[3] DFFS = dry fat-free solids.
[4] ns = not significant.

The body weights of IUGR rat fetuses were 24% less than the control. The total DFFS also were less in IUGR fetuses, but this was related to the body weight. When the DFFS were expressed per unit of body weight, no difference was observed between IUGR and control rats. Body water expressed as percentage of body weight of the IUGR rat was 0.5% more than the control; the difference was significant ($P < 0.005$). Fat content was less in IUGR fetuses (4.5 ± 0.3 versus 6.2 ± 0.6 mg/100 g DFFS). Nitrogen content was reduced (10.1 ± 0.05 versus 12.3 ± 0.7 g/100 g DFFS). Calcium content was lower and phosphorus content was higher in the IUGR than in the control rats, but the difference was not statistically significant. The Ca/P ratio was significantly lower in IUGR fetuses.

The chemical composition of placenta of the IUGR fetuses is noted in table 3. The compositions of the parameters measured were similar in the IUGR and control fetuses.

DISCUSSION

The changes of body composition during the last 5 days of gestation in the rat clearly show that as the rat grows in utero, there is a gradual increase in protein (measured as nitrogen), fat and dry fat-free solids, as well as calcium and phosphorus, with a concomitant contraction of the body water. This pattern of change is similar to the changes in body composition during the immediate postnatal life. Therefore, these findings are consistent with the concept proposed by Spray and Widdowson (1), that the body compositional change in the rat is a continuous process during pre- and postnatal life.

The differences in body composition between the IUGR fetuses and controls are of interest. The smaller body weight in IUGR rats has been demonstrated previ-

TABLE 3

Chemical composition of placenta of intrauterine growth-retarded (IUGR) rat fetuses

	IUGR (21) [1]	Controls (21) [1]
Wt, mg	755 ± 26 [2]	780 ± 20
Total DFFS,[3] mg	114 ± 5	131 ± 7
Water, % wt	84.6 ± 0.26	84.4 ± 0.35
Fat, % wt	0.73 ± 0.10	0.80 ± 0.12
Nitrogen, g/100 g DFFS	12.3 ± 0.5	12.8 ± 0.7
Calcium, mg/100 g DFFS	48.0 ± 6.0	44.0 ± 7.0
Phosphorus, mg/100 g DFFS	699 ± 74	682 ± 84
Ca/P ratio	0.09 ± 0.01	0.08 ± 0.02

[1] Number in parentheses indicates number of fetuses.
[2] Mean ± SEM. None of the differences are statistically different.
[3] DFFS = dry fat-free solids.

ously by Wigglesworth (4) and Blanc.[6] The total DFFS also is smaller in IUGR, but when related to unit body weight, no difference was observed.

The reduction of fat content is a function of decreased fat synthesis, perhaps due to decreased fatty acid transport across the placenta, as a result of reduction of placental blood flow. It is difficult to rule out the possibility that decreased fat content is also a result of increased fat utilization in utero. The observation of decreased subcutaneous tissue in human IUGR infants is confirmed by direct tissue analysis in this animal model.

The lesser protein content of IUGR rat fetuses is consistent with the concept that in IUGR the undernutrition involves all primary nutritional elements, namely, fat, carbohydrate (7) and protein. It has been shown that in human IUGR, the cord-maternal blood ratio of amino acids is lower than that of nonundergrown infants, suggesting the possibility of a decreased amino acid transport across the placenta in such pregnancies (8). This suggests that the observed low protein content in our IUGR rat fetuses may result from reduced amino acid transport and synthesis in utero, secondary to decreased placental blood flow and perfusion.

The calcium and phosphorus content are similar in IUGR and control fetuses. This is consistent with the finding in human IUGR where bone growth is within the normal range. The reason for the difference in the Ca/P ratio is not apparent.

The lack of significant differences in the placental chemical composition between IUGR and control rat fetuses is not surprising. The chemical elements measured, i.e., water, fat, nitrogen, calcium and phosphorus, are final metabolic products stored in the tissue. Since placenta is primarily a transport organ, it is, therefore, not unusual that no difference was observed.

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Vitamin E Deficiency and the Accumulation of Amino Acids in Skeletal Muscle [1,2]

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ABSTRACT The movements of ^{14}C-labeled glycine and α-aminoisobutyric acid (AIB) into and out of skeletal muscle slices from control and vitamin E-deficient rabbits were studied. Vitamin E deficiency increased ^{14}C-amino acid accumulation at 60 minutes of incubation without having any demonstrable effect either on the early phase of entry (zero to 30 minutes) or on the efflux of ^{14}C-amino acids. Inhibition of ^{14}C-glycine incorporation into protein by puromycin had no effect either on the early phase of entry or on the accumulation of ^{14}C-glycine by control or vitamin E-deficient muscle. The early phase of ^{14}C-glycine entry followed first-order kinetics; its rate constant in sec^{-1} was 5.5×10^{-4}. Whereas steady-state distribution ratios of ^{14}C-amino acids were reached by control muscle in about 30 minutes, dystrophic muscle continued to accumulate the ^{14}C-amino acids for 60 minutes or more. Small differences in influx, efflux, or both, can result in significant differences in net accumulation with extended time. Thus, we suggest that vitamin E-deficient muscle has one or more compartments capable of increasing the accumulation of ^{14}C-glycine and ^{14}C-AIB due to a change either in influx or efflux which is too small to demonstrate. These compartments may be located in regenerating muscle cells, or in other cell types more abundant in the dystrophic muscle.

Accompanying the muscle wasting of vitamin E deficiency are abnormalities of amino acid metabolism. Dinning et al. (1) observed that vitamin E deficiency increases the labeling of muscle protein by ^{14}C-glycine in vivo, an observation which subsequently has been confirmed in vivo (2–6) and in vitro (7–9). While investigating possible causes of the increased protein labeling, Diehl and Diehl and Jones (10–12) found that vitamin E-deficient muscle accumulated ^{14}C-glycine and ^{14}C-α-aminoisobutyric acid (AIB) to a greater extent than did control muscle in vivo and in vitro (8, 10, 13). The latter amino acid, AIB, has been shown by Christensen et al. (14) to be nonmetabolizable.

In the present report we have concerned ourselves with the question of how vitamin E-deficient muscle accumulates increased amounts of amino acids. We have attempted to answer this question by measuring the movement of ^{14}C-amino acids into and out of muscle slices from control and vitamin E-deficient rabbits.

We have also asked whether the increased amino acid accumulation is dependent on de novo protein synthesis. This question was answered by measuring amino acid accumulation in the presence of puromycin.

METHODS

White New Zealand rabbits of either sex, initially weighing approximately 500 g, were fed a purified vitamin E-deficient diet. The composition of the diet is as follows: (in grams) sucrose, 294.75; cornstarch, 270; cellulose,[6] 250; casein,[7] 112.50; salt mixture, 22.5; cod liver oil, 22.5; stripped lard,[8] 22.5; vitamin mixture,[9] 3.75; inositol,

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[2] Part of these data were included in a preliminary report at the 52nd Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, 1968.

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[6] Alphacel, Nutritional Biochemicals Corporation, Cleveland, Ohio.

[7] Vitamin-free casein, Nutritional Biochemicals Corporation.

[8] Obtained from Distillation Products Industries, Rochester, N. Y.

[9] Vitamin mixture: (in grams) casein (vitamin-free), 357; and nicotinamide, 15; and (in milligrams) pyridoxine, 375; thiamine·HCl, 375; riboflavin, 375; folic acid, 375; menadione, 190; and biotin, 3.75.

0.75; choline chloride, 0.75; and 45 μg vitamin B_{12}. All rabbits received 0.05% sodium sulfaquinoxaline in the drinking water 5 days of each week to control coccidiosis (5). Control rabbits were given oral supplements of 12 mg *dl*-α-tocopheryl acetate/kg body weight three times weekly.

When the vitamin E-deficient rabbits showed distinct signs of muscular weakness (3 to 5 weeks after starting the vitamin E-deficient diet), they and control rabbits which had received the diet for a comparable length of time were killed for studies in vitro. Longitudinal slices of skeletal muscle were prepared as described previously (9) and were incubated with glycine-1-^{14}C (4.85 mCi/mmole), AIB-1-^{14}C (4.0 to 5.1 mCi/mmole), or inulin-methoxy-^{3}H (164 to 351 mCi/g).[10] Some incubation vessels also contained puromycin dihydrochloride.[11] Other incubation conditions are given in the legends to the figures. Radioactivity was measured with a liquid scintillation spectrometer [12] equipped with an automatic external standard. Sufficient counts were collected so that the error of the counting procedure was less than 5%.

Following incubation the muscle slices were blotted, weighed and homogenized with 10% trichloroacetic acid (TCA). The homogenate was heated at 100° for 30 minutes. To obtain protein samples, the homogenate was centrifuged and washed sequentially with 5% TCA, 70% ethanol, 95% ethanol and diethyl ether. The remaining residue, TCA-precipitable protein, was dissolved in 0.2 N NaOH and diluted with distilled water. Separate aliquots of the final protein solution were used for measurement of radioactivity and for measurement of protein concentration by the biuret method (15). With this procedure we could account for 95% or more of the added radioactivity.

The accumulation of ^{14}C-amino acids in the intracellular water was measured in the following manner. The 10 and 5% TCA fractions were combined, and aliquots of the incubation media and the pooled TCA fractions were counted for radioactivity. Efflux was measured by first incubating the muscle slices in the presence of ^{14}C-amino acid. Then, after carefully removing the slices and gently blotting them, they were incubated in the presence of unlabeled amino acid. Small aliquots of the second incubation medium were withdrawn at various time intervals and used for measurement of radioactivity.

Because Diehl (10) had previously shown that muscle slices weighed the same before and after 60 minutes of incubation, the total water content of muscle slices which had been incubated for 30 minutes was measured by drying to a constant weight at 105°. Muscle slices from control and vitamin E-deficient rabbits gave total water values of 83.6 and 83.0 g/100 g wet weight tissue, respectively. Extracellular space was measured by incubating muscle slices with ^{3}H-labeled inulin. The wet muscle weight and total muscle water were used to calculate the volume of intracellular water (total water minus inulin space).

Significant statistical difference between experimental conditions was obtained using Student's t test. $P < 0.05$ was taken as the limit of statistical significance.

RESULTS

Measurements of the inulin space of control and vitamin E-deficient muscle slices are shown in figure 1. Compared with control muscle, the vitamin E-deficient muscle required a longer incubation time to approach a steady-state value for the inulin space. The difference in the inulin space of control and dystrophic muscle slices was significant after 10 and 20 minutes incubation, but was not significant after 30 or 60 minutes incubation. Longer incubations were not done because Diehl (10) reported that inulin uptake did not increase appreciably after 60 minutes. Because the steady-state inulin space appears to be the best estimate of the true extracellular space, and because there was no difference between the two groups at 60 minutes, the 60-minute values of control and vitamin E-deficient muscle were averaged; this gave an inulin space of 0.41 g extracellular water per gram wet weight muscle slice. Although this value is higher than that reported for the inulin space of skeletal muscle in vivo (16, 17), it agrees well with the inulin space value of skeletal muscle slices found earlier by Diehl (10).

[10] New England Nuclear Corporation, Boston, Mass.
[11] Nutritional Biochemicals Corporation.
[12] Packard Tri-Carb, Packard Instrument Company, Inc., Downers Grove, Ill.

The accumulation of ^{14}C-labeled glycine and AIB is shown as a function of time in figure 2. At an external concentration of 0.5 mM glycine, which is approximately the physiological extracellular concentration (18), vitamin E deficiency had no effect on ^{14}C-glycine accumulation during 10 to 30 minutes of incubation. After 10 minutes of incubation at an external concentration of 6 mM glycine, however, there was less ^{14}C-glycine in dystrophic muscle than in control muscle; this difference was no longer present at the end of 20 minutes of incubation. In agreement with the fore-

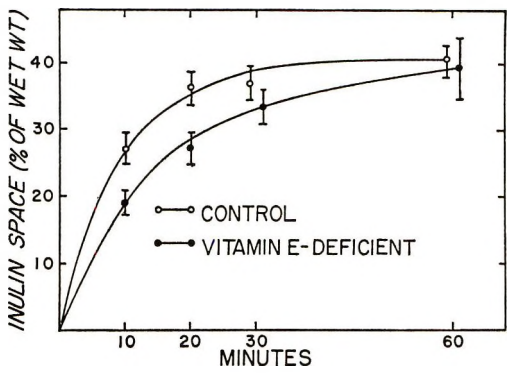

Fig. 1 Inulin space of skeletal muscle slices. Incubation vessels containing 5.0 to 6.0 μCi of ^{3}H-inulin (2.15 to 2.90 mg/100 ml) in 3.0 ml of Krebs-Ringer bicarbonate solution, pH 7.4, were allowed to equilibrate in a Dubnoff metabolic shaking incubator at 37° under an O_2:CO_2, 95:5, gas phase. Incubations were begun by placing an individual blotted skeletal muscle slice (approximately 300 mg wet weight) in each incubation vessel. Incubations were done both in the absence and presence of puromycin (200 μg/ml incubation medium). Because puromycin had no significant effect on the inulin space either of control or vitamin E-deficient muscle, the data obtained from incubations with and without puromycin were combined. The thin vertical lines in this and subsequent figures indicate 1 standard error of the mean. Each point at 10, 20, and 30 minutes is the mean of data from nine rabbits; each point at 60 minutes is the mean of data from four rabbits.

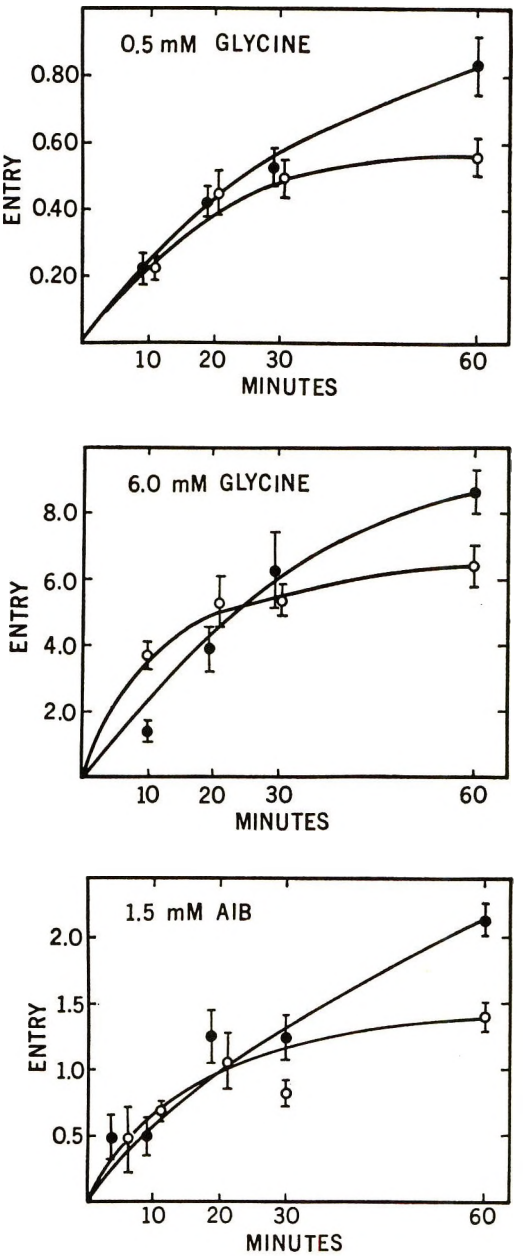

Figure 2

Fig. 2 Time course of glycine and AIB entry. Incubation vessels contained either 3.0 μCi of ^{14}C-glycine or 0.6 μCi of ^{14}C-AIB and the specified total external amino acid concentration. The incubations were done in the absence and presence of puromycin (200 μg/ml incubation medium). Because puromycin had no significant effect on the amino acid entry either into control or vitamin E-deficient muscle, the data obtained from incubations with and without puromycin were combined. Other incubation conditions are given in figure 1. A value of 41% for the inulin space was used in all calculations. The ordinate indicates the total amino acid accumulation in millimoles per liter of intracellular water. Each point is the mean of data from four to six rabbits. Control, ○ - - - ○; vitamin E-deficient, ● - - - ●.

going observations, vitamin E deficiency did not accelerate the early phase of entry of ^{14}C-AIB.

The effect of increasing the external amino acid concentration on the early phase of ^{14}C-glycine entry is shown in figure 3. During the first 20 minutes of incubation (early phase of entry) ^{14}C-glycine entry followed first-order kinetics and was the same in control as in dystrophic muscle; the rate constant in sec^{-1} is 5.5×10^{-4}.

The efflux of ^{14}C-glycine, ^{14}C-AIB and ^{3}H-inulin from muscle slices is shown in figure 4. Vitamin E deficiency had no significant effect on the efflux of any of the three compounds. In each case about 90% of the label was lost from the muscle slices during a 60-minute period of incubation. If vitamin E deficiency does decrease amino acid efflux, its effect is obscured by the large and rapid loss of label plus overlap in the data.

Despite our inability to demonstrate that vitamin E deficiency causes an increase in

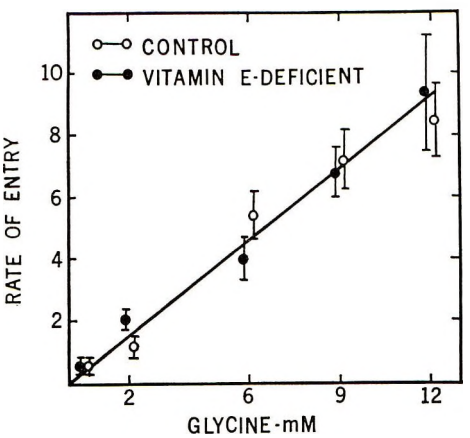

Fig. 3 Effect of external glycine concentration on the rate of glycine entry. The incubation time interval was 20 minutes. The ordinate indicates the amino acid accumulated in millimoles per liter of intracellular water per 20 minutes. Other incubation conditions are given in figures 1 and 2. A value of 41% for the inulin space was used in all calculations. Each point is the mean of data from four to six rabbits.

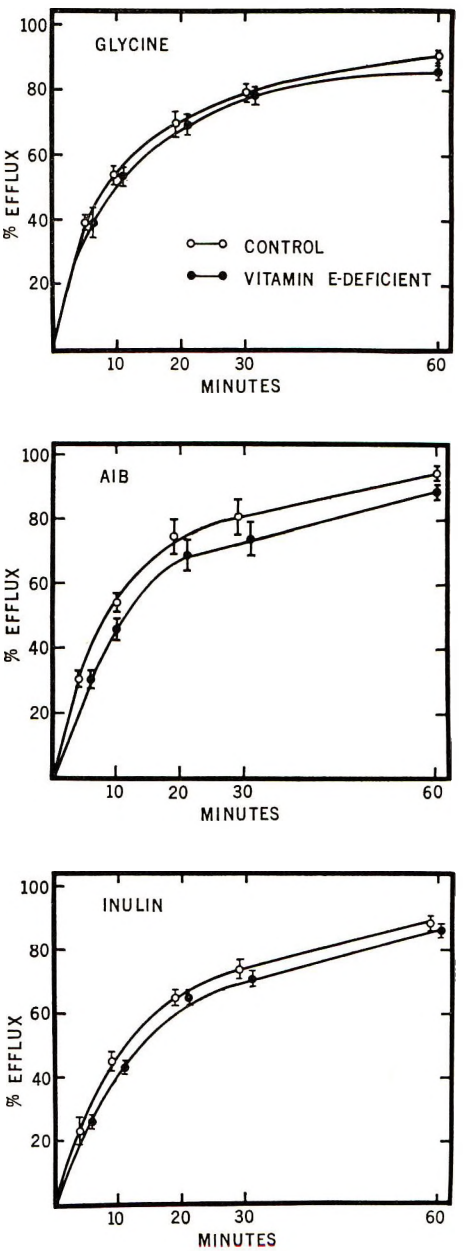

Figure 4

Fig. 4 Efflux of glycine, AIB and inulin. Muscle slices were first incubated in the presence of ^{14}C-glycine (6 mM), ^{14}C-AIB (6 mM) or ^{3}H-inulin (1.45 mg/100 ml) for 20 minutes. Other first incubation conditions are given in figures 1 and 2. The muscle slices were then transferred to a second incubation vessel containing 10.0 ml Krebs-Ringer bicarbonate solution, pH 7.4, and 10 mM unlabeled glycine or AIB. Other incubation conditions were the same as the first incubation. At various time intervals 25 μl were withdrawn and measured for radioactivity. Efflux is expressed as the percentage of label present in muscle at zero time. Each point is the mean of data from five to six rabbits.

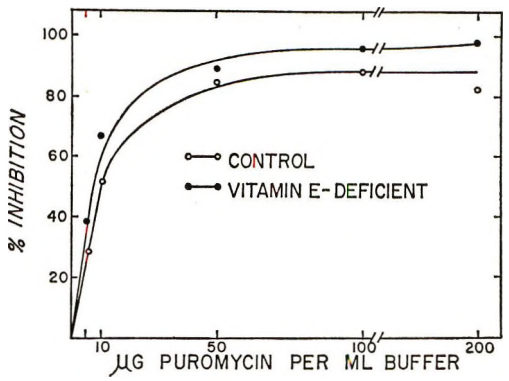

Fig. 5 Concentration of puromycin required to inhibit ^{14}C-glycine incorporation into muscle slice protein. Incubation vessels contained 1.0 μCi of ^{14}C-glycine (64 μM). The incubation time interval was 60 minutes. Other incubation conditions are given in figure 1. The ranges of protein specific activity with no puromycin present were 50 to 120 and 850 to 2200 for control and vitamin E-deficient muscle, respectively. Each point is the mean of data (duplicate incubation vessels) from three rabbits.

the early phase of entry or a decrease in efflux, there still was a significant increase in the accumulation of ^{14}C-labeled glycine and AIB by vitamin E-deficient muscle (fig. 2). After 60 minutes of incubation the distribution ratios (intracellular concentration ÷ extracellular concentration) of the ^{14}C-amino acids had reached steady-state values of approximately 1.0 in control muscle; in dystrophic muscle the distribution ratios were 1.4 or greater and continued to increase. These observations confirm previous reports (8, 10–13) of increased ^{14}C-amino acid accumulation by skeletal muscle of vitamin E-deficient rabbits.

The possibility of a direct effect of the increased incorporation of ^{14}C-glycine into protein on the increased ^{14}C-amino acid accumulation was examined by measuring both processes in the presence of puromycin. The concentration of puromycin required to inhibit the incorporation of ^{14}C-glycine into protein of control and vitamin E-deficient muscle is shown in figure 5. Significant inhibition was observed with as little as 10 μg puromycin/1.0 ml incubation medium and was maximal at 100 to 200 μg/ml.

The effects of puromycin and vitamin E deficiency on ^{14}C-glycine incorporation into muscle protein are shown in figure 6. In agreement with previous reports (1–9) vitamin E deficiency caused a significant increase in muscle protein specific activity; this effect was obtained for all incubation time intervals and glycine concentrations except the 10-minute incubation using 0.5 mM glycine. Puromycin decreased the specific activity of protein from vitamin E-deficient muscle. The low level of radioactivity present in the protein from control muscle makes the effect of puromycin less evident.

Puromycin had no effect on the inulin space or on the accumulation of ^{14}C-glycine by either control or vitamin E-deficient muscle, although it almost completely abolished protein synthesis. The experiments described in figures 1 and 2 were done in the presence and absence of puromycin. In no case did puromycin have any significant effect; therefore, the data obtained from incubations with and without puromycin were combined.

DISCUSSION

One objective of the present study was to determine whether vitamin E deficiency increases the accumulation of ^{14}C-amino acids in muscle by increasing the influx or decreasing the efflux of ^{14}C-amino acids. Since muscle slices were used, the contribution of cut and damaged fibers must be considered in interpreting the data. For example, the large inulin space of 41% (fig. 1) probably is due to the presence of cut muscle fibers. In addition, damage to control muscle fibers possibly explains why the steady-state distribution ratios for ^{14}C-glycine and ^{14}C-AIB did not exceed 1.0 (fig. 2), and why there was so little incorporation of ^{14}C-glycine into protein (fig. 6). On the other hand, cut or damaged muscle fibers of vitamin E-deficient muscle explain neither the increased accumulation of ^{14}C-amino acids (fig. 2) nor the increased incorporation of ^{14}C-glycine into protein (fig. 6).

If there were a general change in the ability of most or all of the cells in vitamin E-deficient muscle to accumulate ^{14}C-amino acids, it should be detectable either as increased influx or decreased efflux of ^{14}C-amino acids. But we found neither abnormality. Small differences in influx or efflux can result in significant differences in net

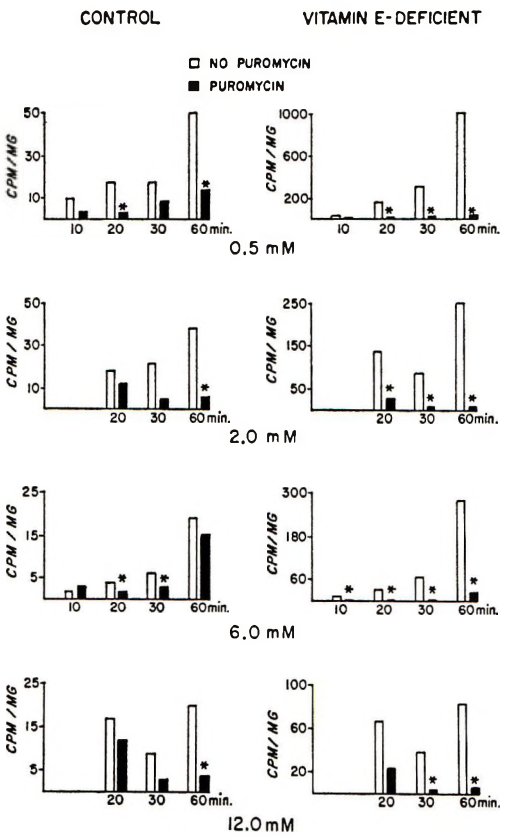

Fig. 6 Effects of puromycin and vitamin E deficiency on ^{14}C-glycine incorporation into muscle slice protein. Incubation vessels contained 3.0 μCi of ^{14}C-glycine. The total external glycine concentrations and incubation time intervals are indicated above. The concentration of puromycin was 200 μg/ml. Other incubation conditions are given in figure 1. Each bar is the mean of data from four to six rabbits. * = Significant puromycin effect.

accumulation with time. Such might be the case if only a relatively small part of the cells in vitamin E-deficient muscle is responsible for the increased accumulation of ^{14}C-amino acids.

In skeletal muscle of vitamin E-deficient rabbits there is hypercellularity composed of degenerating muscle cells, relatively normal muscle cells, inflammatory cells, regenerating muscle cells, and others (19, 20). Some of these cell types are absent from or present only in small numbers in control muscle. If one or more of the abnormally abundant cell types concentrate ^{14}C-amino acids either because small size permits it to escape injury from slicing, or because of a direct effect of vitamin E deficiency, the increased accumulation of ^{14}C-amino acids could be explained. Since muscle from young rats is known to have a greater capacity for concentrating amino acids than muscle from older rats (21), it is probable that the young regenerating muscle cell in vitamin E-deficient muscle is responsible, at least in part, for the increased accumulation of ^{14}C-amino acids.

A second objective of this study was to examine the relationship of increased amino acid accumulation to increased protein synthesis. Pertinent to this objective, we found that puromycin abolished protein synthesis (fig. 6) but had no effect either on the early entry or on the accumulation of ^{14}C-glycine (fig. 2). Furthermore, increased ^{14}C-glycine incorporation into protein occurs prior to increased ^{14}C-glycine accumulation. Thus, the increased accumulation of ^{14}C-glycine is not directly dependent on protein synthesis.

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Unavailability of Cystine from Trypsin Inhibitors as a Factor Contributing to the Poor Nutritive Value of Navy Beans

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ABSTRACT A disproportionate fraction of the total cystine content of the navy bean is found in the isolated trypsin inhibitor. Experiments were undertaken to test the possibility that the poor availability of cystine from navy beans might be due to a failure to utilize the cystine which resides in this protein fraction. Chicks were fed the following diets: 1) basal cystine-deficient diet in which the only source of nitrogen was a mixture of amino acids; 2) basal diet plus 0.15% cystine; 3) basal diet plus 2% unheated navy bean trypsin inhibitor (NBTI); and 4) basal diet plus 2% heated NBTI. The replacement of unheated NBTI by heated NBTI produced essentially the same improvement in growth as the supplementation of the basal diet with cystine. The excretion of cystine, as well as several other representative amino acids (aspartic and glutamic acids, serine, threonine, and proline), was much greater on the diet containing unheated NBTI than on the one with heated NBTI; this difference, however, was confined to the protein-bound amino acids of the excreta. Unheated NBTI produced a pronounced depression in the level of trypsin in the pancreas and intestines with little change in the size of the pancreas. Unheated NBTI, but not heated NBTI, was markedly resistant to digestion by pepsin, trypsin and chymotrypsin. It is concluded that the cystine of NBTI is not readily available for the growth of chicks unless modified by heat; the resistance of unheated NBTI to attack by digestive enzymes appears to be the major factor involved.

The poor nutritive value of raw legumes is well known to nutritionists and has been attributed, at least in part, to the presence of trypsin inhibitors and a deficiency of the sulfur-containing amino acids (1, 2). It has been suggested (3, 4) that the physiological effect of the trypsin inhibitor is to stimulate the pancreas to produce excessive levels of enzymes which are lost to the animal through feces. Barnes et al. (5, 6) have pointed out that since trypsin is rich in cystine, the endogenous loss of this enzyme may account for the cystine deficiency noted in animals fed diets containing raw soybean meal or soybean trypsin inhibitor. With the recent isolation and characterization of pure trypsin inhibitors rich in cystine from a number of legumes (7–11), however, it appears possible that a dietary loss of cystine derived from the inhibitor itself could contribute in a significant fashion to the growth depression of animals fed diets containing raw legumes.

In the present investigation a crude trypsin-inhibitor preparation from the navy bean (*Phaseolus vulgaris*) was used to test the hypothesis that the cystine which resides in this fraction is unavailable to the animal (chick) for growth. To avoid any complications due to a possible influence of the trypsin inhibitor on the availability of cystine from dietary protein, a basal diet was used in which the only source of nitrogen, other than the inhibitor, was a mixture of amino acids. An attempt was also made to minimize the contribution of cystine from pancreatic secretions by taking advantage of the fact that in chicks, unlike rats where pancreatic response to the inhibitor is almost immediate (4), the onset of pancreatic hypertrophy is delayed for several days following the ingestion of the inhibitor (12, 13).

MATERIALS AND METHODS

Trypsin-inhibitor preparation. A partially purified trypsin-inhibitor concentrate (NBTI) was prepared from navy beans

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according to the procedure of Kakade and Evans (14). The trypsin-inhibitor activity of this preparation, determined by the method of Kakade et al. (15), was approximately six times that of the original crude extract, i.e., 1115 and 183 trypsin units inhibited per milligram protein, respectively. The antitryptic activity of NBTI was destroyed by autoclaving at 15 lb/in^2 (120°) for 2 hours, and the preparation so treated is referred to as heated NBTI.

Preparation of diets. The composition of the experimental diets used in this study is shown in table 1. Basal diet A contains 0.15% DL-methionine, a level selected on the basis of preliminary growth experiments that produced the most marked supplementary effect, compared with other levels of methionine (0.25 and 0.35%), when added to a diet containing 0.15% cystine (diet B). Diets C and D contain 2% unheated and heated NBTI, respectively. Based on the amino acid composition data shown in table 2 this level of NBTI provides a final level of 0.12% cystine and 0.026% methionine in these diets. Thus, the total level of sulfur-containing amino acids in diets C and D is approximately equivalent to the cystine content of diet B.

Chick feeding experiments. One-day-old male chicks, originating from a cross of Vantress males and Arbor Acre White Rock females, were fed diet A for 3 days to deplete the endogenous supply of sulfur-containing amino acids. Those chicks, which lost or managed to maintain their weight during the last 2 days of this period, were distributed equally according to body weight into four groups, each of which consisted of three lots of three chicks each. Each group was fed diet A, B, C, or D for a period of 2 days, at the end of which time the weight of each chick was recorded. The animals were killed by cervical dislocation, and the pancreases were excised, weighed, and immediately frozen. The intestinal tract of each animal was ligated at the distal orifice of the gizzard and at the juncture of the egg-yolk sac. This portion of the intestine was then excised, the contents flushed out with distilled water, and the final volume adjusted to 50 ml.

Excreta from each lot of three chicks were collected twice daily on glassine-weighing paper [4] and dried overnight at 95°. The dried excreta were ground into a fine powder which was then analyzed for nitrogen and amino acids.

Trypsin assays. The pancreases from each lot of three chicks were combined;

[4] Eli Lilly and Company, Indianapolis, Ind.

TABLE 1

Composition of experimental diets

Ingredient	Diet			
	A [1]	B [2]	C [3]	D [4]
	%	%	%	%
Amino acid mixture [5]	18.93	18.93	18.93	18.93
Corn oil	15.00	15.00	15.00	15.00
Cellulose	3.00	3.00	3.00	3.00
$NaHCO_3$	1.00	1.00	1.00	1.00
Salt mixture [6]	6.00	6.00	6.00	6.00
Vitamin mixture [7]	0.50	0.50	0.50	0.50
Choline chloride (70%)	0.29	0.29	0.29	0.29
NBTI, unheated			2.00	
NBTI, heated [8]				2.00
L-Cystine		0.15		
Starch	55.28	55.13	53.28	53.28

[1] Basal diet.
[2] Basal diet + cystine.
[3] Basal diet + unheated NBTI.
[4] Basal diet + heated NBTI.
[5] As described by Kelly and Scott (16) except that cystine was omitted and the level of methionine was reduced to provide 0.15% DL-methionine.
[6] Mineral mixture P–6 (17).
[7] Mineral mix P–4 (17).
[8] Heated at 120° for 2 hours.

TABLE 2

Amino acid composition [1] *of navy bean, purified NBTI, and the partially purified NBTI used in these studies*

Amino acid	Navy bean [2]	Purified NBTI [3]	NBTI preparation used in this study [4]
Lysine	5.7	6.9	8.9
Histidine	2.5	6.6	3.9
Arginine	6.1	5.2	5.7
Aspartic acid	10.9	17.1	14.4
Threonine	3.6	7.1	5.8
Serine	3.8	15.8	8.8
Glutamic acid	12.4	10.8	14.8
Proline	3.6	7.9	5.2
Glycine	3.0	1.6	5.3
Alanine	4.3	3.1	6.1
Cystine [5]	1.0	15.5	7.4
Valine	4.9	1.0	4.3
Methionine	1.0	0.6	1.6
Isoleucine	4.3	5.0	4.3
Leucine	7.6	3.4	5.2
Tyrosine	2.3	3.1	3.1
Phenylalanine	5.0	2.9	2.9

[1] Expressed as grams per 16 g N.
[2] Based on nitrogen content of 3.84%. Values taken from Kakade and Evans (23).
[3] Based on nitrogen content of 16.18%. Values taken from Wagner and Riehm (9).
[4] Based on nitrogen content of 13.00%. Experimental data of this study.
[5] Determined as cysteic acid.

they were homogenized in distilled water, diluted to 50 ml, and filtered. To 2 ml of filtrate was added an equal volume of 1% solution of "viodenum" [5] in 0.05 M [tris(hydroxymethyl)amino methane-1,3-propanediol] (Tris) buffer, pH 8.2, containing 0.02 M $CaCl_2$. After incubating for 1 hour at 37°, 5 ml of *N*-benzoyl-DL-arginine-*p*-nitroanilide,[6] prepared in the same buffer, were added and the mixture incubated at 37° for 30 minutes. Following the addition of 1 ml 30% acetic acid, the absorbance of the solution was measured at 410 mμ against a blank prepared in an identical fashion except that the acetic acid was added prior to the addition of the substrate. The tryptic activity of the intestinal contents was assayed in the same manner except that 5 ml of the substrate were added directly to 2 ml of the intestinal contents diluted with an equal volume of water.

Amino acid analysis. Samples were hydrolyzed with 20% HCl for 6 hours at 120° according to the procedure of Evans and Bandemer (18) and analyzed on an amino acid analyzer [7] (19). In all cases cystine was determined as cysteic acid after oxidation with performic acid (20). No attempt was made to differentiate between cysteic acid derived from cystine and any cysteic acid that might have been present as such in the original sample. For the determination of nonprotein amino acids in the feces, 100 mg of dried excreta were homogenized with 5 ml water; 5 ml 10% trichloroacetic acid were added and the suspension filtered. The filtrate was extracted several times with diethyl ether to remove the trichloroacetic acid, and residual ether was eliminated from the aqueous phase by setting the samples in boiling water for 2 minutes. An aliquot of this solution was used for amino acid analysis.

Nitrogen determination. The nitrogen of excreta and diet was determined by the Kjeldahl procedure (21).

In vitro digestion of NBTI. To 1 ml of an aqueous solution or homogenate [8] con-

[5] A desiccated preparation of the duodenum purchased from Viobin Corporation, Monticello, Ill. This preparation serves to activate the zymogens of the pancreas.
[6] Nutritional Biochemicals Corporation, Cleveland, Ohio.
[7] Spinco model 120, Beckman Instruments Inc., Spinco Division, Palo Alto, Calif.
[8] Because of the insolubility of heated NBTI in water, it was necessary to homogenize the sample in a Potter-Elvehjem glass homogenizer.

taining 10 mg of NBTI, unheated or heated, was added 1 ml enzyme solution containing 1 mg of trypsin,[9] chymotrypsin,[10] subtilisin,[11] or pronase [12] dissolved in 0.05 M Tris buffer, pH 8.2, or pepsin [13] dissolved in 0.1 M HCl. Following an incubation period of 4 hours at 37°, 2 ml of 10% trichloroacetic acid were added. The suspension was allowed to stand at room temperature for 1 hour and filtered. The extent of digestion was measured by reading the absorbance of the filtrate at 280 mμ or measuring the free amino groups in the filtrate with 2, 4, 6, trinitrobenzene sulfonic acid [14] (22). The blanks against which these readings were made were samples that had been treated in an identical fashion except that trichloroacetic acid was added prior to the addition of the enzyme. When it was desired to measure the remaining activity of NBTI, digestion was terminated by heating at 80° for 3 minutes in lieu of trichloroacetic acid.

RESULTS

Table 2 shows the amino acid composition of the partially purified preparation of NBTI used in this study compared with the values reported for navy bean protein (23) and chromatographically purified NBTI (9). Although these data show a lack of correspondence between the values reported for pure NBTI and the preparation used in these studies, it is nevertheless important to note that a seven-fold enrichment of the cystine content of the protein (from 1.0 to 7.4%) has been achieved by the purification procedure employed here (14). Since this enrichment of cystine is accompanied by a six-fold increase in trypsin-inhibitor activity, it may reasonably be assumed that the cystine of this preparation is derived primarily from the navy bean inhibitor which, in its pure isolated state, contains 15.5% cystine.

Table 3 presents data pertaining to growth, food intake, and excretion of nitrogen of chicks fed the various experimental diets. The weight loss incurred on the basal diet was effectively overcome by supplementation with 0.15% cystine (diets A versus B). When unheated NBTI was provided as a source of cystine (diet C), the loss in weight was similar to that observed on the unsupplemented basal diet. In contrast to this, when NBTI, which had been heated to destroy its antitryptic activity, was provided as a source of cystine (diet D), growth was comparable to that obtained when the basal diet had been supplemented with 0.15% cystine. Expression of the results in terms of feed efficiency (gram feed intake per gram weight gain) permits the same conclusion to be drawn.

Chicks fed the basal diet excreted a greater proportion of ingested nitrogen (43%) than those receiving the cystine-supplemented diet (29%). The difference in nitrogen, excreted when the basal diets were supplemented with unheated or heated NBTI, 46 and 37%, respectively,

[9] Crystalline preparations from Worthington Biochemical Corporation, Freehold, N. J.
[10] See footnote 9.
[11] Trade-name, Nagarse, purchased from Nagase and Company, Ltd., Osaka, Japan.
[12] Calbiochem, Los Angeles, Calif.
[13] See footnote 9.
[14] Nutritional Biochemicals Corporation.

TABLE 3

Growth response and weight of pancreas of chicks fed experimental diets [1]

	Diet supplements	Weight gain [2,3]	Feed intake [4]	Feed efficiency [3]	Nitrogen excreted [4]	Pancreas wt [2]
		g	*g*		*% of N intake*	*% of body wt*
A	None	−1.0 [a]	9.3	−9.3 [a]	42.7 [b]	0.26 [a]
B	0.15% cystine	4.6 [b]	13.7	3.0 [b]	29.3 [a]	0.29 [ab]
C	Unheated NBTI	−1.8 [a]	8.0	−4.4 [a]	46.2 [b]	0.33 [b]
D	Heated NBTI	3.3 [b]	10.3	3.1 [b]	36.9 [ab]	0.29 [ab]

[1] Values with different superscripts are significantly different ($P < 0.05$) using Duncan's multiple range test (24).
[2] Average of individual weights of nine chicks per group for 2-day periods beginning at 3 days of age.
[3] Feed intake (gram) per gram weight gain.
[4] Average of three lots of three chicks each.

TABLE 4

Amino acids [1] (expressed as a percentage of intake) in the excreta of chicks fed unheated (U) and heated (H) NBTI

Amino acid	Total		Nonprotein		Protein bound [2]	
	U	H	U	H	U	H
Aspartic acid	27.8	10.3	2.4	3.4	25.4	6.9
Threonine	18.2	5.0	3.3	2.7	14.9	2.3
Serine	33.1	11.1	4.3	6.0	28.8	5.1
Glutamic acid	1.5	0.7	0.8	0.7	0.7	0
Proline	17.2	7.2	3.3	3.3	13.9	3.9
Cystine [3]	55.5	23.7	0	0	55.5	23.7

[1] Average of three lots, each lot represented by a sample pooled from three chicks.
[2] Calculated as the difference between the total and nonprotein amino acids.
[3] Determined as cysteic acid.

was not significant; however, neither did these values differ significantly from the unsupplemented basal diet.

The weight of the pancreas, shown in table 3, did not show any large differences among the various diets. The weights of the pancreas from birds receiving unheated NBTI, however, were significantly higher than those fed the basal diet.

Data relating to the excretion of cystine and several other representative amino acids in the excreta of chicks fed unheated and heated NBTI are summarized in table 4. Although cystine was the amino acid of principal interest in these studies, the five amino acids that immediately follow cysteic acid during chromatography (namely, aspartic acid, threonine, serine, glutamic acid, and proline) have been included for comparison. The total excretion of each of the amino acids was greater in chicks fed unheated NBTI than those fed the heated preparation. When the total amino acids of the excreta were further differentiated into protein-bound and nonprotein amino acids, the higher excretion of amino acids by birds fed unheated NBTI was found to reside in the protein fraction of the excreta. Little difference in the excretion of nonprotein amino acids between the two diets was apparent.

The trypsin activity of the intestinal contents and pancreas of chicks fed heated or unheated NBTI is shown in figure 1. The level of trypsin activity present in both the intestinal tract and pancreas was markedly reduced when unheated NBTI was used in place of the heated preparation.

The results of the in vitro digestion experiment are shown in table 5. Heated NBTI was digested to a much greater extent than unheated NBTI by trypsin, chymotrypsin, and pronase. Pepsin, on the other hand, had a similar effect on either the heated or unheated substrate. Subtilisin was capable of digesting unheated and heated NBTI to about the same degree. Although unheated NBTI was digested by trypsin and chymotrypsin to some extent, the inhibitory activity of NBTI was not affected. The digestion that is observed is most likely due to the proteolytic degradation of contaminating proteins rather than

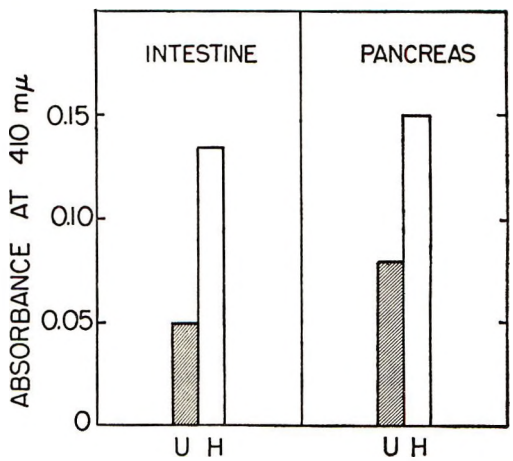

Fig. 1 Level of trypsin activity in intestinal contents and pancreas of chicks fed diets containing unheated NBTI (U) and heated NBTI (H). Trypsin activity denoted by increase in absorbance at 410 mμ using *N*-benzoyl-DL-arginine-*p*-nitroanilide as substrate. Each value is the average of three lots, each lot represented by a sample pooled from three chicks.

NBTI itself, although it is conceivable that partially degraded NBTI may retain its activity.

DISCUSSION

Partially purified preparations of trypsin inhibitors from the lima bean, *Phaseolus lunatus* (25) and the navy bean (14) have been shown to depress the growth of animals. Both these inhibitors, when isolated in a high state of purity, have been shown to be very rich in cystine, 15.5% and 18.5% for the navy bean (9) and lima bean (7), respectively. From the data compiled in table 6 it is possible to estimate how much of the total cystine of these two bean proteins is derived from the trypsin inhibitor. Although comprising only about 2.5% of the bean protein, the trypsin inhibitor contributes approximately 32 and 40% of the total cystine of the protein of the lima and navy bean, respectively. The results of the growth experiments (table 3) and excretion studies (table 4) support the contention that in the case of the navy bean inhibitor the cystine which it contains is not available to the chick for growth, at least not during the short period of study deliberately chosen to minimize pancreatic effects. Studies were not carried out for longer periods of time to test the possibility that some adaptation to the effects of the trypsin inhibitor would have eventually taken place.

One or more of the following reasons might be invoked to explain the unavailability of cystine from unheated NBTI: 1) an interference in the extent to which NBTI can be digested in vivo; 2) an impairment in the absorption of amino acids, including trypsin, from the intestinal tract; and 3) degradation of cystine by intestinal microflora.

Of these various possibilities the first would appear to be the most likely since the in vitro experiments showed that unheated NBTI was more refractory to attack by trypsin and chymotrypsin than

TABLE 5

In vitro digestion of heated and unheated NBTI

Enzyme	Percentage of original activity of NBTI		Extent of proteolysis [1]					
			Method A [2]			Method B [3]		
	Unheated	Heated	Unheated	Heated	Δ [4]	Unheated	Heated	Δ [4]
Pepsin	100	0	0.115	0.055	−0.060	0.410	0.325	−0.085
Trypsin	100	0	0.050	0.320	0.270	0.035	0.165	0.130
Chymotrypsin	100	0	0.050	0.420	0.370	0.045	0.440	0.395
Subtilisin	44	0	0.520	0.450	−0.070	1.350	1.300	−0.050
Pronase	53	0	0.680	0.910	0.230	1.800	2.000	0.200

[1] Expressed as units of enzyme activity where one unit is defined as an increase of 1.0 absorbance unit at 280 mμ (method A) or 346 mμ (method B).
[2] Based on absorbance at 280 mμ.
[3] Based on absorbance at 346 mμ after reaction with 2,4,6-trinitrobenzene sulfonic acid.
[4] Value for heated NBTI − value for unheated NBTI.

TABLE 6

Cystine and trypsin inhibitor (TI) content of navy beans (Phaseolus vulgaris) and lima beans (Phaseolus lunatus)

	Cystine content		TI content of protein	Contribution of TI cystine to total	
	Beans	TI		Protein	Cystine
	g/16 g N	*g/16 g N*	%	%	%
Navy beans	1.0 [1]	15.5 [1]	2.6 [2]	0.40	40
Lima beans	1.4 [3]	18.5 [4]	2.5 [4]	0.45	32

[1] Taken from table 2.
[2] Based on the determination of the trypsin inhibitor activity of a crude extract of navy bean used in these studies and the specific activity of purified NBTI reported by Wagner and Riehm (9).
[3] Reported by Aykroyd and Doughty (26).
[4] Based on data reported by Jones et al. (7).

heated NBTI (table 5). This resistance to enzymatic attack is probably due to the stability of the molecule produced by a large number of disulfide bonds (2, 11), and the effect of heat is to cause an unfolding of the molecule resulting in the exposure of peptide bonds susceptible to enzymatic cleavage. A number of investigators (27–30) have postulated the existence in raw soybeans of a cystine-containing protein fraction which is resistant to tryptic attack unless modified by heat. The most likely candidates for this fraction are the Bowman-Birk inhibitor (11) and the 1.9S inhibitor isolated by Yamamoto and Ikenaka (10) which contain 17 and 16% cystine, respectively.

In addition to the incomplete digestion of NBTI itself, the effect that the antitryptic activity of this fraction would have had on the availability of amino acids from an exogenous supply of protein merits comment. The fact that the inclusion of raw NBTI in the diet caused a depression of the level of active trypsin present in the intestines (fig. 1) would probably be an important factor contributing to the incomplete digestion of dietary protein if such had been provided in the diet. In the case of chicks fed raw soybeans, an inhibition of intestinal proteolysis has in fact been reported (31–33).

The data in table 4 show that a much greater proportion of the ingested amino acids was excreted when unheated NBTI replaced heated NBTI in the diet. This difference, however, was confined to the protein-bound fraction. Since there was no difference in the excretion of free amino acids, it may be concluded that unheated NBTI exerts little effect on the absorption of free amino acids. This conclusion is at variance with the report by DeMuelenaere (34) that the trypsin inhibitor of soybeans interfered to some extent with the intestinal absorption of amino acids in the rat. In addition to the protein-bound amino acids derived from undigested NBTI, a high proportion of the protein fraction of the excreta must have originated from the free amino acids of the diet, perhaps as a result of microbial synthesis; or, as suggested by DeMuelenaere (34) for the soybean inhibitor, from a sloughing off of the intestinal mucosa. In any event, it is the excretion of unabsorbed cystine which would be expected to exert the most critical effect on the growth of the animal.

The bacterial degradation of cystine in the gut has been proposed as a factor contributing to the unavailability of this amino acid in animals fed raw soybeans (5, 6, 28, 35). The data in table 5 show, however, that bacterial proteases, as represented by subtilisin and pronase, are capable of digesting raw as well as heated NBTI. Furthermore, if cystine from unheated NBTI had been lost as a result of bacterial degradation, one would have expected less cystine to be excreted in the feces with unheated NBTI than heated NBTI, whereas the opposite effect was in fact observed.

Finally, it may be argued that the cystine of NBTI might have been available, in the sense that it was absorbed from the gastrointestinal tract, and that the high level of cystine excreted by the chicks fed unheated NBTI could have been of endogenous origin, that is, from the trypsin secreted by the pancreas as in the case of the soybean inhibitor (5, 6, 35). Experiments similar to the one described here have been conducted with rats by Khayambashi and Lyman (36) employing amino acid diets containing the soybean trypsin inhibitor. It is important to note, however, that under their experimental conditions pancreatic hypertrophy was observed, and this condition was accompanied by high levels of protein-bound cystine and proteolytic activity in the intestines. The lower level of tryptic activity in the pancreas and intestines of chicks fed unheated NBTI under the experimental conditions described here are presumably a consequence of the fact that a short experimental period was deliberately chosen to take advantage of the delay in the development of pancreatic hypertrophy in the chick (12, 13, 31–33). This point was verified by the observation that there was no significant difference in the size of the pancreas of chicks fed unheated or heated NBTI (table 3). Thus, the contribution by the pancreas to the cystine excreted by the chicks in our experiment is believed to be minimal.

In summary, it is proposed that the trypsin inhibitor isolated from navy beans accentuates a deficiency of cystine, which already exists in navy beans, by virtue of the fact that it contains a disproportionate fraction of the cystine, which the animal cannot use to meet its requirements for growth. In addition, it is possible that, under certain conditions, this deficiency might be further intensified by the ability of the trypsin inhibitor to inhibit the intestinal proteolysis of dietary protein and to stimulate the production of pancreatic enzymes, which leads to an endogenous loss of cystine.

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Amino Acid Patterns During Digestion in the Small Intestine of Ducks [1]

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ABSTRACT Total nitrogen and amino acid patterns were studied in five equal segments of intestine of ducks fed rations of known amino acid composition ad libitum. The largest quantities of free amino acids were found in the middle segment extending from 41 to 60% of intestinal length. Total quantity of nitrogen was highest in the first two segments and decreased markedly in more distal segments. The pattern of free amino acids in the intestinal segments reflected the pattern of the dietary protein, particularly in the mid-20% of the intestine. This was demonstrated by feeding diets in which the protein was either a combination of corn and soybean meal or of corn and corn gluten meal. The pattern of amino acids in segments more distal to the midpoint did not reflect the dietary amino acid pattern as well as that in upper portions, but was similar to that observed when a nitrogen-free diet was fed. This suggests that digestion of dietary proteins occurs before that of endogenous protein. The dilution of dietary protein by endogenous protein was insufficient to mask the amino acid pattern of ingested protein.

Nasset (1), in interpreting results obtained by himself and his colleagues while studying protein digestion in nonruminant animals, has concluded that the presence of endogenous nitrogen in the intestinal tract acts as a homeostatic device to prevent wide fluctuations in the amino acid mixture available for absorption. He also reported that the amino acid mixture produced during digestion remained relatively constant regardless of the composition of the ingested protein. These conclusions have interested not only physiologists and nutritionists but also parasitologists studying the biology of intestinal helminths. Read et al. (2) extended Nasset's conclusions by emphasizing that the membrane transport systems of intestinal helminths would be exposed to constant molar ratios of amino acids. These authors also implied that the pattern of the constant molar ratios of amino acids in the intestinal lumen might be a significant factor in determining whether an animal offers a suitable environment for a given intestinal helminth, or not.

The aims of the work described in this paper were to investigate protein digestion in ducks and to discover if variations in the composition of the dietary protein had any effect on the amino acid patterns in the intestine of the bird. Special attention was given to different regions of the intestine including the section extending from 61 to 80% of the distance along the intestine. This region is the principal environment of *Polymorphus minutus*, an acanthocephalan parasite of ducks (3).

MATERIALS AND METHODS

The principle of the method used to investigate protein digestion in ducks was to analyze samples of intestinal contents obtained from known regions of the intestine for free amino acids, percentage nitrogen and percentage chromic oxide when this compound has been included in the diet.

General procedure. Khaki Campbell ducks of ages varying from 8 to 11 weeks, and of known sex and weight, were allowed to feed ad libitum on water and rations of known composition which are given in table 1. Rations were presented in the form of pellets with the exception of the nitrogen-free ration, which had to be offered in less regular fragments. Samples of intestinal contents for amino acid analysis were obtained from 31 ducks and samples for the determination of percentage nitro-

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TABLE 1

Composition of the rations used for feeding ducks

Ingredient	Soybean ration	Corn gluten ration	Nitrogen-free ration
	%	%	%
Alfalfa meal	2.50	2.50	—
Cellulose	—	—	5.00
Corn (yellow)	60.84	53.84	—
Corn gluten meal	—	37.00	—
Soybean meal (50% protein)	30.00	—	—
Corn oil	1.00	1.00	3.00
Dicalcium phosphate	2.00	2.00	2.00
Limestone	1.20	1.20	1.20
Mineral mixture [1]	1.46	1.46	1.46
Starch	—	—	86.34
Vitamin mixture [2]	1.00	1.00	1.00

[1] The mineral mixture supplied the following: (in grams per kilogram final diet) 5.0 $KHCO_3$; 6.0 NaCl; 0.33 $MnSO_4 \cdot H_2O$; 0.33 $FeSO_4 \cdot 7H_2O$; 3.0 $MgSO_4$ (anhydrous); 0.00267 KI; 0.0167 $CuSO_4 \cdot 5H_2O$; 0.0623 ZnO; 0.0017 $CoCl_2 \cdot 6H_2O$; 0.0083 $NaMoO_4 \cdot 2H_2O$; and 0.0001 Na_2SeO_4.

[2] The vitamin mixture supplied the following: (in milligrams per kilogram final diet) thiamine·HCl, 15.0; riboflavin, 15.0; nicotinamide, 50.0; D-calcium pantothenate, 20.0; pyridoxine·HCl, 6.0; folic acid, 6.0; biotin, 0.6; menadione sodium bisulfite, 1.52; choline chloride, 1400; inositol, 250.0; and butylated hydroxytoluene, 100; (in IU) vitamin D_3, 4500; vitamin A, 5000; and vitamin E, 110; and (in micrograms) vitamin B_{12}, 20.

gen and percentage chromic oxide were obtained from an additional 22 ducks. Ducks feeding on the soybean ration were found to be suitable hosts for *P. minutus*.

Sampling procedures. Each duck was killed between 8:45 and 9:15 AM on the day samples were required. Its intestine was stretched out and divided into five sections designated A, B, C, D and E in the text. Section A extended from zero to 20% of the intestinal length, section B from 21 to 40% of the intestinal length, and so on. Since the average intestinal length of ducks at the age used is about 150 cm, each intestinal segment was about 30 cm long. The contents of each section were gently squeezed into a clean glass beaker and weighed. The time taken to kill a duck, collect and weigh samples, either freeze them or incorporate analytical reagents, was about 8 minutes. Throughout the work, care was taken to avoid contamination of the samples with blood and other tissue. During the collection of samples food was observed to be present in the ventriculi of all the ducks examined.

Analytical procedures. Five milliliters of distilled water were added to each sample for amino acid analysis and the resulting slurry was then treated as described by Stein and Moore (4). A known volume of the final sample, together with 1 ml of norleucine as internal standard (0.25 μmole/ml), was analyzed by means of an amino acid analyzer.[3] The results were calculated initially as milligrams amino acid per gram wet weight of intestinal contents, but they are expressed in tables 3, 4 and 5 as ratios to leucine, the concentration of which was assigned a value of 1.00 for each analysis undertaken. Percentage nitrogen and percentage chromic oxide were estimated by the methods of Nesheim and Carpenter (5).

RESULTS

General observations on protein digestion. Examples of the concentrations of free amino acids detected in the intestinal contents are given in table 2. The amount of amino acid per unit of intestinal contents was variable but tended to be highest in section A, B and C. Due to the greater quantity of material present in section C than in A and B, however, the highest total quantities of free amino acids were found in section C which extends from 41 to 60% of the length of the intestinal tract. This result is represented diagrammatically in figure 1(a) and it can be seen that the amount of free amino acid in section C is about three times as great as that in sections A and B and about one and one-half times as great as that in section D. The distribution of nitrogen along the intestinal tract is shown in figure 1(b). In section A,

[3] Technicon Auto-analyzer, Technicon Corporation, Ardsley, N. Y.

TABLE 2

Means and ranges of concentration of certain free amino acids of ducks feeding on a soybean ration

Amino acid	Concentration in sections of intestine				
	A	B	C	D	E
	mg/g wet wt intestinal contents				
Alanine	0.117 [1]	0.368	0.214	0.049	0.127
	0.055–0.195 [2]	0.099–0.416	0.059–0.263	0.013–0.097	0.028–0.045
Glutamic acid	0.165	0.580	0.552	0.141	0.231
	0.113–0.225	0.161–0.666	0.121–0.325	0.110–0.407	0.038–0.084
Leucine	0.258	0.463	0.309	0.086	0.142
	0.067–0.426	0.109–0.448	0.080–0.431	0.029–0.188	0.026–0.046
Lysine	0.250	0.386	0.240	0.081	0.125
	0.057–0.496	0.089–0.563	0.067–0.299	0.023–0.198	0.034–0.054
Methionine	0.067	0.114	0.061	0.231	0.010
	0.021–0.096	0.058–0.124	0.016–0.110	0.014–0.036	0.003–0.007
Phenylalanine	0.154	0.301	0.225	0.0484	0.056
	0.044–0.240	0.077–0.293	0.045–0.243	0.018–0.080	0.013–0.021
Serine	0.130	0.250	0.233	0.057	0.123
	0.044–0.279	0.076–0.323	0.042–0.344	0.032–0.013	0.020–0.062
Valine	0.101	0.237	0.186	0.056	0.103
	0.025–0.235	0.062–0.254	0.045–0.214	0.016–0.156	0.020–0.034

[1] Each mean is result of determinations on samples from five or six ducks, except in section D where up to nine samples were involved.
[2] Range.

the nitrogen level rose from the dietary level of 3% to about 4.4%; this level was maintained until section C was reached, where the nitrogen level fell to about 2.6%. Thus, the rise in the amount of free amino acids and the fall in percentage nitrogen in section C indicated that most amino acids are released from protein digestion and absorbed into the hepatic portal system in this portion of the intestine. There was no evidence of a massive dilution of exogenous nitrogen by endogenous nitrogen; the data in figure 1(b) suggest that the endogenous contribution is about 50% of the exogenous intake.

The distribution of chromic oxide, which was present in the ration at a level of about 0.3%, is shown in figure 1(c). The steady increase in chromic oxide content along the intestine is to be expected since nutrients are absorbed along the tract and peristaltic contractions are stronger in the proximal than in the distal portion of the tract. The ratio of percentage nitrogen to chromic oxide is shown in figure 1(d). In section C, the decrease in the ratio results from the decrease in the nitrogen level and the increase in the level of chromic oxide; both processes indicate that nitrogenous nutrients are being absorbed in this part of the intestine. All the data reported above apply to ducks feeding ad libitum on a soybean ration of the type used commercially.

Free amino acid patterns in the intestine. The patterns of free amino acids, expressed as ratios to leucine, for different portions of the alimentary tract, are given in table 3. These values, with few exceptions, are not markedly different from those which appear to exist in the soybean ration. Since the dilution of exogenous protein by endogenous protein was only about 50%, the amino acid pattern of the exogenous protein would not be affected greatly by the endogenous pattern. Therefore, the amino acid in section C was examined after ducks had been allowed to feed ad libitum for 2 to 4 days on a ration containing corn gluten as the chief protein. Since a high level of leucine and a low level of lysine exist in corn gluten, it was predicted that the ratios to leucine for amino acids in section C should be much lower for ducks eating the corn gluten ration than for those eating the soybean ration if the endogenous pro-

TABLE 3

Patterns of free amino acids found in different sections of the intestine of ducks feeding on a soybean ration

Amino acid	Section of intestine					Soybean ration
	A	B	C	D	E	
	ratio to leucine					
Aspartic acid	0.54 [1]	0.50	0.72	0.67	0.78	1.03
	0.11 [2]	0.13	0.18	0.32	0.43	
	0.05 [3]	0.05	0.08	0.13	0.25	
Threonine	0.72	0.59	0.86	0.75	1.14	0.40
	0.25	0.11	0.23	0.15	0.16	
	0.11	0.04	0.09	0.06	0.09	
Serine	0.57	0.53	0.67	0.64	0.91	0.52
	0.12	0.15	0.23	0.14	0.38	
	0.05	0.06	0.09	0.06	0.22	
Glutamic acid	1.09	1.17	2.07	1.78	1.76	2.07
	0.44	0.31	0.91	1.41	1.28	
	0.22	0.12	0.37	0.53	0.74	
Proline	0.56	0.36	0.52	0.56	0.52	0.89
	0.24	0.18	0.24	0.12	0.01	
	0.13	0.07	0.10	0.04	0.00	
Glycine	0.30	0.35	0.58	0.75	0.85	0.46
	0.03	0.11	0.24	0.28	0.52	
	0.01	0.04	0.09	0.09	0.30	
Alanine	0.68	0.73	0.67	0.59	0.91	0.54
	0.01	0.19	0.17	0.13	0.21	
	0.05	0.07	0.07	0.04	0.12	
Valine	0.42	0.48	0.56	0.62	0.68	0.59
	0.13	0.07	0.15	0.11	0.11	
	0.06	0.03	0.06	0.04	0.06	
Methionine	0.28	0.28	0.23	0.27	0.12	0.11
	0.09	0.06	0.11	0.08	0.11	
	0.03	0.03	0.04	0.03	0.06	
Isoleucine	0.52	0.40	0.47	0.52	0.59	0.48
	0.15	0.07	0.12	0.09	0.06	
	0.06	0.03	0.05	0.03	0.03	
Leucine	1.00	1.00	1.00	1.00	1.00	1.00
	—	—	—	—	—	
	—	—	—	—	—	
Tyrosine	0.79	0.60	0.65	0.63	0.56	0.42
	0.20	0.12	0.11	0.07	0.13	
	0.08	0.05	0.05	0.02	0.06	
Phenylalanine	0.61	0.63	0.62	0.58	0.47	0.54
	0.15	0.09	0.17	0.09	0.08	
	0.06	0.03	0.07	0.03	0.04	
Lysine	0.92	0.84	0.75	0.99	1.12	0.72
	0.21	0.26	0.10	0.18	0.25	
	0.08	0.10	0.05	0.06	0.12	
Histidine	0.19	0.24	0.33	0.30	0.33	0.44
	0.07	0.12	0.07	0.12	0.16	
	0.03	0.05	0.04	0.04	0.08	
Arginine	1.06	0.91	0.85	0.92	0.83	0.89
	0.19	0.23	0.22	0.17	0.25	
	0.76	0.09	0.11	0.06	0.15	

[1] Mean ratios are obtained from six, six, six, nine and five samples for sections A, B, C, D and E, respectively.
[2] SD.
[3] SE.

tein was having little effect on the pattern of free amino acids. Section C was chosen for investigation because the greatest quantities of free amino acids were found there (fig. 1), and the amino acid pattern was similar to that of the ration (table 3).

The pattern of free amino acids detected in section C of the intestine of ducks feeding on the corn gluten ration is given in table 4. The amino acid ratios are similar to those present in the ration. No evidence was found in support of the hypothesis that the secretion of endogenous protein is great enough to result in its own amino acid pattern masking that of the ingested protein. It was concluded that the amino acid content of the diet directly affects the free amino acid pattern in the intestine when ducks are allowed to feed ad libitum.

Samples were also obtained from section C of the intestine of two other ducks feeding on the corn gluten ration. The intestinal contents collected from these ducks were posterior to 53% of the distance along the intestine and were, therefore, near section D. The ratios of free amino acids found in these samples were higher than those given in table 4, although still lower than the values obtained from section C in ducks feeding on the soybean ration. If the amino acid pattern of the endogenous protein was similar to that of the soybean ration, its possible effect on the amino acid pattern of section D in ducks feeding on the soybean ration would have escaped detection. It was postulated, therefore, that section D might include that part of the intestine where free amino acids, produced by the hydrolysis of endogenous protein, are liberated and absorbed. Consequently, the amino acid patterns in sections C and D of the intestine of ducks feeding on a nitrogen-free ration were determined and assumed to indicate the pattern produced from endogenous protein. These data, and those obtained from section D both for ducks feeding on the corn gluten ration and for ducks eating a commercial poultry feed (BOCM baby chick crumbs),[4] are compared in table 5. If the patterns detected in sections C and D from ducks feeding on a nitrogen-free ration represent the pattern of amino acids in the endogenous secretion, the higher ratios in section D than in section C for ducks feeding on the corn gluten ration may result from the mixing of the endogenous amino acid pattern with the exogenous fraction, which was either unabsorbed or incompletely digested in section C. The high quantities of free amino acids of exogenous origin in section C normally conceal the endogenous amino acids, and when the amino acid patterns of exogenous and endogenous proteins are similar, this effect will not readily be detected. The amino acid pattern in section D, from ducks feeding on the BOCM ration, is similar to the pattern produced in the soybean ration. This result is to be expected because both rations are suitable for normal growth of poultry and contain similar proportions of amino acids. The data given in table 5 support the suggestion that amino acids of endogenous origin are

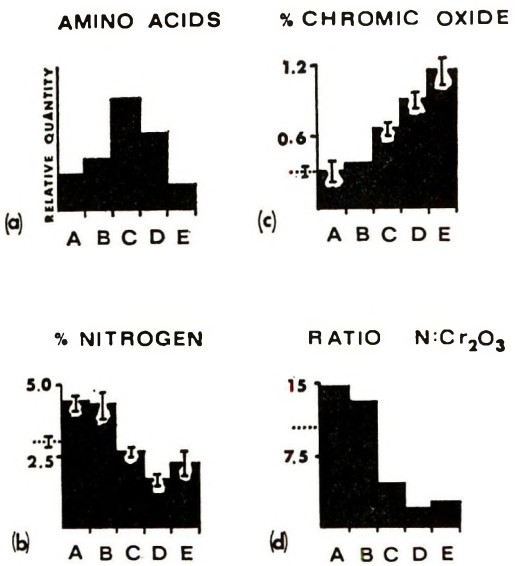

Fig. 1 These histograms apply to ducks receiving the soybean ration. The letters on the abscissa represent the sections of intestine in each figure (see text); (a) shows the distribution of relative quantities of free amino acids in each intestinal segment; (b) shows the distribution of nitrogen along the intestine; (c) represents the distribution of chromic oxide in the intestine; and (d) shows the ratio of $N:Cr_2O_3$ in the intestinal segments. I = standard deviation of individual observations shown. The point on the ordinate shown by ... = value of the parameter measured for the diet.

[4] British Oil Cake Manufacturers.

TABLE 4

Pattern of free amino acids found in section C of the intestine of ducks feeding on a corn gluten ration

Amino acid	Section C	Corn gluten ration
	ratio to leucine	
Aspartic acid	0.09 [1]	0.38
	0.03 [2]	
	0.01 [3]	
Threonine	0.42	0.20
	0.12	
	0.04	
Serine	0.37	0.29
	0.09	
	0.03	
Glutamic acid	0.37	1.37
	0.67	
	0.07	
Proline	0.26	0.68
	0.17	
	0.07	
Glycine	0.15	0.16
	0.07	
	0.02	
Alanine	0.44	0.54
	0.03	
	0.03	
Valine	0.27	0.14
	0.06	
	0.03	
Cystine	0.48	
	0.11	
	0.07	
Methionine	0.15	0.17
	0.03	
	0.01	
Isoleucine	0.23	0.28
	0.07	
	0.02	
Leucine	1.00	1.00
	—	
	—	
Tyrosine	0.46	0.36
	0.06	
	0.02	
Phenylalanine	0.46	0.40
	0.06	
	0.02	
Lysine	0.19	0.14
	0.06	
	0.05	
Histidine	0.11	0.18
	0.03	
	0.01	
Arginine	0.34	0.34
	0.12	
	0.04	

[1] Mean ratios are obtained from seven samples.
[2] SD.
[3] SE.

either absorbed or are more conspicuous in section D. These amino acids may not be liberated as quickly during digestion because, unlike the exogenous protein, their protein source has not been subjected to the digestive processes of the proventriculus and ventriculus. Consequently, hydrolysis of endogenous protein will take longer than that of exogenous protein, and peristalsis will move the majority of the resulting free amino acids into section D for absorption.

DISCUSSION

Evidence has been obtained during this study of protein digestion in ducks to show that the amino acid composition of the diet directly affects the pattern of free amino acids in the intestine, and that the central portion of the intestinal tract appears to be the principal site of amino acid absorption into the hepatic portal system. Wilson (6) considered that this function could be ascribed to the middle region of the intestine in mammals.

The influence of the amino acid pattern of the diet on that of the intestinal contents is of interest because it does not appear to agree with the observations and interpretations discussed by Nasset (1). It may be argued that the results from ducks cannot be compared with those from small mammals, although functioning of the intestinal tract and protein digestion in birds and mammals are unlikely to be fundamentally different. The difference between our results from ducks and the earlier work on dogs and rats probably rests in the differences in experimental procedure. Nasset and his collaborators usually fasted their animals for 24 hours and then obtained samples 1.5 hours after feeding. Thus, it was possible that digestion and absorption of the exogenous protein had nearly been completed and most of the remaining protein in the intestine was of endogenous origin, a situation which would explain the high dilution of exogenous protein by endogenous protein. The implication that endogenous protein is not digested as rapidly as exogenous protein is supported not only by evidence presented in this paper, but also by the results of Twombly and Meyer (7) working with

TABLE 5

Patterns of free amino acids found in sections C and D of the intestine of ducks feeding on various rations

Amino acid	Nitrogen-free		Corn gluten	BOCM	BOCM ration
	Section of intestine				
	C	D	D	D	
	ratio to leucine				
Aspartic acid	0.58 [1]	0.65	0.38	0.50	1.12
	0.11 [2]	0.10	0.17	0.17	
	0.06 [3]	0.07	0.10	0.09	
Threonine	0.59	0.59	0.77	0.80	0.50
	0.22	0.11	0.28	0.11	
	0.13	0.07	0.16	0.06	
Serine	0.39	0.78	0.72	0.69	0.61
	0.05	0.03	0.31	0.11	
	0.03	0.02	0.17	0.06	
Glutamic acid	1.48	1.35	0.70	1.16	2.54
	0.18	0.13	0.02	0.28	
	0.11	0.09	0.01	0.16	
Proline	0.41	0.20 [4]	1.05	0.48	1.08
	0.09	—	0.55	0.09	
	0.05	—	0.31	0.05	
Glycine	0.43	0.40	0.43	0.58	0.83
	0.06	0.02	0.06	0.10	
	0.04	0.01	0.03	0.06	
Alanine	0.83	0.70	0.72	0.66	0.76
	0.58	0.13	0.22	0.07	
	0.04	0.09	0.13	0.04	
Valine	0.49	0.54	0.47	0.58	
	0.14	0.10	0.05	0.06	
	0.08	0.07	0.03	0.03	
Methionine	0.28	0.07	0.22	0.24	0.25
	0.01	—	0.01	0.02	
	—	—	0.00	0.01	
Isoleucine	0.52	0.35	0.45	0.49	0.52
	0.02	0.06	0.06	0.02	
	0.01	0.04	0.03	0.01	
Leucine	1.00	1.00	1.00	1.00	1.00
	—	—	—	—	
	—	—	—	—	
Tyrosine	0.74	0.49	0.51	0.63	0.45
	0.03	0.06	0.09	0.04	
	0.02	0.04	0.05	0.02	
Phenylalanine	0.57	0.28 [4]	0.43	0.48	0.58
	0.02	—	0.08	0.04	
	0.01	—	0.05	0.02	
Lysine	0.97	0.88	0.52	0.93	0.64
	0.05	0.05	0.33	0.08	
	0.03	0.03	0.23	0.05	
Histidine	0.27	0.23 [4]	0.31 [4]	0.29	0.30
	0.07	—	—	0.10	
	0.04	—	—	0.05	
Arginine	1.01	0.82	0.52	0.73 [4]	0.83
	0.06	0.02	0.27	—	
	0.03	0.01	0.19	—	

[1] Mean ratios are obtained from three samples.
[2] SD.
[3] SE.
[4] Mean ratios obtained from only one sample.

rats, and in theory by Gitler (8). Fasting may also affect the quantity of endogenous secretions released immediately from the stimulus of refeeding. The levels of endogenous amino acids in Nasset's experiments thus may have been elevated, compared with those found during the current studies.

Throughout this investigation, the possible effects of the intestinal amino acid pattern on parasitic worms of ducks were considered. If amino acids liberated from the digestion of endogenous protein are absorbed in the region extending from 61 to 80% of the intestinal length, which is also the environment of *P. minutus*, this parasite will be living in an area where a potentially constant amino acid mixture is available for absorption. Other parasites which live in the anterior part of the tract, however, will experience changing amino acid patterns when their hosts' diet varies during different times of the year. These parasites may, therefore, change position in the intestinal tract to locate a region where the amino acid mixture is compatible with the absorptive mechanisms.

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Effects of Magnesium Deficiency, Dietary Sulfate and Thyroxine Treatment on Kidney Calcification and Tissue Protein-bound Carbohydrate in the Rat [1]

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ABSTRACT Three experiments were conducted to investigate relationships between calcium accumulation in the kidney and changes in ground substance of the magnesium-deficient young male albino rat. It was found that a modest dietary sulfate deficiency did not prevent kidney calcification, nor did it prevent the lowered liver protein-bound hexose/hexosamine ratio of magnesium-deficient animals. Dietary L-thyroxine (L-T_4) prevented kidney calcification, lowered total serum protein and the percentage of glycoprotein in the serum protein. Magnesium deficiency lowered total protein in serum but not glycoprotein content of the serum protein. Retention of ^{35}S in serum and bone 48 hours after dosing was decreased by magnesium deficiency and especially by L-T_4 treatment. Kidney ^{35}S retention 48 hours after a single dose was greater in animals fed a magnesium-deficient diet for 14 days than for 4 days except in L-T_4 treated animals. Kidney calcification and ^{35}S retention were not affected by intraperitoneal dosage with D-T_4. On the basis of these experiments it appears that the initial stages of kidney calcification arising from magnesium deficiency do not involve changes in ground substance, although such changes may contribute to the later, more extensive calcification.

The extensively documented calcification of soft tissues, particularly renal tissue, of magnesium-deficient rats (1–4) provides a convenient tool for studies of mechanisms leading to soft tissue calcification. It has been observed that this calcification of magnesium deficiency is accompanied by the appearance of para-aminosalicylic acid-positive material (5) and is prevented by administration of thyroxine (6–8). The state of renal ground substance has been implicated in the initiation of kidney calcification in other studies (9–11). The involvement of sulfated mucopolysaccharides in the process of calcification has also been demonstrated in several investigations related to atherosclerosis and aortic plaque formation (12–15). A deficiency of dietary sulfate has been demonstrated to affect aortic strength, suggesting a role in formation of connective tissue constituents of the blood vessels (16).

Three experiments were designed to study the nature of the relationship between calcium accumulation and changes in ground substance of the kidney of magnesium-deficient rats. The qualitative and quantitative alterations in mucopolysaccharide and glycoprotein components that might occur during magnesium deficiency and thyroxine (T_4) administration were also investigated in an attempt to establish whether renal calcification was associated with kidney ground substance changes that would provide nucleation sites for mineralization of that tissue.

EXPERIMENTAL PROCEDURE

Male weanling rats of the Sprague-Dawley strain were used: 40 animals in each of the first two studies and 60 in study 3. They were housed individually in stainless steel cages at a controlled temperature of 23 to 25°. Weekly body weights and daily food intakes were recorded. Demineralized water was provided.

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Experiment 1 was conducted to investigate the effect of low sulfate intake on the development of soft tissue calcification of magnesium deficiency. The composition of the basal diet is given in table 1. Alterations were made in the basal diet at the expense of glucose to increase magnesium from 80 to 465 ppm and sulfur from 0.004 to 0.035%, using magnesium carbonate and sodium sulfate. All diets contained 0.5 to 0.6% Ca and a 1:1, Ca:P ratio. At the end of a 4-week feeding period (ad libitum) the animals were decapitated; blood, liver, kidney and tibias were taken for analysis.

In experiment 2, the effect of T_4 treatment and magnesium deficiency on the glycoprotein and acid mucopolysaccharide levels in serum and urine were determined in an attempt to relate the observed changes to kidney calcification. L-Thyroxine (sodium salt) [3] and magnesium carbonate were added to the basal diet (table 1) alone and combined to give a final concentration of 20 ppm T_4 and 514 ppm Mg. Forty-eight hours prior to termination of the experiment the animals were placed in metabolism cages for collection of urine. Several animals from each treatment were killed after 2, 3 and 4 weeks of the experiment, and blood, urine and kidneys were obtained for analyses. The results from all three experimental periods were pooled, since there was little variation in results obtained between periods.

TABLE 1

Percentage composition of the basal diets

	Exp. 1	Exps. 2 and 3
Casein [1]	15.00	15.00
DL-Methionine	0.10	0.50
Corn oil	8.00	8.00
Cellulose [2]	3.00	3.00
Cod-liver oil [3]	0.50	0.50
Salt mix	2.89 [4]	2.66 [5]
Vitamin mix [6]	5.00	5.00
Glucose [7]	65.51	65.34

[1] Vitamin-free test casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

[2] Solka Floc, Brown Company, Boston, Mass.

[3] Parke-Davis standardized cod liver oil containing 2000 USP units/g vitamin A and 250 USP units/g vitamin D.

[4] Contained: (in percent) NaCl, 8.64; K_2CO_3, 16.59; $MnCl_2 \cdot 4H_2O$, 0.62; $FeCl_2 \cdot 4H_2O$, 0.31; $ZnCl_2$, 0.17; $CuCl_2 \cdot 2H_2O$, 0.05; $4MgCO_3 \cdot Mg(OH)_2 \cdot nH_2O$, 1.04; KI, 0.01; and $CaHPO_4$, 72.57.

[5] Contained: (in percent) NaCl, 14.00; K_2CO_3, 18.90; $FeSO_4 \cdot 7H_2O$, 1.50; $MnSO_4 \cdot H_2O$, 0.454; $CuSO_4 \cdot 5H_2O$, 0.227; KI, 0.014; $ZnCO_3$, 0.074; $4MgCO_3 \cdot Mg(OH)_2 \cdot nH_2O$, 1.030; and $CaHPO_4$, 63.801.

[6] Contained: (in grams per kilogram) thiamine·HCl, 0.20; riboflavin, 0.12; pyridoxine·HCl, 0.08; Ca pantothenate, 0.32; biotin, 0.004; nicotinic acid, 0.50; folic acid, 0.010; B_{12} in mannitol (0.1% B_{12}), 0.40; choline chloride, 30.0; menadione, 0.007; and glucose, 968.359.

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

The alterations in incorporation of labeled sulfur into kidney mucopolysaccharides during the course of magnesium deficiency and concomitant intraperitoneal administration of L- and D-T_4 were studied in experiment 3. We included the D-isomer of T_4 because a preliminary experiment showed it to be active against kidney calcification when included in the diet. The basal diet was similar to that in experiment 2 but contained less magnesium (63 ppm). The control diet contained 593 ppm Mg. The L- and D-T_4 were administered intraperitoneally on alternate days starting with day 1 of the experiment, at a level of 10 μg/injection and increasing through 15 to 20 μg. Animals were killed on day 5 and at the end of 2 weeks. Forty-eight hours prior to killing, the rats were injected intraperitoneally with an isotonic carrier-free solution of $Na_2{}^{35}SO_4$, each receiving 1 μCi ^{35}S/g body weight. An interval of 48 hours was chosen because maximum fixation or uptake of radioactive sulfate in skin of rats has been reported to occur between 24 and 48 hours (17). After injection of the isotope the animals were placed in metabolism cages for urine and feces collection. Serum, kidney, urine and tibia samples were obtained at the time of killing. Radioactivity in these samples was measured in a liquid scintillation spectrometer,[4] as described by Mahin and Lofberg (18).

Chemical analyses for calcium and magnesium in diets and tissues were performed with an atomic absorption spectrophotometer,[5] according to routine procedures. The sulfur content of the diets was determined by the method of Letonov and Reinhold (19). Glycoproteins were determined as protein-bound hexose by the orcinol test on acetone powder extracts of kidney and liver and on an aliquot of serum according to the procedure of Winzler (20). Urine glycoproteins were determined by the

[3] Sigma Chemical Company, St. Louis, Mo.

[4] Packard Tri-Carb, model series 3000, Packard Instrument Company, Inc., Downers Grove, Ill.

[5] Perkin-Elmer model 303, Perkin-Elmer Corporation, Norwalk, Conn.

method of Engel and Catchpole (21). Hexosamine levels in kidney and liver acetone powders were measured by the method of Boas (22). Serum hexosamine was estimated by the method of Winzler (20). Serum and urine proteins were measured by the procedure of Lowry et al. (23). Urine and kidney acid mucopolysaccharides were determined as outlined by Di Ferrante and Rich (24) and by Bollet et al. (25), respectively.

Analysis of variance and Tukey's multiple range test were used to evaluate the data statistically. Where significant differences are cited in the text they are at the $P < 0.01$ level.

RESULTS

Experiment 1. Rats fed the low magnesium diet in this experiment gained an average of 4.3 g daily (table 2), there being no influence of sulfur levels on weight gain. The gains of the magnesium-adequate animals average 6.5 ± 0.17 g (SE) and 7.1 ± 0.10 g for the low and normal sulfur treatments, respectively. On this basis it appears that the low sulfur diet was slightly inhibitory to weight gain (via reduced feed intake) but that this characteristic was undetectable in the presence of the more severe magnesium deficiency induced by the low magnesium diet.

Tissue calcium and magnesium concentrations were not affected by dietary sulfate level although the typical reduction in serum and bone magnesium and gross exaggeration of kidney calcium was observed as a result of magnesium deficiency.

The nature of the liver glycoprotein was altered by magnesium deficiency but not by sulfate intake. This is seen as a decrease in protein-bound hexose and an increase in protein-bound hexosamine in livers from magnesium-deficient rats. Changes in kidney protein-bound carbohydrate were not remarkable.

Serum glycoprotein hexose was reduced both by magnesium deficiency and low sulfate intake, but these treatments were not additive.

Experiment 2. The data obtained in this experiment are shown in table 3. The weight gains were obviously reduced by magnesium deficiency and by T_4 adminis-

TABLE 2

Effects of low sulfate and low magnesium diets on ground substance components and minerals in selected tissues

Treatment: Magnesium	−	−	+	+
Sulfur	−	+	−	+
Wt gain, g/day	4.3 ±0.11 [1]	4.2 ±0.22	6.5 ±0.17	7.1 ±0.10
Serum calcium, mg/100 ml	10.2 ±0.18	10.1 ±0.33	9.6 ±0.17	10.1 ±0.15
Serum magnesium, mg/100 ml	0.74±0.015	0.74±0.015	2.33±0.035	2.19±0.037
Tibia magnesium, % of ash	0.31±0.008	0.29±0.010	0.74±0.008	0.71±0.012
Liver glycoprotein, mg hexose/g acetone powder	4.6 ±0.83	4.8 ±0.21	8.0 ±0.39	10.1 ±0.71
Liver glycoprotein, mg hexosamine/g acetone powder	4.2 ±0.15	3.9 ±0.17	3.5 ±0.14	3.1 ±0.11
Kidney glycoprotein, mg hexose/g acetone powder	4.0 ±0.04	4.0 ±0.08	3.2 ±0.10	4.1 ±0.06
Kidney glycoprotein, mg hexosamine/g acetone powder	8.9 ±0.31	7.7 ±0.25	8.7 ±0.17	8.5 ±0.17
Serum glycoprotein, mg hexose/100 ml	99.1 ±1.7	105 ±2.5	103 ±3.7	116 ±2.9
Kidney calcium, mg/g dry wt	26.1 ±5.33	19.9 ±3.65	0.35±0.042	0.35±0.014

[1] Mean ± SE.

TABLE 3

Effect of dietary magnesium and thyroxine on protein-bound carbohydrate of serum and urine and on kidney calcium

Treatment: Magnesium	−	−	+	+
T_4	−	+	−	+
Wt gain, g/day	4.2	3.0	6.0	3.1
Serum protein, %	7.2 ±0.23	6.5 ±0.23	8.3 ±0.24	7.0 ± 0.24
Protein-bound hexose [1]	1.63±0.028	1.34±0.030	1.67±0.107	1.37± 0.030
Protein-bound hexosamine [1]	1.97±0.138	1.65±0.183	2.01±0.143	1.36± 0.096
Urine protein, mg/day	73 ±7.1	91 ±8.6	83 ±7.0	102 ±14.4
Urine protein-bound hexose [2]	3.35±0.254	2.95±0.474	3.85±0.208	3.07± 0.482
Kidney calcium, μg/g dry wt	32.9 ±8.0	0.42±0.02	0.41±0.02	0.36± 0.01

[1] Percentage of serum protein.
[2] Percentage of urine protein.

tration, although the effect of magnesium deficiency was masked by the more severe reduction brought on by the calorigenic effect of T_4.

The total serum protein was decreased both by magnesium deficiency and by T_4 treatment. The protein-bound hexose and hexosamine, expressed as percentage of the serum protein, were each decreased in T_4-treated animals, but not by magnesium deficiency. The kidney calcification of magnesium-deficient animals was prevented by T_4 administration. The concentration of hexose bound to urine protein showed a tendency toward reduction in T_4-treated animals, but the effect was not statistically significant, nor were there significant treatment effects in total urinary protein excretion.

Experiment 3. As shown in table 4 there were no treatment effects at 4 days on incorporation of ^{35}S into serum, bone or kidney. Excretion of ^{35}S in the urine of magnesium-deficient animals treated with L-T_4 was decreased during period 1, but this effect was not seen in magnesium-adequate animals, or in any animals whose treatments extended to 14 days.

In period 2, magnesium deficiency and also L-T_4 treatment decreased the incorporation of ^{35}S in serum and in bone, L-T_4 having the larger effect (a 50% reduction). Kidney ^{35}S levels were not affected by magnesium status. The increased retentions seen at 14 days, however, did not occur in T_4-treated animals.

The serum calcium and magnesium data shown in table 5 reveal the usual hypercalcemia and hypomagnesemia seen in magnesium deficiency. These values were not affected by T_4 treatment. The kidney calcium concentration is seen to be markedly increased by magnesium deficiency, the increase being prevented by L-T_4 injection, but at best only delayed slightly by D-T_4 injection.

Although urine and kidney acid mucopolysaccharides were determined, they were not affected by treatment and averaged 0.08 mg/24 hours and 0.07% of the dry kidney weight, respectively, as uronic acid.

DISCUSSION

Although the low sulfur diet used in experiment 1 appeared to be slightly deficient to maximize weight gains it did not affect the kidney calcification arising from magnesium deficiency. Faragalla and Gershoff (26) reported that diets low in sulfur afforded partial protection against oxalate stone formation in rats receiving vitamin B_6-deficient diets. They were not, apparently, dealing with magnesium deficiency nephrocalcinosis, since their basal diets contained 400 ppm Mg and the stones produced were largely oxalate rather than phosphate. In magnesium deficiency the calcification involves an increase in calcium and phosphorus in the kidney in about a 2:1 ratio.

The decrease in liver and serum protein-bound hexose, and the increase in liver hexosamine provides support for the hypothesis that biosynthesis of glycoproteins was altered by the lack of magnesium which is an activator of enzymes involved in protein biosynthesis.

The liver is the primary site for the synthesis of both the hexose and hexosamine

TABLE 4

Distribution of ^{35}S in serum, bone, kidney and urine

Treatment: Magnesium	−	−	+	+	+
L-T_4	0	+	0	+	0
D-T_4	0	0	0	0	+
Period 1. ^{35}S given at 2 days, samples taken at 4 days					
Serum, % dose/100 ml	1.7 ±0.17[1]	1.4 ±0.11	1.7 ±1.09	1.4 ±0.21	1.4 ±0.06	2.3 ±0.26
Bone, % dose/10 g	4.2 ±0.14	5.7 ±1.04	5.8 ±0.72	6.7 ±1.30	5.8 ±0.65	10.6 ±1.67
Kidney, % dose/10 g	0.64±0.037	0.64±0.024	0.70±0.062	0.69±0.067	0.60±0.039	0.73±0.059
Urine, % dose/48 hr	47 ±4.1	32 ±1.9	41 ±1.2	44 ±1.5	41 ±2.6	43 ±1.9
Period 2. ^{35}S given at 12 days, samples taken at 14 days						
Serum, % dose/100 ml	3.7 ±0.45	1.6 ±0.14	3.0 ±0.32	4.3 ±0.40	2.2 ±0.15	1.0 ±0.36
Bone, % dose/10 g	4.6 ±0.71	2.2 ±0.21	3.7 ±0.47	6.4 ±0.50	3.1 ±0.55	5.8 ±0.80
Kidney, % dose/10 g	1.16±0.10	0.60±0.042	0.90±0.058	0.93±0.058	0.63±0.075	0.96±0.083
Urine, % dose/48 hr	61 ±3.9	59 ±3.1	62 ±1.8	58 ±2.5	46 ±5.4	53 ±4.2

[1] Mean ± SE.

components of plasma glycoproteins (27) and for the attachment of these carbohydrates to the developing protein molecule (28). Thus, it is possible that the alteration in glycoprotein synthesis in liver due to magnesium deficiency is reflected in the altered serum glycoproteins. Many distinct proteins contribute to the total protein-bound hexose of plasma and these different proteins may react differently and even oppositely in pathological and abnormal conditions. Bunce and Poe [6] reported an elevated seromucoid level in magnesium-deficient rats compared with controls. The absolute value of seromucoid in magnesium-deficient rats (15 mg/100 ml) obtained by these authors was several times less than the protein-bound hexose (glycoprotein) values found in this study (116 mg/100 ml). The components measured in the two experiments were obviously different.

The T_4 effects on total serum protein are similar to those observed by Gershoff et al. (29), and the decreased protein-bound hexose resulting from T_4 treatment is similar to that reported earlier from this laboratory (7). Urine glycoprotein excretion was not affected by treatments even though treatments did lower serum glycoproteins. The source of the urinary glycoproteins has not been confirmed with any degree of certainty. They could originate from the serum, being filtered through the glomerulus, which acts as a molecular sieve. In conditions associated with destructive and proliferative processes, serum glycoproteins have been shown to be increased, as was their excretion in the urine (29, 30). The other source of urine glycoproteins is the external glycoprotein layer of the kidney tubule cell membranes where they are synthesized and then cast into the urine (31). Studies of the composition of calcium deposits in kidney have shown an organic matrix containing macromolecules which stained positively for polysaccharides (10, 32). Meyer [7] demonstrated the presence of increased amounts of PAS-positive material in areas where calcium

[6] Bunce, G. E., and C. D. Poe 1967 Serum seromucoid in magnesium deficiency. Federation Proc., 26: 634 (abstract).

[7] Meyer, D. L. 1967 Mineral and endocrine relationships in renal calcification. Ph.D. thesis, University of Illinois at Urbana-Champaign.

TABLE 5

Serum (mg/100 ml) and kidney (mg/g dm) mineral levels after 4 and 14 days of a magnesium-deficient diet with and without L- *and* D-*thyroxine administered intraperitoneally*

Treatment: Magnesium	−	−	−	+	+	+
L–T_4	0	+	0	0	+	0
D–T_4	0	0	+	0	0	+
			Period 1 (4 days)			
Serum calcium	10.0 ±0.36 [1]	9.2 ±0.14	8.3 ±0.40	8.2 ±0.36	8.7 ±0.23	8.8 ±0.15
Serum magnesium	0.76±0.04	0.73±0.08	0.77±0.05	2.07±0.08	2.16±0.08	2.15±0.07
Kidney calcium	2.68±1.00	0.52±0.01	0.74±0.18	0.47±0.03	0.44±0.01	0.42±0.01
			Period 2 (14 days)			
Serum calcium	10.1 ±0.15	9.4 ±0.45	9.5 ±0.11	8.9 ±0.16	8.4 ±0.09	8.9 ±0.24
Serum magnesium	0.64±0.03	0.67±0.04	0.66±0.04	2.46±0.03	2.40±0.03	2.60±0.06
Kidney calcium	4.03±1.06	0.48±0.04	4.21±1.83	0.37±0.01	0.42±0.02	0.46±0.06

[1] Mean ± SE.

deposition had occurred in kidneys from magnesium-deficient animals.

The data of experiment 3 show that the calcification process is initiated during the first few days of magnesium deficiency and that this may be prevented by intraperitoneal injection of L-T_4 and to a small extent by D-T_4. The continued administration of L-T_4 protected the kidney against calcium deposition. Intraperitoneally administered D-T_4 was not effective during this 14-day period, in contrast to its effectiveness when included in the diet.[8] Further investigation of this discrepancy is being made.

On the basis of the experimental data, renal calcification appears to precede any change in sulfate uptake by the kidney tissue. This would suggest that the initial increase in calcium is not accompanied by any alteration in the amount of renal ground substance. With the extended duration of magnesium deficiency there was a significant increase in ^{35}S incorporation in the kidneys of all but the L-T_4-treated animals, indicating that changes in the organic matrix had occurred. The beneficial effects of L-T_4 may thus be related to its prevention of increased sulfate retention in the kidney ground substance. Calcification was initiated by 4 days, however, at which time no T_4 effects on ^{35}S retention were detectable. The ground substance is the extracellular amorphous matrix interspersed between the tubular cells and it is the medium through which metabolites and ions are continuously being exchanged. Electron-microscopic studies have shown that the calcium deposits in kidneys of magnesium-deficient rats lie free in the cytoplasm or are found within lysosome-like bodies (33), whereas the calcium casts associated with atherosclerosis are extracellular in location. Thus, it appears reasonable to conclude that calcium accumulation commences intracellularly in the very early stages after instituting a magnesium deficiency, possibly due to the altered ionic composition of the tubular fluid. With the continued passage of this abnormal tubular fluid through the extracellular phase it is possible that the physical characteristics and chemical composition of the ground substance components are changed, causing degenerative changes predisposing to further extensive calcification.

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Failure of Men to Select a Balanced Amino Acid Mixture [1]

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ABSTRACT Six healthy young men were given a choice between balanced and deficient amino acid mixtures containing 7 g N/day with a formula diet adequate in all other nutrients to see if they would select the balanced diet. For the 12 meals during which they were observed, the men demonstrated no ability to select the balanced mixture and actually chose the mixture deficient in methionine and phenylalanine somewhat more frequently. The study gave no indication that humans have the capacity to discriminate between amino acid mixtures on the basis of their ability to supply adequate quantities of indispensible amino acids.

Investigators in both biological and social sciences have suggested that animals can discriminate between poorly balanced and well-balanced diets and will select the diet which supplies a nutrient in which they are deficient if they have not been conditioned to allow nonphysiological factors to influence their choice (1, 2). Harriman (3) found that adrenalectomized rats had a preference for salt solutions over glucose solutions unless they had been allowed to develop a preoperative preference for glucose, in which case they continued to consume glucose and died. The same author (4) also showed that vitamin A-deficient rats offered a choice of diets would select the one which would alleviate the deficiency. Davis (5) reported that three infants who had received only breast milk for 7.5 to 9 months before entering the study could self-select balanced diets from a variety of simple natural foods and continue to grow and develop normally for as long as 1 year.

More recently, Harper (6) reported that rats fed amino acid-imbalanced mixtures ad libitum chose a correctly balanced mixture over an imbalanced mixture although the small quantity of amino acid added to achieve balance would not have materially affected flavor or odor. The question of whether or not adult humans can discriminate and will select a balanced diet in contrast to an inferior one is difficult to answer because of the variety of factors influencing choice of food. Since humans are not accustomed to eating mixtures of purified amino acids, however, such a source of essential nitrogen should provide a means of determining whether or not humans can select a properly balanced amino acid mixture when physiological response is not strongly overridden by cultural and other psychological factors.

EXPERIMENTAL

Six healthy men were confined to a metabolic unit for 3 days. They received, in equal quantities four times daily at 0830, 1230, 1730, and 2130, a protein-free formula that supplied adequate amounts of all macrominerals and 1440 kcal from safflower oil and a mixture of carbohydrates. Additional protein-free calorie sources offered were 78 g of orange-flavored beverage powder and, ad libitum, weighed amounts of protein-free rusks,[3] biscuits [4] and salt-free margarine.[5] Two servings each of decaffeinated coffee and soluble tea and unlimited amounts of deionized water were provided. Vitamins and trace minerals were given in capsule form (7).

At breakfast each day, two coded, preweighed cups of amino acid mixtures were positioned randomly with each man's table

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setting. The allowance for each meal was one-fourth of the total 7 g N provided per day. The complete essential amino acid pattern was based on Rose et al. recommendations (8) plus cystine and tyrosine. The deficient mixture differed in that it lacked methionine and phenylalanine. The amino acid mixtures provided the following: (in grams per day) L-tryptophan, 0.290; L-threonine, 0.580; L-isoleucine, 0.812; L-valine, 0.928; L-lysine, 0.928; L-methionine, 1.10 (or 0); L-phenylalanine, 1.10 (or 0); L-leucine, 1.276; L-cystine, 1.276; L-tyrosine, 1.276; L-glutamic acid, 20.95; L-alanine, 12.67; and glycine, 10.68.

Subjects were instructed to taste both amino acid mixtures at the start of each meal and to eat all of the one that they preferred. The men had 12 opportunities to discriminate between patterns. At the end of the study, the men were asked if they could distinguish between the mixtures and the reasons for their choices, if any.

RESULTS AND DISCUSSION

As shown in table 1, the men were not able to select the better balanced amino acid mixture and actually chose the deficient mixture more frequently. None of the men developed a consistent preference for a particular diet, and the choice of diet did not appear to be influenced by the time of day or the length of time on the experiment (table 2).

The design of the formulas provided that proper diet selection had to be only somewhat better than chance to supply adequate amounts of both methionine and phenylalanine for balance over longer periods than intervals between meals. Even with the amounts ingested during tasting, average daily intakes of both methionine and phenylalanine were only 0.28 to 0.67 g. With the exception of one subject who consumed 0.28 g, all intakes should have fulfilled adequately the "safe" minimum recommendation for methionine in the presence of cystine. Only one subject, however, met the "safe" minimum recommendation for phenylalanine in the presence of adequate tyrosine.

TABLE 1

Individual preference for balanced and deficient amino acid mixtures for 12 meals [1]

Subject	Age	Body wt	Number of times chosen	
			Balanced	Deficient
		kg		
1	23	82.2	3	9
2	24	59.5	5	7
3	24	55.4	5	7
4	31	66.4	5	7
5	26	85.6	5	7
6	24	56.3	7	5

[1] Men received four meals per day for 3 days.

TABLE 2

Daily group preferences for balanced and deficient amino acid mixtures

Day	Mixture	Meal time				Daily total
		0830	1230	1730	2130	
1	Balanced	3 [1]	1	3	4	11
	Deficient	3	5	3	2	13
2	Balanced	2	3	3	1	9
	Deficient	4	3	3	5	15
3	Balanced	3	3	1	3	10
	Deficient	3	3	5	3	14

[1] Number of men indicating preference, of the group of six.

Upon being questioned at the end of the study, all the men felt that they had been able to distinguish between the two mixtures and had attempted to choose the one they preferred. Most of the subjects looked for the mixture which tasted "less sweet" or "more sour"—sensations which they also associated with "feeling better" after the meal. Four of the men said that they never made their selection on the basis of guessing, and two men guessed less than one-fourth of the time. The significance of any taste difference between the amino acid mixtures appears minor in view of the fact that no one consistently selected one particular mixture.

If humans have a homeostatic mechanism with respect to appetite and amino acid balance similar to rats fed low nitrogen diets *ad libitum*, the present experimental conditions should have produced a physiological response ensuring the selection of a properly balanced amino acid mixture. Rats trained to eat one or a few meals a day (9, 10) no longer show keen discrimination between balanced and imbalanced amino acid mixtures. Perhaps these rats provide a better experimental model for comparing human and animal responses with subtle

changes in dietary amino acid composition. Although the diets used in the present study did not meet the exact specifications for an amino acid imbalance (6), human subjects showed no evidence of being able to choose a properly balanced amino acid mixture, despite the removal of the usual biasing factors in diet selection such as appearance, taste, and smell of the food.

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Effect of Protein Deprivation of Swine During All or Part of Gestation on Birth Weight, Postnatal Growth Rate and Nucleic Acid Content of Brain and Muscle of Progeny

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ABSTRACT It was previously shown that gilts deprived of dietary protein during gestation are capable of producing grossly normal, viable young. The present experiment was designed to study the effect of dietary protein deprivation during various intervals of gestation in the gilt on subsequent growth of the progeny and on nucleic acid content of the brain and muscle of the progeny during young adulthood. Eighteen pregnant gilts were divided at 3 to 4 days after breeding into four groups as follows: 1) protein-free diet to parturition; 2) protein-free diet to day 16, then control diet days 16 to 20 (period of implantation of blastocyst), then protein-free diet to parturition; 3) control diet to day 24, then protein-free diet to parturition; and 4) control diet to parturition. All gilts fed the protein-free diet during any part of gestation lost weight; all control gilts gained weight. Litter size (number of live plus stillborn pigs) was not significantly affected by treatment. Birth weight and postnatal growth rate were significantly reduced in progeny of gilts fed the protein-free diet to parturition (group 1) but not in those of gilts deprived from day 24 to parturition (group 3). The birth weight and postnatal growth rate of progeny of gilts given transitory protein at implantation (group 2) were greater than those of gilts deprived of protein throughout (group 1), supporting the concept of a beneficial effect of transitory protein at implantation on the development of the fetus. Fresh weights of cerebrum and cerebellum plus medulla of the progeny at a slaughter weight of approximately 90 kg were not affected by prenatal treatment but percentage dry matter was greater in brains of control progeny than in those of progeny of protein-deprived gilts. Neither DNA concentration nor total DNA content of either organ were affected by treatment. RNA per gram of protein or total RNA per organ was greater in progeny of controls than in those of protein-deprived gilts, suggesting greater protein synthetic activity in the brains of the former. No treatment differences were noted in dry matter, protein, DNA or RNA concentration of skeletal muscle (longissimus dorsi) of progeny. It is concluded that dietary protein deprivation of the gilt throughout pregnancy results in reduced birth weight and postnatal weight gain of the progeny but does not permanently affect DNA content (cell number) of the cerebrum or cerebellum. RNA concentration and total RNA in these organs in the young adult progeny may be decreased by maternal dietary protein deprivation during gestation, suggesting an effect on brain protein synthetic activity. DNA concentration of skeletal muscle at 90 kg body weight was not affected by treatment but RNA/DNA was significantly reduced in progeny of protein-deprived gilts. The depressed postnatal weight gain in progeny of protein-deprived gilts suggests an effect on metabolism not clearly reflected by these parameters.

The ability of the pig to maintain pregnancy while being deprived of dietary protein during all or part of gestation has been demonstrated in this laboratory (1). Berg (2) has shown that the rat can also maintain pregnancy on a protein-free diet when transitory supplements of protein are provided at mating (days zero to 2) and at the time of implantation of the blastocyst (days 5 to 9). Under these conditions, litter size was unaffected, but birth weight was reduced. Several reports in rats indicate an adverse effect of prenatal malnutrition on later development (3–5).

The present report describes the effect of dietary protein deprivation during vari-

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ous intervals of gestation in the gilt on subsequent growth and development of the progeny.

METHODS AND MATERIALS

Eighteen pregnant gilts were divided at 3 to 4 days after breeding into four groups as follows: 1) protein-free to parturition (PF); 2) protein-free to day 16, then control (C) days 16 to 20, then protein-free to parturition (PF-C-PF); 3) control to day 24, then protein-free to parturition (C-PF); and 4) control to parturition (C). The protein-free (PF) and control (C) diets were the same as those used earlier (1). Groups 1 (PF) and 3 (C-PF) were included to provide a comparison of the effect of the duration of protein deprivation during gestation on reproductive performance. Previous work (1) had suggested that protein deprivation during only the final three-fourths of pregnancy resulted in far less adverse effects on the offspring than protein deprivation throughout pregnancy, but this was not tested in a single experiment. Group 2 (PF-C-PF) was included to test the effect of supplying protein only at the time of implantation of the blastocyst (day 18 in the pig) on growth of the fetus and its subsequent development. It has been shown in the rat (2) that pregnancy can be maintained by transitory feeding of protein during this stage of gestation.

During gestation gilts were kept in indoor pens with concrete floors, and were fed 1.82 kg feed/animal daily in two equal morning and afternoon portions. At day 112 of pregnancy gilts were moved to individual farrowing pens where they were kept with their litters to weaning at 5 weeks of age. Beginning on the day of parturition all gilts from all treatment groups were fed the control diet throughout lactation. Thus, any effects on lactation were carry-over effects from the diet sequence during gestation.

A blood sample was obtained from each gilt during weeks 5 and 12 of pregnancy and at parturition for total serum protein determination (6). Blood was also obtained from newborn pigs before suckling in some litters for serum protein (6) and hemoglobin determination (7). There was approximately 30% mortality from birth to weaning, a rate typical under normal swine husbandry conditions. The deaths were scattered among all groups and did not appear to be related to sow gestation diet. At weaning, a total of 92 randomly selected pigs from the four treatment groups (21, 20, 30 and 21 from groups 1 through 4, respectively) were transferred in groups of 7 to 10 to 3m by 3 concrete-floored pens and fed a standard corn-soybean meal-type diet ad libitum until slaughter at approximately 90 kg body weight. They were removed individually as they reached slaughter weight and from approximately one-half the randomly selected animals in each group the following measurements were made on the cerebrum and cerebellum plus medulla and on a 2-cm cross section of the longissimus dorsi muscle taken from between the 10th and 11th ribs: fresh weight, percentage dry matter, percentage protein in the dry matter and RNA and DNA content of the fresh tissue. Protein was determined by the Kjeldahl procedure (N × 6.25) and RNA and DNA by a modification of the procedure of Schmidt and Thannhauser (8). RNA and DNA content of cerebrum and cerebellum plus medulla were then expressed as RNA/DNA, milligrams per gram of protein and as total milligrams per organ (concentration × organ weight). RNA and DNA content of longissimus dorsi were expressed as RNA/DNA and as milligrams per gram of protein. The results were treated statistically by analysis of variance for each criterion (9).

RESULTS AND DISCUSSION

The reproductive performance is summarized in table 1. All gilts fed PF diets during any part of gestation lost weight with the greatest loss in those fed the PF diet from day 3 to 4 onward (group 1). The control (C) gilts (group 4) gained an average of 22.4 kg. There was no significant difference in litter size (number alive plus stillborn) among treatments, although the C group had fewer stillbirths per litter than other groups. Statistical treatment of litter size and number of stillbirths has little value in view of the small number of litters available. Birth

TABLE 1

Reproductive performance of gilts deprived of dietary protein during various intervals of gestation

Group no.	1	2	3	4
Gestation diet sequence [1]	PF	PF–C–PF	C–PF	C
No. of gilts	5	5	5	3
Body wt at breeding, kg	127.9	131.7	124.7	127.0
Body wt 1 day postpartum, kg	106.7	121.4	119.2	149.4
Total pigs per litter	10.0	11.0	9.2	10.7
No. of stillborn per litter	1.6	1.2	1.4	0.8
Birth wt of progeny, g [2]	750	880	1090	1120

[1] PF = protein-free diet to parturition; PF–C–PF = protein-free diet to day 16, then control diet to day 20, then protein-free diet to parturition; C–PF = conrol diet to day 24, then protein-free diet to parturition; C = control diet to parturition.
[2] PF significantly less than other three groups; PF–C–PF significantly less than C–PF and C ($P < 0.05$).

weight of individual pigs was significantly reduced ($P < 0.05$) in group 1 (PF). Progeny in group 2 (PF-C-PF) were significantly larger at birth than those in group 1, but smaller than those in groups 3 (C-PF) and 4 (C). There was no statistically significant difference between average birth weights of progeny in groups 3 and 4. The higher birth weight of progeny of gilts fed the PF-C-PF sequence (group 2) than of progeny of gilts fed PF (group 1) supports the observation of Berg (2) in rats, indicating a beneficial effect of transitory protein at the time of implantation on the development of the fetus. The mode of action of this apparent beneficial effect on the growth of the fetus is not known, but, if not a chance occurrence in this experiment, it may involve an improved vascularity of the placenta mediated endocrinologically during the critical time of implantation of the blastocyst. The similarity in birth weight of progeny of C-PF (group 3) and C (group 4) gilts agrees with the previous observation (1), but needs further study since this period of gestation corresponds to the time of greatest absolute increase in size of the fetus. It is important to recognize that the preexperimental nutritional status of the gilts used in the present experiment was excellent and that this could be an important factor in determining the response to long-term protein deprivation during gestation.

The weight loss of protein-deprived gilts during gestation (groups 1, 2 and 3) agrees with the earlier observation (1) and substantiates the concept of a "labile protein reserve" for maintenance of pregnancy during dietary protein deprivation. As shown in table 2, serum protein concentration was depressed in protein-deprived gilts. The severity of the depression, however, was strikingly small, indicating that this blood parameter tends to be maintained at a high level in the pregnant animal even after prolonged depletion. Total serum protein concentration has also been shown to be a rather insensitive index of protein nutriture in the nongravid young adult rat (10). Levels of free amino acids in the blood plasma of the protein-deprived pregnant pig would be of importance in terms of the amino acid pattern presented to the placenta for transfer to the fetus. The serum protein and hemoglobin levels of newborn pigs before suckling were not strikingly different among treatments. The mean serum protein (grams per 100 ml)

TABLE 2

Serum protein levels of pregnant pigs deprived of dietary protein during various intervals of gestation

Group no.	1	2	3	4
Gestation diet sequence [1]	PF	PF–C–PF	C–PF	C
	g/100 ml			
No. of gilts	5	5	5	5
Week 5 [2]	6.5	6.9	7.4	7.6
Week 12 [3]	6.6	7.4	7.0	8.9
Postpartum [4]	6.4	6.6	7.0	8.4

[1] See footnote 1, table 1.
[2] PF significantly less than C–PF and C ($P < 0.05$).
[3] PF significantly less than C ($P < 0.05$).
[4] PF, PF–C–PF and C–PF significantly less than C ($P < 0.05$).

concentrations of 12, 14, 3 and 3 pigs which were sampled before suckling from PF, PF-C-PF, C-PF and C dams, respectively, were 4.5, 3.5, 6.1 and 4.9. Corresponding values for hemoglobin (grams per 100 ml) were 8.1, 8.1, 7.4 and 12.3. These values among pigs from protein-deprived dams do not represent striking manifestations of protein malnutrition.

Postnatal weight gains are summarized in table 3. Body weight at weaning (5 weeks) was significantly depressed in progeny of protein-deprived gilts (groups 1, 2 and 3) compared with that of controls (group 4). The design of the experiment did not permit separation of the relative effects of prenatal nutrition versus carry-over effects of gestation diet sequence on lactation of the dam. Previous work (1), in which reciprocal transfer was made of one-half the litters from paired control and protein-deprived dams during the suckling period, indicated that lactation performance was affected in dams deprived of dietary protein from day 24 to 28 of pregnancy to parturition. Progeny of these gilts, however, were not stunted in weight at weaning, if transferred to a control gilt during the suckling period, nor was their postweaning rate of body weight gain adversely affected (11). However, birth weight was not significantly depressed in those pigs. In the present experiment the more severe protein depletion of dams in group 1 (PF to parturition) and in group 2 (PF to parturition except for control diet days 16 to 20) resulted in a significant reduction in birth weight. Therefore, the lower weaning weight of progeny of these two groups of gilts in this experiment was probably associated with both a smaller birth weight and a smaller milk supply. In contrast, the lower weaning weight of progeny of gilts in the C-PF group must be a result of a smaller milk supply since birth weight was not significantly different from that of control progeny, in agreement with previous observations (1).

Daily weight gain from weaning to slaughter weight at approximately 90 kg was also significantly different among groups ($P < 0.01$). It should be pointed out that progeny of control gilts were younger at slaughter than progeny of protein-deprived gilts because of the weight-constant slaughter schedule. As shown in table 3, the objective of standard slaughter weight was not achieved as progeny of control gilts exceeded the mean slaughter weight of the other groups. The higher slaughter weight of the control progeny was accompanied by an approximately 2-week younger age. This should be considered in interpreting the results of tissue composition measurements in the present experiment. Progeny of gilts deprived of protein to parturition (group 1, PF) had the lowest daily weight gain (490 g) compared with progeny of the controls (group 4, C) (602 g). Progeny of groups 2 and 3 were intermediate. The superior postnatal weight gains of progeny of group 2 (PF-C-PF) compared with progeny of group 1 (PF) again suggests the importance of transitory dietary protein at the time of implantation on the subsequent development of the progeny, as reported by Berg (2) in the rat. An endocrine effect on maintenance of pregnancy in the rat

TABLE 3

Postnatal weight gain of progeny of gilts deprived of dietary protein during various stages of gestation

Group no.	1	2	3	4
Gestation diet sequence [1]	PF	PF–C–PF	C–PF	C
No. of progeny observed	21	30	20	21
Body wt, 5 weeks, kg [2]	5.42	5.11	5.85	7.42
Daily gain, birth to 5 weeks, g [2]	133	121	136	180
Daily gain, 5 weeks to slaughter, g [3]	568	657	658	705
Daily gain, birth to slaughter, g [3]	490	552	553	602
Slaughter wt, kg	92	94	95	107

[1] See footnote 1, table 1.
[2] C significantly greater than other three groups.
[3] PF significantly different than C ($P < 0.005$).

in the absence of dietary protein has been suggested (12, 13). The importance of an endocrine response to the transitory protein fed to gilts in the present experiment needs to be studied.

The effect of duration and time sequence of dietary protein deprivation during gestation on dry matter, protein and nucleic acid content of brain (cerebrum and cerebellum plus medulla) and skeletal muscle (longissimus dorsi) of the progeny during young adulthood is summarized in tables 4, 5 and 6. The fresh weight of the cerebrum and cerebellum plus medulla was not permanently affected by protein deprivation of the dam during

TABLE 4

Effect of protein deprivation of gilts during gestation on weight, dry matter, protein, RNA and DNA content of the cerebrum of the young adult progeny

Group no.	1	2	3	4
Gestation diet sequence [1]	PF	PF–C–PF	C–PF	C
No. of animals	10	13	10	9
Fresh wt, g	82.4	79.3	83.8	82.5
Dry matter, %	20.6	20.9	21.1	21.8
Protein in dry matter, %	50.5	49.5	49.8	50.3
RNA per gram protein, mg [2]	10.3	9.5	10.5	10.9
Total RNA, mg [2]	88.4	78.1	93.0	96.3
DNA per gram protein, mg	6.6	6.2	6.6	5.6
Total DNA, mg	54.7	51.3	61.9	52.6
RNA/DNA	1.6	1.7	1.6	1.9

[1] See footnote 1, table 1.
[2] PF and PF–C–PF significantly less than C–PF and C ($P < 0.05$).

TABLE 5

Effect of protein deprivation of gilts during gestation on weight, dry matter, protein, RNA and DNA content of the cerebellum plus medulla of the young adult progeny

Group no.	1	2	3	4
Gestation diet sequence [1]	PF	PF–C–PF	C–PF	C
No. of animals	10	13	10	9
Fresh wt, g	18.5	17.9	17.8	16.1
Dry matter, % [2]	22.8	22.4	23.0	24.6
Protein in dry matter, %	44.6	45.3	45.2	43.0
RNA per gram protein, mg	9.0	10.6	10.7	10.3
Total RNA, mg	16.7	19.1	19.9	18.2
DNA per gram protein, mg	14.1	15.4	15.9	15.1
Total DNA, mg	26.2	27.4	29.2	25.3
RNA/DNA	0.64	0.69	0.68	0.72

[1] See footnote 1, table 1.
[2] C significantly greater than other three groups ($P < 0.05$).

TABLE 6

Effect of protein deprivation of gilts during gestation on dry matter, protein, RNA and DNA content of the longissimus dorsi muscle of the young adult progeny

Group no.	1	2	3	4
Gestation diet sequence [1]	PF	PF–C–PF	C–PF	C
No. of animals	10	13	10	9
Dry matter, %	29.0	29.5	29.0	29.1
Protein in dry matter, %	80.9	74.7	77.4	79.8
RNA per gram protein, mg	5.3	4.9	4.5	5.3
DNA per gram protein, mg	2.8	2.8	3.0	2.6
RNA/DNA [2]	1.9	1.7	1.5	2.1

[1] See footnote 1, table 1.
[2] C significantly greater than other three groups ($P < 0.05$).

pregnancy. The percentage of dry matter, however, was higher among progeny of control gilts (group 4) than among those of protein-deprived gilts. This difference was significant ($P < 0.05$) for the cerebellum-medulla and approached significance for the cerebrum. The protein concentration, as a percentage of dry matter, was not affected by treatment. DNA, expressed either as milligrams DNA per gram of protein or as total DNA per organ was not significantly affected by treatment. If the timing of the nutritional insult is of importance in producing an effect on subsequent development, maximal effects on a given organ might be expected to coincide with maximal growth of the organ. Davison and Dobbing (14) have compared rates of brain growth and development in several species and report that maximal growth of the brain of pigs occurs earlier than that of rats. This would suggest the importance of prenatal malnutrition on subsequent development in the pig. A reduction in DNA content of rats and pigs fed protein-deficient diets during early postnatal life has been demonstrated (15–17). More recently, Zamenhof et al. (18) showed a reduction in DNA (and, therefore, cell number) and protein content of the cerebrum of newborn progeny of rats fed an 8% protein diet compared with a 27% protein diet before and during pregnancy. Measurements were not made of the subsequent DNA and protein content of the brains from these rats, so it is not known whether this effect noted at birth was a permanent effect that could be demonstrated in later life. The results of the present experiment demonstrate that protein deprivation of the pregnant gilt, though reducing fetal and postnatal growth rate, has no effect on the DNA content (and presumably total cell number (18)) of the brain of the progeny following feeding to young adulthood on an adequate diet. A small number of DNA measurements of brains from stillborn pigs in the present experiment failed to show differences related to prenatal treatment. Further studies are needed in the viable newborn pig to verify this.

The RNA content of the cerebrum, expressed either as RNA per gram of protein or as total RNA, was significantly higher in the progeny of control gilts (group 4) than in the progeny of protein-deprived gilts (groups 1, 2 and 3). Similar trends were observed in the cerebellum plus medulla, although the differences among treatments did not reach statistical significance. These differences are interpreted to indicate reduced protein synthetic activity in the cerebrum of progeny of protein-deprived gilts. The significance of this, in relation to the behavioral changes associated with protein deficiency during early postnatal life in the rat and pig (19, 20), is not known.

The percentage dry matter and percentage protein in the dry matter of the longissimus dorsi of the progeny were not significantly affected by dietary regimen of the dam during gestation. Differences in RNA and DNA concentration (milligrams per gram of protein) did not reach statistical significance, but RNA/DNA was significantly ($P < 0.05$) greater in the control progeny than in progeny of protein-deprived gilts. This suggests the possibility of greater protein synthetic activity in skeletal muscle of control progeny. The total longissimus dorsi was not dissected from the carcass nor was total muscle mass determined. Conventional slaughterhouse carcass measurements of physical parameters, however, revealed no reduction in backfat thickness, carcass length or cross-sectional area of the longissimus dorsi at the 10th rib of progeny of protein-deprived gilts. These physical measurements are highly correlated with chemically determined protein and fat content of animal bodies, and the lack of a detrimental effect of maternal diet on these parameters taken from animals slaughtered at similar body weights supports the lack of differences noted in dry matter and protein concentration of the longissimus dorsi.

Lee and Chow (3) have shown derangements in protein metabolism of progeny of rats restricted in energy during gestation and lactation. No measures were made of nitrogen retention or identity of forms of excreted nitrogen in the present experi-

ment; nor was it feasible to obtain information on total feed consumed or efficiency of utilization of feed during the growing period in the present experiment. It has been suggested (21) that maternal protein restriction is of more importance in producing the depressed weight gain of progeny of rats than restriction in energy, vitamins and minerals. Whether or not there is a common basis for the reduced growth rate of progeny of protein-deprived gilts in the present experiment and that of progeny of protein-energy restricted rats reported by Hsueh et al. (21) remains to be determined.

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Effect of Histidine and Certain Other Amino Acids on the Absorption of Iron-59 by Rats [1]

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ABSTRACT Reports in the literature have indicated that several amino acids can increase the absorption of ferrous iron. The objectives of the experiments reported here were to determine if certain amino acids would increase absorption of ^{59}Fe supplied in the ferric form and, if so, to investigate the mechanism of action of these amino acids. Of six amino acids tested, histidine and lysine significantly increased ^{59}Fe absorption from ligated, in vivo, duodenal segments, whereas glutamine, glutamic acid, methionine, and glycine did not. Further, histidine was not effective if administered intraperitoneally or if put into an intestinal segment adjacent to the one in which ^{59}Fe was administered. When histidine was added to an ^{59}Fe solution which contained ascorbic acid, it increased absorption over and above that observed with ascorbic acid alone. In all of these studies, histidine increased ^{59}Fe uptake only if histidine and ^{59}Fe were administered in the same solution. This suggests some direct reaction between iron and histidine and is consistent with the hypothesis that an amino acid-iron chelate is formed and subsequently absorbed. Since histidine is a product of protein hydrolysis in the gastrointestinal tract, it may be involved in the normal absorption of iron.

The level of protein in the diet significantly affects the absorption of iron (1). The reasons for the depression in iron absorption that accompanies a low protein diet are not known; however, at least a part of this depression may be due to a decreased availability of amino acids when low protein diets are consumed. Circumstantial evidence for this possibility is provided by work showing that several amino acids increase the intestinal absorption of ferrous iron (2, 3).[3]

The mechanisms proposed for the amino acid-induced increase in iron absorption can be divided into three primary categories: 1) Groen et al. (2) suggested that amino acids and certain other organic acids increase iron absorption by buffering the pH of the intestinal contents and delaying the rise in pH of the intestinal contents toward neutrality, where the iron is oxidized and forms insoluble precipitates; 2) Kroe et al. (3) [4] have suggested that the formation of iron-amino acid chelates may increase iron absorption; and 3) Kroe et al. (4) have also presented some evidence that neither the buffering of luminal pH nor chelation mechanisms adequately account for the effect of amino acids on iron absorption, and proposed an amino acid stimulation of iron transport systems.

In the experiments of Kroe et al. (3, 4),[5] iron was provided as ferrous iron; however, under the conditions that prevail in the small intestine (pH 7.0 or higher), ionic ferrous iron is probably oxidized to the ferric form which would be expected to form insoluble ferric hydroxides (solubility product of $Fe(OH)_3$ is approximately 10^{-36}) unless the ferric iron is kept in solution by complexing or chelating agents. The primary objectives of the experiments reported here were to determine the effects of certain amino acids on the absorption of iron supplied in the ferric form and to determine where these amino acids exert their effects.

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[3] Kroe, D., N. Kaufman, T. D. Kinney and J. V. Klavins 1963 The effect of histidine on iron absorption. Federation Proc., 23: 511 (abstract).

[4] See footnote 3.

[5] See footnote 3.

MATERIALS AND METHODS

General. Male rats of the Sprague-Dawley strain were used in these experiments. They were housed in stainless steel cages with raised wire floors and were fed a commercial pelleted ration [6] which was analyzed by atomic absorption [7] and found to contain 200 ppm iron. According to the supplier's label, this diet provides a minimum of 25% protein. Male rats, weighing between 200 and 350 g, were used and were allocated to treatments in a randomized block design on the basis of body weight.

Dosing techniques. Two different procedures for estimating iron absorption were used in these experiments. Some of the experiments utilized an isolated, in situ, segment technique. When this technique was used, the solutions to be tested were injected into an intestinal segment approximately 7 cm long. The preparation and use of these segments has been described previously (5, 6). A second technique involved perfusion of the test solutions through a segment of intestine. In this procedure, an inflow cannula was inserted through the pyloric sphincter, via a small slit in the stomach wall, and secured with a ligature in the proximal end of the duodenum. The outflow cannula was inserted through a small slit in the duodenal wall about 7 cm distal to the pylorus. Solutions were pumped through this duodenal segment with a constant flow pump which delivered 0.28 ml/minute, and the effluent was collected in tubes which were changed every 10 minutes.

In those experiments in which serial blood samples were obtained, they were taken from the tail. Additionally, in those experiments in which other tissues were sampled, a terminal sample of blood was taken by heart puncture, and samples of liver, kidney, and spleen were also taken and counted in a well-type scintillation counter.[8] Blood volume was estimated at 7% of body weight, and this figure was used to estimate the percentage of dose in the blood.

In some of the studies, radioactivity in the carcass was measured. When this was done, rats were killed with an overdose of ether; the segment in which the ^{59}Fe had been administered was removed, and the remainder of the carcass was counted in a small animal whole-body counter.[9] A rat, in which an identical dose of radioactive iron had been placed in a ligated segment, was killed at the end of 30 minutes and counted intact to serve as a standard.

Solutions. The ^{59}Fe was obtained as ferric chloride [10] and had an initial specific activity of 8.97 mCi/mg. The ^{59}Fe and amino acids were administered in a modified Krebs-Ringer phosphate buffer solution. In the first three experiments reported in this paper, the buffer solution contained only one-third of the amounts of calcium and magnesium used in the original preparations. The amounts of calcium and magnesium were lowered to eliminate the precipitation of calcium and magnesium phosphates. Hopping and Ruliffson (7) reported that omission of calcium and magnesium decreased precipitation problems and increased iron absorption, therefore, these elements were deleted from the solutions used in experiment 4. In all cases, buffer solutions were adjusted to pH 7.0; ^{59}Fe was then added and the pH was readjusted to 7.0.

The solutions administered to the rats in the ligated segment experiments provided each rat with 0.4 μg iron in 0.5 ml. When amino acids were administered along with the iron, they were present in the solutions in concentrations of 0.1 M. In experiments where ^{59}Fe solutions were perfused through an intestinal segment, the iron concentration was 1 μg/ml and histidine was 0.1 M. In experiments in which ascorbic acid was administered, it was present in the dosing solutions at a concentration of 1.0 μmole/ml.

When ^{59}Fe was administered by syringe, the standard was also prepared with a syringe, i.e., 0.5 ml of the ^{59}Fe solution was drawn into a syringe and then ejected into

[6] Big Red Dog Chow, Agway, Inc., Syracuse, N. Y. (Trade names and company names are included for the benefit of the reader and do not imply any endorsement or preferential treatment of the product listed by the U. S. Department of Agriculture.)

[7] Model 303 atomic absorption spectrophotometer, Perkin-Elmer Corporation, Norwalk, Conn.

[8] Model 4218, automatic gamma counting system, Nuclear-Chicago Corporation, Des Plaines, Ill.

[9] Model 4350, large sample gamma counting system, Nuclear-Chicago Corporation.

[10] Union Carbide Corporation, Tuxedo, N. Y.

a counting vial. A second 0.5 ml was then drawn into the syringe and given to the rat. Thus, a counting standard was prepared for each rat, and the standard aliquot was taken with the same syringe used for dosing the rat.

Statistical analyses. Results of all experiments were subjected to an analysis of variance and individual means were compared by a multiple range test (8). Statements of significance in this paper are based on odds of at least 19 to 1.

RESULTS

Experiment 1. The rats in these experiments were dosed with ^{59}Fe either in a buffer alone or in a buffer solution containing one of the six different amino acids that were tested. The iron and amino acids were both injected into a duodenal segment that was ligated at the pyloric sphincter and at a site about 7 cm distal to the pylorous. At the end of 3 hours, the rats were killed and blood and tissue samples were taken for counting. The uptake of ^{59}Fe by the selected tissues and the disappearance of ^{59}Fe from the ligated intestinal segment are presented in table 1. Using either of these parameters as an index of absorption, the only amino acids that increased ^{59}Fe uptake were lysine and histidine. When lysine was administered, it increased ^{59}Fe absorption about two and one-half times over that of the controls. If histidine was administered along with the ^{59}Fe, absorption was four to five times greater than that in the controls. The other amino acids produced only small, nonsignificant, changes in ^{59}Fe uptake. These results are not in complete agreement with some reports in the literature, and several possible reasons for this will be considered in the discussion.

TABLE 1

Effect of amino acids on the absorption of ^{59}Fe from ligated duodenal segments

Amino acid administered	^{59}Fe in sampled tissues [1,2]	Disappearance of ^{59}Fe from segment
	% of dose	%
None	7.5 [a 3,4]	15.8 [a]
Histidine	35.6 [b]	84.1 [b]
Lysine	19.9 [c]	56.5 [c]
Methionine	9.6 [a]	19.9 [a]
Glutamic acid	8.9 [a]	23.5 [a]
Glutamine	8.9 [a]	22.5 [a]
Glycine	8.1 [a]	29.0 [a]

[1] Percentage of administered dose recovered in blood, kidneys, liver, and spleen.
[2] Each value is the mean of seven observations.
[3] Values in any column that are not followed by the same letter are significantly different.
[4] Experimental period was 3 hours.

Experiment 2. In this series of experiments, two adjacent intestinal segments were ligated and various combinations of control and histidine solution were administered. One segment of each pair began at a point 14 to 15 cm distal to the pylorus and was about 7.0 cm long. The second segment was located immediately distal to the first and was also about 7.0 cm long. Iron-59 absorption from jejunal segments is slower than from duodenal segments; however, they were used in this experiment because of the desirability of having two segments that were as nearly alike as possible. The treatment combinations were designed so that half of the segments containing the control ^{59}Fe solution were paired with segments that contained a control buffer solution; the other half were paired with segments that contained histidine. Similarly, half of the histidine-^{59}Fe-containing segments were paired with a segment containing buffer alone, and the other half were paired with segments containing histidine. The results of these experiments are given in tables 2 and 3.

In table 2, the disappearance of ^{59}Fe, administered either in buffer alone or along with histidine, is presented. When histidine-^{59}Fe was administered and histidine was put into the adjacent segment,

TABLE 2

Absorption of ^{59}Fe from ligated intestinal segments. Effects of histidine on disappearance of ^{59}Fe from either the same or from an adjacent segment.

^{59}Fe-solution administered	Solution in adjacent segment	Disappearance of ^{59}Fe from segment [1]
		%
Histidine-^{59}Fe	Histidine	45.5 [a 2,3]
Histidine-^{59}Fe	Control	49.4 [a]
Control-^{59}Fe	Histidine	1.7 [b]
Control-^{59}Fe	Control	3.7 [b]

[1] Each value is the mean of 14 observations.
[2] Values not followed by the same letter are significantly different.
[3] Experimental period was 3 hours.

45.5% of the dose disappeared from the segment. When control solution with no histidine was put into the adjacent segment, an average 49.4% of the dose left the segment. Comparable values for uptake in those rats dosed with control-^{59}Fe solutions were 1.7% when histidine was put into the adjacent segment and 3.7% when no histidine was put into the adjacent segment. It is clear that the uptake of ^{59}Fe solutions from either histidine-^{59}Fe solutions or control-^{59}Fe solutions was not affected by putting histidine into an adjacent segment. Additional support for this conclusion is provided by the data in table 3. In this table, the ^{59}Fe content of the carcasses, exclusive of the intestinal segments, is presented. Rats having histidine-^{59}Fe solution put into both segments had 52% of the ^{59}Fe in the carcass at the end of 3 hours. Comparable figures for those rats that had control-^{59}Fe solution in both segments were 1.6%, and carcass uptake in rats that had one control segment and one histidine segment averaged 29.6%. It is clear from the data of tables 2 and 3 that histidine had an effect when it was given along with the ^{59}Fe, but had no effect when administered in an adjacent intestinal segment.

Experiment 3. This study was designed, as was experiment 2, to determine the effect of remotely administered histidine on ^{59}Fe absorption. The ^{59}Fe-containing solutions were perfused through a duodenal segment, and histidine was administered in two different ways. In some rats the histidine was included in the ^{59}Fe solution that was perfused through the intestinal segment. In others, the histidine was administered by intraperitoneal injection. Blood samples were taken from the tail at predetermined intervals and counted. The ^{59}Fe levels in the blood are presented in figure 1. It is evident from this figure that histidine did not increase ^{59}Fe uptake when given intraperitoneally and, as in experiment 2, was effective only when mixed with the ^{59}Fe prior to administration.

The effluent solutions that had been perfused through the segment were collected at 10-minute intervals and these fractions were counted. They were quite variable, however, and with the small number of animals involved (six rats per treatment), they were of little value as indicators of absorption. Large amounts of radioiron from the control solutions, however, were apparently adsorbed to the intestinal wall. When histidine-^{59}Fe solutions were administered, less than 1% of the total ^{59}Fe that had been pumped through the segment had adhered to the segment. When the control-^{59}Fe solution was used, however, 20 to 25% of the ^{59}Fe that had been pumped through was adhered to the intestinal segment. It was apparent from blood and

TABLE 3

Carcass uptake of ^{59}Fe from ligated segments. Effect of histidine when administered in the same segment as the ^{59}Fe and when administered in an adjacent segment.

No. of rats	Treatment		^{59}Fe in carcass [1]
	Segment 1	Segment 2	
			% of dose
7	Histidine-^{59}Fe	Histidine-^{59}Fe	52.0 [a 2,3]
7	Histidine-^{59}Fe	Control-^{59}Fe	29.6 [b]
7	Control-^{59}Fe	Histidine-^{59}Fe	
7	Control-^{59}Fe	Control-^{59}Fe	1.6 [c]

[1] Exclusive of ligated segments.
[2] Values not followed by the same letter are significantly different.
[3] Experimental period was 3 hours.

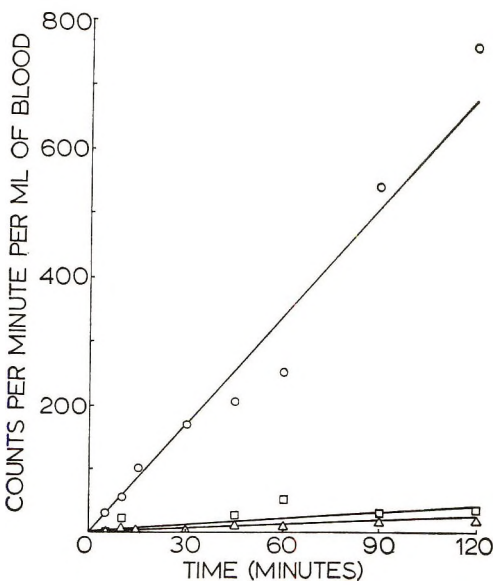

Fig. 1 Effect of intraperitoneally and intraduodenally administered histidine on blood levels of ^{59}Fe. △———△, control, no histidine; □——□, histidine administered intraperitoneally; ○——○, histidine administered intraduodenally.

tissue levels that very little of this ^{59}Fe from the control solutions was actually absorbed and transported across the wall of the gut. The 20 to 25% of the dose that had adhered to the intestinal segment probably was adsorbed to the mucosal surface, or if it had actually entered the mucosal cells, it either precipitated or was bound in some nonabsorbable form.

Experiment 4. Ferric iron is only slightly soluble at the pH normally encountered in the intestine (7.0 to 8.0) and apparently forms high molecular weight nonabsorbable aggregates under these conditions (9). Thus, the histidine-induced increase in ferric iron absorption from intestinal segments could be due to the prevention of aggregate formation by histidine. Since ferrous iron does not aggregate under these conditions (9) and is much more soluble at pH 7.0 than ferric iron, these experiments were conducted to see what effect ascorbic acid would have on absorption of ^{59}Fe from ligated duodenal segments and, further, to see if histidine would have an effect that was additive to that of ascorbic acid. The results of these experiments are presented in figure 2 and in table 4.

Figure 2 illustrates the blood levels of ^{59}Fe at various intervals after dosing. Ascorbic acid significantly increased the blood levels of ^{59}Fe over those of the controls; however, a much larger increase was observed in those animals that received a solution containing both histidine and ascorbic acid along with the ^{59}Fe. Histidine given intraperitoneally did not increase iron uptake. The data in table 4 are in good agreement with figure 2, and it can be seen that the addition of ascorbic acid gave an 8- to 10-fold increase in ^{59}Fe uptake. When both histidine and ascorbic acid were given intraduodenally, however, absorption was almost double that observed when ascorbic acid alone was given.

DISCUSSION

Previous reports indicate that some nine amino acids, including histidine, glutamine, glutamic acid, and methione, improved the uptake of iron from ligated, in vivo, intestinal segments (3). In contrast, only histidine and lysine were effective in

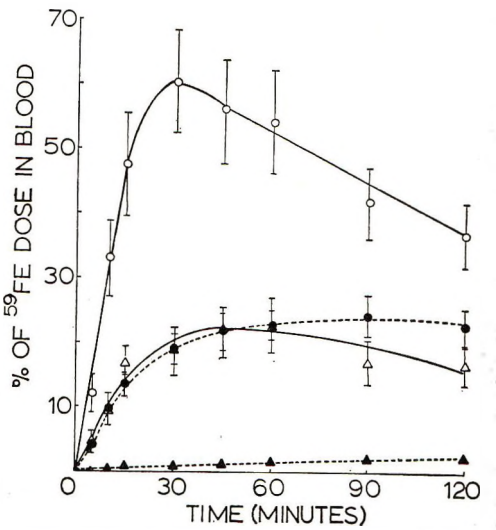

Fig. 2 Effect of ascorbic acid and histidine on blood uptake of ^{59}Fe from ligated duodenal segments. ▲-----▲, control, no ascorbic acid, no histidine; ●-----●, ascorbic acid given intraduodenally, no histidine; △——△, ascorbic acid given intraduodenally, histidine given intraperitoneally; ○——○, ascorbic acid and histidine both given intraduodenally.

TABLE 4

Effect of ascorbic acid and histidine on the absorption of ^{59}Fe from ligated duodenal segments

Treatment		^{59}Fe in sampled tissues [1,2]	Disappearance of ^{59}Fe from segment
Intraduodenal	Intraperitoneal		
		% of dose	*%*
Control	Control	2.5 [a 3,4]	6.0 [a]
Ascorbic acid	Control	26.3 [b]	42.6 [b]
Ascorbic acid	Histidine	21.1 [b]	43.7 [b]
Ascorbic acid + histidine	Control	46.1 [c]	73.1 [c]

[1] Percentage of the administered dose recovered in blood, kidneys, liver, and spleen.
[2] Each value is the mean of at least eight observations.
[3] Values in any column that are not followed by the same letter are significantly different.
[4] Experimental period was 2 hours.

the studies reported here. The exact reasons for this discrepancy are not known; however, there are several differences in experimental materials and procedures that could explain some of the differences in results.

Kroe et al. (3, 4) [11] administered ferrous iron in their experiments, whereas we used ferric iron. A number of amino acids, including several of those used in these studies, will bind ferrous ions fairly readily. Histidine, apparently, binds ferrous iron more strongly than any of some 20 amino acids tested by Albert (10, 11). Measurement of the avidity of these same amino acids for ferric ion is difficult, if not impossible, because of the tendency of ferric salts to precipitate at pH 3.0 or above; thus, data on stability constants for ferric-amino acid complexes are not readily available. Some indirect evidence for the strength of the histidine-ferric complex, however, is provided by a report that histidine is more effective than any other amino acid in speeding the movement of ferric iron along a paper chromatogram running in phenol (12). On the basis of this report and Albert's results with ferrous iron, it seems likely that histidine chelates ferric iron more effectively than any of the other amino acids tested in these experiments. Thus, while many amino acids could effectively chelate ferrous iron and perhaps increase its absorption, only an amino acid that could prevent precipitation of ferric iron at pH 3.0 and above would be expected to have any effect on ferric iron. Since histidine apparently is the strongest chelator of ferric iron, it should be the most effective of the amino acids tested in preventing precipitation or formation of suspended aggregates of ferric iron. Hence, differences in the reactions of ferrous and ferric ions with amino acids may explain some of the differences between our results and the previous ones.

In our experiments, the pH of the buffer solution was adjusted to 7.0 before adding the ^{59}Fe. According to Jacobs (9), ferric ions condense at increasing pH to form complex ions having molecular weights greater than 5000. Furthermore, Conrad and Schade (13) have demonstrated that the sequence in which additions and pH adjustments are made to iron solutions is important. Addition of ascorbic acid to a ferric iron solution before raising the pH above 3.0 prevented precipitation of the iron; however, when the pH was raised until the iron precipitated before ascorbic acid was added, the iron did not go back into solution. In the experiments reported here, iron was added to the buffer solutions after the pH had been adjusted to 7.0; thus, a relatively strong chelating agent would be required to prevent precipitation. Some of the amino acids that were tested may chelate ferric ion too weakly to be effective under these conditions.

Groen et al. (2) suggested that a number of compounds increase iron absorption by increasing the length of time that the gastrointestinal contents remain acidic after entering the small intestine. That would not explain the increased absorption seen with histidine and lysine in these experiments, since the pH of the solutions was adjusted to 7.0 prior to administration. In the experiments where the terminal pH of the contents of ligated segments was determined,[12] values were generally between 7.0 and 8.0, and there was no significant difference between contents of control rats and histidine-treated rats.

Kroe et al. (4) concluded that chelation alone did not explain the influence of histidine and glutamine on iron absorption, and suggested that their effects were due in part to "amino acid stimulation of specific or nonspecific pathways of intestinal absorption." If this is true, administration of histidine or glutamine at sites remote from the one in which iron is administered conceivably could also be stimulatory and increase iron uptake. The experiments in which histidine was administered, either in an adjacent segment or intraperitoneally, were designed to test this possibility. Remotely administered histidine was not effective, however, and, in these studies, histidine increased iron absorption only when administered in the same solution as the ^{59}Fe.

The observation that ^{59}Fe and histidine must be administered in the same solution

[11] See footnote 3.

[12] Van Campen, D. R., and E. Gross, unpublished observations.

if histidine is to have any effect suggests a direct reaction between the two, i.e., formation of a histidine-iron chelate; or, possibly, histidine prevents formation of either iron precipitates or impermeable but suspended aggregates. The last experiment reported in this paper was an attempt to discriminate between these two general mechanisms. Ferrous iron does not form aggregates at pH 7.0 (9) and is much more soluble than ferric iron at this pH (solubility products are approximately 10^{-36} for $Fe(OH)_3$ and 1.64×10^{-14} for $Fe(OH)_2$). Thus, any histidine effect on the absorption of ferrous iron presumably would not be due to prevention of precipitate or aggregate formation. The last experiment reported here tested the effects of intraduodenally and intraperitoneally administered histidine on the absorption of iron from an ^{59}Fe solution that contained ascorbic acid. Intraperitoneal histidine had no effect on iron uptake, but when histidine was administered along with the ^{59}Fe, it almost doubled the uptake observed when ascorbic acid alone was administered. The interpretation of these results is made more difficult by reports that ascorbic acid acts not only as a reducing agent but also as an iron chelating agent (13, 14). They do indicate, however, that histidine increased iron uptake in a system where low solubility was not considered to be a factor.

The results of the experiments reported here are consistent with the hypothesis that the histidine-induced increase in ferric-^{59}Fe absorption is due to formation of iron-histidine chelates. This general mechanism fits in well with the proposal of Helbock and Saltman (15) that low molecular weight chelates are necessary to maintain iron in a soluble and permeable form. Histidine, as a product of protein hydrolysis in the intestine, may well play such a role under normal conditions, and may also have some potential for increasing iron absorption when this is desirable.

ACKNOWLEDGMENTS

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Effect of Excess Amino Acids on the Utilization of the First Limiting Amino Acid in Chick Diets

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ABSTRACT A crystalline amino acid reference standard diet (RS) was modified in various ways to examine the effect of excess amino acids on the utilization of the first limiting amino acid. When a basal diet containing 60% of the amino acids in the RS was supplemented with graded amounts of an amino acid mixture (RS minus lysine), neither weight gain nor efficiency of lysine utilization was adversely affected. In contrast, supplementing a diet containing 50% of the amino acids in the RS with graded amounts of an amino acid mixture (RS minus isoleucine) progressively depressed weight gain and food intake. Nevertheless, gain was commensurate with isoleucine intake. Chicks fed a low nitrogen diet (60% RS with a void of isoleucine) consumed more feed and grew much better than chicks fed a high nitrogen diet (RS minus isoleucine) at comparable concentrations of isoleucine at or below 0.4%. Efficiency of isoleucine utilization, as measured by the slope-ratio technique over the linear part of the response curves, was identical in both series. An assay of the same type involving leucine yielded comparable results. Efficiency of isoleucine utilization was found to be identical whether growth was depressed by an imbalanced mixture of amino acids or by an anorexic agent (*Quillaja* saponin). In all instances reported here, gain in weight was a function of the absolute intake of the first limiting amino acid.

Inherent in the formulation of diets suitable for determining the availability of amino acids in proteins is the presence of excess amino acids not needed to maximize gain. It has been suggested that amino acids in excess of the amount required by the animal would drain the pool of the most limiting amino acid, and thus, adversely affect its utilization (1–4). It has been shown by Fisher et al. (5), however, that while supplementing a lysine-deficient purified diet (sesame meal-glucose) with excess nitrogen from individual amino acids or combinations of amino acids depressed weight gain by virtue of accentuating the lysine deficiency, the imbalance did not impair the utilization of the first limiting amino acid (lysine) as measured by gain per unit of lysine consumed. Subsequently, it was shown by Huston,[1] using the slope-ratio technique, that the utilization of leucine, arginine, isoleucine or threonine as well as lysine was not impaired by the excess amino acids present in crystalline amino acid diets formulated to be deficient to varying degrees for each of the aforementioned amino acids. This was true whether the diets contained the pattern of amino acids represented in the reference standard (RS) or 60% of this standard (table 1). Moreover, it has recently been demonstrated by Sugahara et al. (6) that the pattern of amino acids, in excess of that required to maximize weight gain in the chick, will dictate whether the adverse effect of excess amino acids on gain will be superimposed on that of an amino acid deficiency.

The present study was undertaken to ascertain whether the excess amino acids in the assay diet modified the utilization of the first limiting amino acid. Two approaches were used for this purpose. In the first, the concentration of the most limiting amino acid was held constant while the amount of the excess amino acids was varied without changing the pattern of amino acids therein. The second approach indirectly reduced the concentration of the excess amino acids by supplementing the deficient diet with increments of the first limiting amino acid.

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[1] Huston, R. L. 1967 Amino acid imbalance studies in the chick using a crystalline amino acid diet: Effect of dietary amino acid interactions on growth and pattern of amino acids in blood plasma and other tissues. Doctoral thesis, University of Illinois, Urbana.

TABLE 1

Pattern of crystalline amino acids in reference standard mixture (RS) expressed as a percentage of the diet

Amino acid	%
L-Arginine·HCl	1.21
L-Histidine·HCl·H_2O	0.41
L-Lysine·HCl	1.19
L-Tyrosine	0.45
L-Tryptophan	0.15
L-Phenylalanine	0.50
DL-Methionine	0.35
L-Cystine	0.35
L-Threonine	0.65
L-Leucine	1.20
L-Isoleucine	0.60
L-Valine	0.82
Glycine	1.20
L-Proline	0.20
L-Glutamic acid	10.00
Total	19.28

EXPERIMENTAL PROCEDURE

Progeny from a mating of New Hampshire males and Columbian females were used in all assays. Information concerning the duration of assays, number and sex of chicks involved is indicated in tabular footnotes. The care of chicks during the pretest period and their selection and assignment to treatment groups has been previously described (7). The pattern of crystalline amino acids in the reference standard is given in table 1 and details concerning the composition of the remaining parts of the basal diet can be found elsewhere (8).

The availability of the amino acid under study was estimated by using the slope-ratio technique for all but experiment 1. The regression coefficient was calculated using average gain per chick per day, and the absolute intake of the limiting amino acid as the independent variables. The intake of the first limiting amino acid was used as the variable rather than the dietary concentration of the limiting amino acid since it has been shown that the former is more highly correlated with gain than the latter (9, 10). As Griminger et al. (9) point out, when the amino acid requirement is expressed as a percentage of the diet, a factor which translates the requirement into absolute amounts, namely food intake, is disregarded.

The basal diet used in experiment 1 contained 60% of the amino acids present in the reference standard (RS) (table 1). This diet was supplemented with increments of an amino acid mixture identical to the pattern in the RS except that lysine was omitted. Thus, the L-lysine·HCl content of all diets was 0.714%.

The design of experiment 2 was essentially the same as experiment 1, except that isoleucine was the limiting amino acid and the basal diet contained 50% of the amino acids in the RS. Since the imbalancing amino acid mixture (RS minus isoleucine) was devoid of isoleucine all diets contained 0.3% of this amino acid. Supplementing the basal diet with 50% of the imbalancing mixture would increase the concentration of all amino acids, except for isoleucine, to that of the RS.

In experiment 3, the basal diets contained the amino acids represented in the RS and 60% RS except for a complete void of isoleucine. Both the high nitrogen and low nitrogen basal diets were supplemented with graded amounts of isoleucine. Experiment 4 was identical to 3 in design except that leucine was the variable amino acid rather than isoleucine.

In experiment 5, the basal diet containing 50% of the amino acids in the RS was supplemented with either graded amounts of the imbalancing mixture (RS minus isoleucine) as in experiment 2, or graded levels of *Quillaja* saponin which is known to be an anorexic agent.

In experiment 6, the effect of the imbalanced mixture of amino acids (RS minus isoleucine) in the absence and presence of *Quillaja* saponin on growth and isoleucine utilization was compared.

RESULTS AND DISCUSSION

Only minor differences in gain resulted when the crystalline amino acid diet (60% RS) was supplemented with graded amounts of a mixture of amino acids from which lysine had been excluded. Nor were there significant differences in lysine utilization as measured by gain per unit of lysine consumed (table 2), even though diet 5 contained a high concentration (7.2%) of the "imbalanced" amino acid mixture. In contrast, weight gain progressively declined, as did feed consump-

tion, when the basal diet, containing 0.3% isoleucine, was supplemented with graded amounts of an imbalanced amino acid mixture lacking isoleucine (table 3). Nevertheless, despite large differences in gain that resulted from the imbalance, regression analysis indicated that isoleucine utilization was not impaired. Clearly, the marked variation in gain could be accounted for by the differences in isoleucine consumption. It was the contrasting results noted in these two assays that led Sugahara et al. (6) to examine in greater detail the effect of various "imbalanced" amino acid mixtures on chick growth.

In experiment 3, the amount of excess amino acids in the diets was altered by varying the concentration of isoleucine from zero to the requirement level and above, for both RS and 60% RS basal diets. Under these conditions each increment of isoleucine, below the requirement level, would have the effect of reducing the concentration of amino acids not needed for growth. Gain in weight was maximized at a concentration of 0.6% isoleucine in the high nitrogen diet and 0.4% in the low nitrogen diet (table 4). Chicks fed the low nitrogen diet consumed more feed than those fed the high nitrogen diet at each corresponding level of isoleucine in the first four treatments. This is reflected in the superior gain of chicks fed the low nitrogen diet. The difference was greatest at the 0.2% level of isoleucine where gain on the low nitrogen diet was eight times that of chicks fed the high nitrogen diet, but this difference decreased rapidly as the concentration of isoleucine in the latter diet approached the requirement level of the chick. Because 0.2% isoleucine represents 33 and 50% of the requirement of the chick for the high and low nitrogen diets, respectively, it follows that the former con-

TABLE 2

Effect of an imbalanced mixture of amino acids on growth and lysine utilization (exp. 1)

Modification of amino acid mixture in basal diet [1]	Gain per chick [2]	Lysine consumed per chick	LER [3]
	g	*g*	
None	31.5	0.443	71.0
Basal + 10% amino acid mix [4]	34.4	0.458	75.2
Basal + 20% amino acid mix	35.6	0.453	78.1
Basal + 30% amino acid mix	31.7	0.420	75.4
Basal + 40% amino acid mix	28.7	0.395	72.7

[1] Basal diet contained 60% of the amino acid mixture represented in the reference standard (RS).
[2] Average for quadruplicate groups of 10 male chicks for the period 9 to 13 days.
[3] Lysine efficiency ratio (gain per gram of lysine consumed).
[4] Expressed as a percentage of the reference standard mixture and having the same pattern of amino acids as in the RS minus lysine.

TABLE 3

Effect of an imbalanced mixture of amino acids on growth and isoleucine utilization (exp. 2)

Modification of amino acid mixture in basal diet [1]	Gain per chick per day (y) [2]	Feed consumed per chick	Isoleucine consumed per chick (x)
	g	*g*	*mg*
None	6.97	89.6	269
Basal + 10% amino acid mix [3]	5.74	83.4	250
Basal + 20% amino acid mix	4.40	73.7	221
Basal + 30% amino acid mix	3.78	65.6	197
Basal + 40% amino acid mix	2.83	56.9	171
Basal + 50% amino acid mix	2.51	52.6	158
		$y = -3.84 + 0.0389\ x$ [4]	

[1] Basal diet contained 50% of the amino acid mixture represented in the reference standard (RS).
[2] Average for quadruplicate groups of 10 female chicks for the period 8 to 14 days.
[3] Expressed as a percentage of the reference standard mixture and having the same pattern of amino acids as in the RS minus isoleucine.
[4] Regression equation.

tained a greater amount of excess amino acids than the latter. Although the differences in gain were large, regression equations calculated over the linear part of the response curve would indicate that efficiency of isoleucine utilization was not altered by varying the concentration of excess amino acids. Moreover, the coefficients for the results obtained with the high and low nitrogen diets do not differ appreciably from one another.

While unrelated to the main objective of the present study, it was of more than passing interest to note that maximum gain, gain-to-feed ratio and efficiency of protein utilization (PER) were reached at the same concentration of isoleucine (0.6%) in the high nitrogen diet. Although gain in weight continued to increase beyond the 0.4% level of isoleucine, gain per unit of isoleucine consumed tended to decrease beyond this concentration. Results comparable to those noted above were observed when chicks were fed the low nitrogen diet also (table 4).

In experiment 4 (table 5), where leucine rather than isoleucine was varied in the high and low nitrogen diets, chicks fed the low nitrogen diet consumed more feed and grew better than those fed the high nitrogen diet, when compared at comparable concentrations of dietary leucine. Again, although differences in feed intake and growth were large, gain in weight was proportional to the absolute intake of leucine throughout the linear part of the response curve. Weight gain on the reference standard plateaued at 1.0% leucine, but it had been shown in prior assays [2] that the leucine content of the reference standard could be lowered from 1.2% to 1.0% without adversely affecting growth or efficiency of diet utilization. Again, as in the isoleucine assay (exp. 3), maximum gain, efficiency of feed and protein utilization were achieved at the same concentration of leucine (1.0% in the high nitrogen and 0.6% in the low nitrogen diet). Likewise, the gain per unit of leucine consumed was greater at the preceding level of leucine.

In those instances where the mixture of excess amino acids has impaired weight gain, there is a commensurate decrease in voluntary feed consumption, and hence,

[2] Scott, H. M., unpublished observations.

TABLE 4

Response of chicks to increments of isoleucine at two levels of dietary nitrogen (exp. 3)

L-Isoleucine	Gain per chick per day (y) [1]	Feed consumed per chick	Gain/feed	PER [2]	Isoleucine consumed per chick (x)	IER [3]
%	g	g			mg	
			High nitrogen diet (RS)			
None	−1.55 [4]	21.9	−0.43	−3.01	0 [4]	—
0.1	−0.78 [4]	27.5	−0.18	−1.19	28 [4]	−0.167
0.2	0.28 [4]	35.6	0.05	0.33	71 [4]	0.023
0.3	2.46 [4]	53.2	0.28	1.94	160 [4]	0.092
0.4	5.91 [4]	74.6	0.48	3.30	298 [4]	0.119
0.5	9.75 [4]	98.2	0.60	4.12	491 [4]	0.119
0.6	11.79	106.6	0.66	4.57	640	0.111
0.7	11.74	105.8	0.67	4.56	741	0.095
0.8	11.16	101.7	0.66	4.49	814	0.082
			Low nitrogen diet (60% RS)			
None	−1.47 [5]	25.0	−0.35	−4.32	0 [5]	—
0.1	−0.29 [5]	34.5	−0.05	−0.62	34 [5]	−0.050
0.2	2.26 [5]	57.4	0.23	2.85	115 [5]	0.112
0.3	6.35 [5]	92.4	0.41	4.94	277 [5]	0.138
0.4	7.75	100.9	0.46	5.48	404	0.115
0.5	7.42	97.0	0.46	5.41	485	0.092
0.6	7.38	98.4	0.45	5.26	590	0.075

[1] Average for duplicate groups of 10 female chicks for the period 8 to 14 days.
[2] Gain per gram of protein consumed.
[3] Gain per milligram of isoleucine consumed.
[4] Values used in calculating the regression equation $y = -1.374 + 0.023\ x$.
[5] Values used in calculating the regression equation $y = -1.265 + 0.028\ x$.

a decrease in the absolute intake of the first limiting amino acid. Although this accentuates the deficiency, the utilization of the first limiting amino acid is not altered. In experiment 5, weight gain and voluntary feed consumption were depressed by adding graded amounts of *Quillaja* saponin to the basal diet (50% RS) in one series and increments of an imbalancing mixture of amino acids (RS minus isoleucine) in a second series, thus affording a direct comparison between two means of depressing weight gain. While the results recorded in table 6 show that gain and feed intake were sharply reduced in both series, isoleucine utilization was not impaired since the chicks grew in proportion to the amount of isoleucine consumed. In experiment 6 (table 7), the severity of the growth depression induced by an imbalancing mixture of amino acids was accentuated in one series by adding 0.6% *Quillaja* saponin to each of the diets. Despite large differences in gain and feed consumption, regression analysis, using the slope-ratio technique, again illustrates that the chicks grew in proportion to the isoleucine consumed.

In examining the relationship between chick growth and requirement for lysine, Griminger and Scott (11) modified weight gain by supplementing a sesame meal-glucose diet with *Quillaja* saponin. We have applied the slope-ratio technique of analysis to their data and find that the coefficients are identical for the series supplemented with saponin and the nonsupplemented series. When the data of Smith and Scott (12) are subjected to the same type of analysis, it is clear that while the imbalancing mixture of amino acids simulating fishmeal depressed gain, the utilization of the first limiting amino acid (isoleucine) was not impaired.

In experiment 1 of the present study the amino acid efficiency ratio (AER) was used to measure utilization of the first limiting amino acid (lysine). The use of AER for this purpose should be restricted to those treatments where gain in weight does not differ greatly between treatment groups, as was the case in experiment 1. When, how-

TABLE 5

Response of chicks to increments of leucine at two levels of dietary nitrogen (exp. 4)

L-Leucine	Gain per chick per day (y) [1]	Feed consumed per chick	Gain/feed	PER [2]	Leucine consumed per chick (x)	LER [3]
%	g	g			mg	
			High nitrogen diet (RS)			
None	−0.86 [4]	21.8	−0.24	−1.72	0 [4]	—
0.1	−0.40 [4]	23.9	−0.10	−0.73	24 [4]	−0.100
0.2	0.02 [4]	28.0	0.00	0.03	56 [4]	0.002
0.3	0.38 [4]	33.2	0.07	0.50	99 [4]	0.023
0.4	1.36 [4]	39.8	0.21	1.46	159 [4]	0.051
0.6	4.63 [4]	62.5	0.43	3.14	375 [4]	0.074
0.8	9.99 [4]	96.6	0.62	4.35	772 [4]	0.078
1.0	11.50	107.7	0.64	4.45	1077	0.064
1.2	11.50	105.1	0.66	4.52	1261	0.055
1.4	11.38	104.9	0.65	4.44	1469	0.046
			Low nitrogen diet (60% RS)			
None	−0.69 [5]	27.2	−0.15	−1.88	0 [5]	—
0.1	−0.35 [5]	31.2	−0.07	−0.82	31 [5]	−0.068
0.2	0.51 [5]	39.0	0.08	0.95	78 [5]	0.039
0.3	1.67 [5]	51.0	0.20	2.36	153 [5]	0.065
0.4	4.07 [5]	70.2	0.35	4.15	281 [5]	0.087
0.5	7.93	102.4	0.47	5.50	512	0.093
0.6	8.23	109.0	0.45	5.32	654	0.076

[1] Average for duplicate groups of 10 female chicks for the period 8 to 14 days.
[2] Gain per gram of protein consumed.
[3] Gain per milligram of leucine consumed.
[4] Values used in calculating the regression equation $y = -0.79 + 0.014\ x$.
[5] Values used in calculating the regression equation $y = -0.81 + 0.017\ x$.

TABLE 6

Growth depressing effect of either an imbalanced amino acid mixture or Quillaja saponin on the utilization of isoleucine (exp. 5)

Modification of amino acid mixture in basal diet [1]	Gain per chick per day (y) [2]	Feed consumed per chick	Isoleucine consumed per chick (x)
	g	*g*	*mg*
None	6.24	89.9	270
Basal + 10% amino acid mix [3]	5.76	80.4	241
Basal + 20% amino acid mix	3.63	64.2	193
Basal + 30% amino acid mix	1.80	51.0	153
Basal + 40% amino acid mix	1.58	48.3	145
Basal + 50% amino acid mix	0.88	41.2	124
	$y = -4.03 + 0.039\,x$ [4]		
+ 0.2% *Quillaja* saponin	6.10	83.8	251
+ 0.4% *Quillaja* saponin	4.74	71.3	214
+ 0.6% *Quillaja* saponin	3.48	60.8	182
+ 0.8% *Quillaja* saponin	2.29	51.6	155
+ 1.0% *Quillaja* saponin	1.44	43.5	131
	$y = -3.72 + 0.039\,x$ [4]		

[1] See table 3, footnote 1.
[2] Average for duplicate groups of 10 male chicks for the period 9 to 14 days.
[3] See table 3, footnote 3.
[4] Regression equation.

TABLE 7

Growth and isoleucine utilization of chicks fed diets supplemented with graded amounts of an imbalanced mixture of amino acids in the absence and presence of Quillaja saponin (exp. 6)

Modification of amino acid mixture in basal diet [1]	Supplemental *Quillaja* saponin: None			Supplemental *Quillaja* saponin: 0.6%		
	Gain per chick per day (y) [2]	Feed consumed per chick	Isoleucine consumed per chick (x)	Gain per chick per day (y) [2]	Feed consumed per chick	Isoleucine consumed per chick (x)
	g	*g*	*mg*	*g*	*g*	*mg*
None	6.90	93.2	280	—	—	—
Basal + 10% amino acid mix [3]	6.37	86.6	260	3.88	62.3	187
Basal + 20% amino acid mix	5.48	76.7	230	2.80	50.3	151
Basal + 30% amino acid mix	3.70	62.8	188	2.33	45.1	135
Basal + 40% amino acid mix	3.58	59.0	177	2.42	44.7	134
Basal + 50% amino acid mix	2.35	48.9	147	1.58	38.0	114
	$y = -2.61 + 0.034\,x$ [4]			$y = -1.85 + 0.031\,x$ [4]		

[1] See table 3, footnote 1.
[2] Average for duplicate groups of 10 male chicks for the period 8 to 14 days.
[3] See table 3, footnote 3.
[4] Regression equation.

ever, large differences in response are induced, as was true in experiments 2 through 6, the use of AER could lead to serious errors in estimating the efficiency with which the first limiting amino acid was being utilized. AER does not take into consideration the maintenance requirement of the chick for the amino acid under study. Regression analysis is not subject to this criticism.

It would appear that excess amino acids, with a variety of patterns and concentrations, do not impair the utilization of the first limiting amino acid even though their presence in the diet depresses feed intake, and hence, weight gain. While short-term isotope studies have demonstrated an increased incorporation of the limiting amino acid in liver protein, such was not the case for muscle protein (13, 14). Thus, it would appear that for purposes of weight gain, the utilization of the limiting amino acid remains unaltered. In all instances reported here, gain in weight was a function of the absolute intake of the first limiting amino acid.

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Effect on Nitrogen Balance of Adult Man of Varying Source of Nitrogen and Level of Calorie Intake [1,2]

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ABSTRACT Six young men were fed semipurified diets containing 6.28 g of total nitrogen per day of which 5.50 g was furnished by the nitrogen source under investigation. Nitrogen balance was not significantly different when intact casein was fed than when 18 crystalline essential and nonessential L-amino acids simulating casein were fed. Nonessential amino acids, however, supplied by intact casein or by crystalline L-amino acids patterned as in casein, were superior to nonessential nitrogen supplied by glycine and diammonium citrate or by glycine, diammonium citrate and glutamic acid. Glycine, diammonium citrate and glutamic acid were no better as sources of nonessential nitrogen than glycine and diammonium citrate. Although no significant difference was obtained between the periods in which intact casein and the mixture of 18 essential and nonessential amino acids were given, raising the calorie intake by 500 kcal/day increased nitrogen retention from both sources.

Data concerning the effectiveness of various sources of nitrogen, as compared with intact proteins, should be most valid when all or almost all of the dietary nitrogen is supplied by the sources being studied, and when the ratio of essential to nonessential nitrogen is the same as that which occurs in the intact protein. Among the few human studies which meet these criteria is that of Rose et al. (1), who reported that when men were given 35 kcal/kg body weight per day whole casein was superior to enzymatically hydrolyzed casein, acid-hydrolyzed casein supplemented with tryptophan or 20 crystalline amino acids recognized as components of casein. Adult rats utilized acid-hydrolyzed casein or a mixture of essential and nonessential amino acids simulating casein as efficiently as intact casein (2). Nitrogen retention of human subjects was better when whole egg rather than a mixture of amino acids simulating egg protein was given [4] (3). In other studies in which only a small portion of the total dietary nitrogen was furnished by the sources being compared, whole egg (4, 5), milk and peanut butter (6) and oats (7) were slightly better than equivalent amounts of the purified amino acids patterned as in the foods.

In all studies in which the major portion of the dietary nitrogen was supplied by the source being compared, however, subjects retained more nitrogen when fed an intact protein than when fed mixtures of essential amino acids patterned after the protein and various combinations of glycine, diammonium citrate and urea (1, 3). Conflicting results have been obtained concerning the effectiveness of nonessential amino acids versus various combinations of glycine, diammonium citrate, glutamic acid and urea, but differences in total nitrogen intake and in the isomeric form in which the amino acids were given may have been responsible for the lack of agreement among investigators [5] (1, 3).

Calorie intake may influence the relative effectiveness of different sources of dietary

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nitrogen. The data of Rose et al. (1) suggest the magnitude of superiority of casein over hydrolyzed casein and mixtures of amino acids was affected by the calorie intake, but these data are not in agreement with those obtained from rats (8).

The purpose of the present investigation was to determine the effect of different sources of dietary nitrogen on nitrogen balance of adult man. The sources studied were: 1) intact casein versus a mixture of 18 L-isomers of essential and nonessential amino acids equivalent to those in the casein with both sources fed at two calorie intakes; and 2) intact casein or the 18 essential and nonessential amino acids simulating casein versus a mixture of essential amino acids equivalent to those in the casein when glycine and diammonium citrate or glycine, diammonium citrate and glutamic acid supplied the nonessential nitrogen.

EXPERIMENTAL PROCEDURES

Diets. Human subjects were fed diets in which 89% of the total daily nitrogen intake of 6.28 g was furnished by casein; by 18 L-amino acids simulating casein supplemented with diammonium citrate in amounts to compensate for the casein nitrogen not accounted for by the 18 amino acids; or by essential amino acids patterned as in casein but with the nonessential nitrogen provided by diammonium citrate and glycine or by diammonium citrate, glycine and glutamic acid. The study lasted 50 days and was composed of a 3-day depletion period, 5-day adjustment period, and six 7-day experimental periods.

The sources and amounts of dietary nitrogen fed to the subjects during the depletion period, the adjustment period and each of the six experimental periods are shown in table 1. Throughout the study, with the exception of the depletion period, the quantities of essential amino acids in the diets met or exceeded the recommended intake of Rose (11); these quantities were twice the minimum requirement of Rose. The major portion of the daily nitrogen of diets 1 and 5 was provided by 38.7 g of casein; small quantities of tryptophan and methionine were added to meet Rose's recommended intake for these amino acids. The essential amino acids and cystine and tyrosine of diets 2, 3, 4 and 6 were provided by crystalline L-isomers in amounts equivalent to those provided by diets 1 and 5. The pattern for the essential and nonessential amino acids of casein was taken from Orr and Watt (12). The nonessential amino acid nitrogen of diets 2 and 6 was provided by eight crystalline nonessential amino acids equivalent to those contained in 38.7 g casein and by diammonium citrate added to compensate for the 0.51 g of unidentified nitrogen of casein. The nonessential amino acids given were: L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, L-histidine, L-proline, L-serine and glycine. Citrulline and hydroxyproline, although known to be components of casein, were not included in the nonessential amino acid mixture. The nonessential amino acid nitrogen of diet 3 was provided by isonitrogenous amounts of glycine and diammonium citrate, and that of diet 4 by isonitrogenous amounts of glycine, diammonium citrate and glutamic acid. In all diets, 35% of the nitrogen was furnished by essential amino acids, 54% by nonessential amino acids and diammonium citrate and 11% by the ordinary foods, which are listed in footnote 4 of table 1.

During the adjustment period an attempt was made to determine the amount of calories required by each subject for weight maintenance. The calorie intake was then held constant for each subject during the experimental periods when diets 1 through 4 were fed; the calorie intake varied from 35 to 52 kcal/kg body weight among the subjects. When the subjects were fed diets 5 and 6, the calorie intake of each individual was increased by 500 kcal/day; the intake varied among the subjects from 41 to 60 kcal/kg body weight. The additional calories were provided by sucrose, fat and jelly. A constant ratio of carbohydrate to fat calories was maintained.

Arrangement of experimental diets. Diets providing the lower calorie intake (diets 1 through 4) were fed during the first four experimental periods, and the diets providing the higher calorie intake (diets 5 and 6) were fed during the last two experimental periods. The sequence

TABLE 1

Sources and amounts of dietary nitrogen

Name of diet	Source of nitrogen							
	Casein [1]	EAA [2]	NE [3]	Gly [3]	Glu [3]	Dac [3]	Other foods [4]	Total nitrogen intake
	g/day	*g/day*	*g/day*	*g/day*	*g/day*	*g/day*	*g/day*	*g/day*
Depletion	0.00	1.10	0.00	0.72	0.00	0.97 [5]	0.64	3.43
Adjustment	0.00	2.21	0.00	1.43	0.00	1.95 [5]	0.64	6.23
1	5.50 [6]	0.10 [7]	0.00	0.00	0.00	0.00	0.69	6.29
2	0.00	2.21	2.87 [8]	0.00	0.00	0.51 [5]	0.69	6.28
3	0.00	2.21	0.00	1.43	0.00	1.95 [5]	0.69	6.28
4	0.00	2.21	0.00	0.96	0.96	1.46 [5]	0.69	6.28
5 [9]	5.50 [6]	0.10 [7]	0.00	0.00	0.00	0.00	0.69	6.29
6 [9]	0.00	2.21	2.87 [8]	0.00	0.00	0.51 [5]	0.69	6.28

[1] Vitamin-test casein, General Biochemicals Incorporated, Chagrin Falls, Ohio.

[2] EAA = the eight essential amino acids, cystine and tyrosine given as crystalline L-amino acids.

[3] NE = nonessential amino acids; eight crystalline amino acids were given and were L-alanine, L-arginine, L-aspartic acid, glycine, L-glutamic acid, L-histidine, L-proline, and L-serine; Dac = diammonium citrate; Gly = glycine; Glu = glutamic acid.

[4] These values are means for all subjects. The natural foods were composed of the following: (in grams per day) applesauce, 100; peaches, 100; pears, 100; tomatoes, 100; green beans, 100; and dill pickle, 50. Other foods given were: wafers made of cornstarch, fat, sucrose, mineral supplement, baking powder (9), and mucilose flakes; carbonated beverage; jelly; sucrose; butteroil; butterballs made of butteroil, confectioner's sugar, artificial coloring and flavoring; and tea or coffee. A commercial vitamin supplement was given daily and contained the following: 5000 IU vitamin A acetate and 400 IU vitamin D calciferol; and (in milligrams per day) thiamine·HCl, 3; riboflavin, 2.5; pyridoxine·HCl, 1; niacinamide, 20; ascorbic acid, 50; D-panthenyl alcohol, 1; and cyanocobalamin, 0.001. The mineral supplement (10) provided the following: (in grams per day) Ca, 1.003; P, 1.001; Mg, 0.199; Fe, 0.015; Cu, 0.002; I, 0.00015; Mn, 0.002; and Zn, 0.0009.

[5] This value included 0.51 g nitrogen from diammonium citrate which compensated for the unidentified nitrogen of casein.

[6] Casein was incorporated into the cornstarch wafer.

[7] Nitrogen from methionine and tryptophan supplements.

[8] The amount of glycine corresponding to that in 38.7 g casein was included in the nonessential amino acids given in this period.

[9] Diets 5 and 6 supplied 500 kcal more per day than diets 1 and 2; in other respects diet 5 was the same as diet 1, and diet 6 was the same as diet 2.

of feeding diets 1 through 4 was arranged at random for each subject. No more than two subjects received the same diet at the same time. Diets 5 and 6 were presented in a simple switchback design in which three of the subjects were fed diet 5 in one period and the other three were fed diet 6, and in the other period the subjects were fed the diets in the reverse order.

Subjects. The subjects were six young male students at the University of Wisconsin. The age, height, weight and calorie intake of the subjects are presented in table 2. During the study the subjects pursued their usual activities, academic and otherwise.

Methods. Complete 24-hour urine collections were made throughout the study. Nitrogen (13) and creatinine (14) were determined daily on fresh urine samples. Feces were collected and composites for the depletion period, the adjustment period and each experimental period were made. Aliquots of feces, food, casein, and amino acid mixtures were analyzed for nitrogen. Blood was obtained from the antecubital vein of each subject before breakfast on day 1 of the investigation and on day 7 of each of the six experimental periods. The subjects then consumed one-third of the daily allotment of foods within 20 minutes; 1 and 2 hours after the beginning of the meal additional venous blood was obtained from three of the subjects. The plasma was deproteinized by adding 15% sulfosalicylic acid in amounts so that resulting sulfosalicylic acid concentration was 3%, and centrifuging for 15 minutes at 28,000 × *g* at 5°. A 10-ml aliquot of the 24-hour urine volume, collected on the days when the blood samples were obtained, was made to a final volume of 25 ml at pH 2.0. The diluted urine contained 0.1% ethylenediaminetetraacetate. The individual blood and urine samples were analyzed for urea content using an amino acid analyzer.[6]

[6] Beckman model 120C, Beckman Instruments, Inc., Spinco Division, Palo Alto, Calif.

TABLE 2

Age, height, weight and calorie intakes of the subjects

Subject no.	Age	Height	Initial wt	Daily calorie intake			
				Diets 1 through 4		Diets 5 and 6	
	yr	*cm*	*kg*	*kcal*	*kcal/kg*	*kcal*	*kcal/kg*
1	22	191	81.9	3115	38	3615	44
2	23	185	66.9	3119	47	3619	54
3	21	173	89.0	3118	35	3618	41
4	20	183	73.2	3117	43	3617	49
5	24	173	67.6	3131	46	3631	54
6	19	179	64.1	3321	52	3821	60

RESULTS

The mean nitrogen balance for the individual subjects and the mean for all subjects during each experimental period are presented in figure 1. When the subjects were fed the diets supplying the lower calorie intake, there was no significant difference between the mean nitrogen retentions when intact casein and crystalline essential and nonessential amino acids simulating casein were fed. Significantly more nitrogen was retained, however, when subjects were fed the intact casein (diet 1) than when they were fed the essential amino acids patterned after casein and the nonessential nitrogen supplied by glycine and diammonium citrate (diet 3; $P < 0.05$) or by glycine, diammonium citrate and glutamic acid (diet 4; $P < 0.05$). Subjects retained significantly greater amounts of nitrogen when the nonessential nitrogen was provided by the nonessential amino acids patterned as in casein (diet 2) than when it was provided by glycine and diammonium citrate (diet 3; $P < 0.05$); the subjects also retained more nitrogen when given the nonessential amino acids than when given glycine, diammonium citrate and glutamic acid, and this difference closely approached significance ($P < 0.10$). A mixture of glycine, glutamic acid and diammonium citrate was no better as a source of nonessential nitrogen than a mixture of glycine and diammonium citrate.

When the calorie intake of the subjects was increased by 500 kcal/day (diets 5 and 6), significantly more nitrogen ($P < 0.01$) was retained than when the lower calorie intake was given (diets 1 and 2), regardless of whether the amino acids were provided in the bound or in the free form. At the higher calorie intake the subjects retained 0.53 g more nitrogen per day when casein was fed and 0.37 g more when essential and nonessential amino acids simulating casein were fed. Although the subjects retained 0.30 g more nitrogen when casein was given than when amino acids simulating casein were given at the higher calorie intake, the difference was not statistically significant.

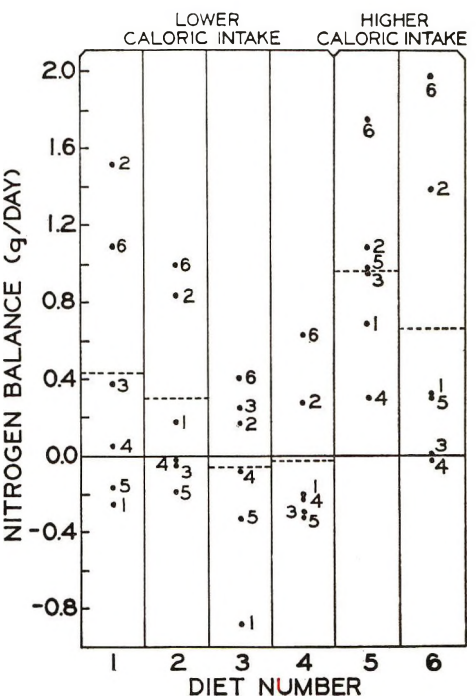

Fig. 1 Effect on nitrogen balance of men by varying the source of nitrogen and the level of calorie intake when total nitrogen was 6.28 g daily. The dashed line represents the mean daily nitrogen balance of all subjects and the dots, the daily nitrogen balance of individual subjects for the last 5 days of each 7-day experimental period.

Subjects 2 and 6 retained more nitrogen throughout all experimental periods than the other subjects. When diets 1, 2, 5 and 6 were fed, the average amount of nitrogen retained by these two subjects was from 0.69 to 1.54 g greater per day than that retained by the other four; and when diets 3 and 4 were fed, subjects 2 and 6 retained slightly more nitrogen than the other subjects. The fact that these two subjects received more calories per kilogram of body weight than the others may have been responsible for the larger amounts of nitrogen retained by them. On the basis of body weight and lean body mass, as evidenced by creatinine excretion, they received the most nitrogen.

During three of the four experimental periods when subject 1 was given the lower calorie intake, he was in negative nitrogen balance. He was in positive nitrogen balance only when he was fed the diet containing crystalline amino acids simulating casein. The slight weight loss of this subject, which occurred during the 28 days he was given the lower calorie intake, indicates that 38 kcal/kg body weight was not adequate; when his calorie intake was increased to 44 kcal/kg body weight, he achieved nitrogen balance and maintained weight.

Although subject 5 was given 46 kcal/kg body weight during the first four experimental periods, he was in negative nitrogen balance; but, when his calorie intake was increased to 54 kcal/kg body weight, he retained nitrogen. His weight remained fairly constant throughout all six experimental periods regardless of the calorie intake.

During the period of low calorie intake, both the nitrogen balance and weight of subject 3 fluctuated; when he was losing a small amount of weight, he was in negative nitrogen balance and when he tended to maintain weight, he retained nitrogen. A slight weight loss over the 4 weeks indicated that 35 kcal/kg body weight was insufficient for this subject. During the last two experimental periods, when he was given 41 kcal/kg body weight, he was in positive balance, but he continued to show a slight weight loss.

The mean nitrogen balances for subject 4 were about the same during all experimental periods regardless of the calorie intake or the source of dietary nitrogen. He was subject to occasional sore throats of unknown etiology. During the study three such episodes occurred and were accompanied by a substantial increase in urinary nitrogen. Although the values for the urinary nitrogen obtained during the time the subject complained of the sore throat were omitted from the average values, the fact that this individual suffered such episodes may have been the reason he did not respond in a manner similar to that of the other subjects.

Mean 24-hour urinary urea values for all subjects and individual values for each subject during each experimental period are presented in figure 2; mean and individual plasma concentrations of urea for three subjects are presented in figure 3. The amount of urinary urea appeared to be related to nitrogen balance; subjects excreted the most urea when nitrogen retention was lowest (diets 3 and 4; $P < 0.01$) and the least when nitrogen retention was highest (diets 5 and 6; $P < 0.01$). Mean urinary urea nitrogen values for all subjects were 3.55, 3.55, 4.31, 4.28, 2.72 and 2.97 g/24 hours, respectively, when they were fed diets 1 through 6. The fasting concentration of plasma urea was not significantly affected by diet and ranged from means of 181 to 231 μmoles/100 ml of plasma during the various experimental periods. Plasma urea at 1 and 2 hours postprandially was essentially the same as the fasting value. When the subjects consumed self-selected diets, the fasting concentration of plasma urea was 520 μmoles/100 ml plasma and was significantly higher than the values obtained when the subjects were fed the experimental diets ($P < 0.01$).

DISCUSSION

In the present investigation, casein or the sources of nitrogen being compared with casein supplied 89% of the dietary nitrogen, and the ratio of essential to nonessential nitrogen was the same as that of the protein being studied. Only in three other investigations was the major portion

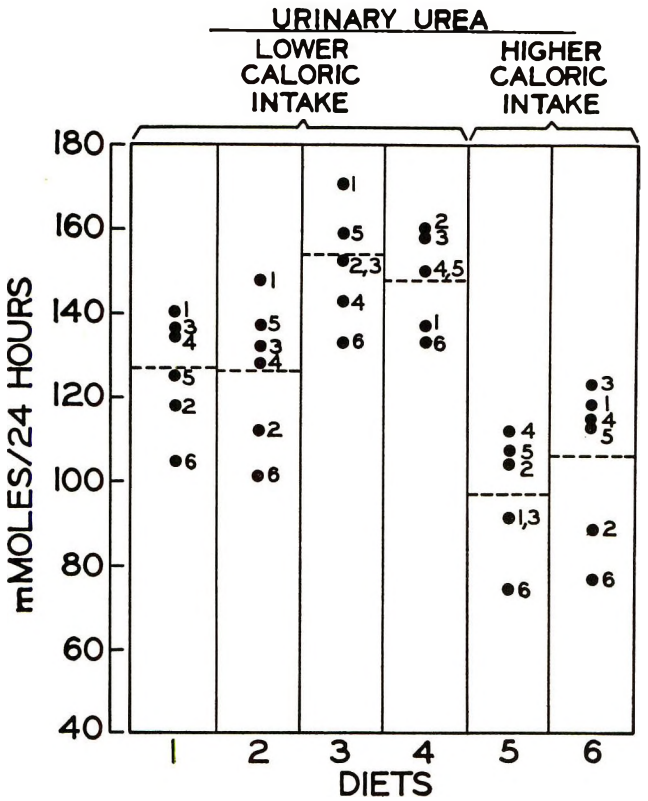

Fig. 2 Effect on urinary urea of men by varying the source of nitrogen and level of calorie intake when total nitrogen was 6.28 g daily. The dashed line represents the mean urea value of all subjects and the dots, the urea value of the individual subjects for the last day of each 7-day experimental period.

of the dietary nitrogen supplied by the sources being compared[7] (1, 3). In contrast, in several other studies (3–7) about 30% or less of the dietary nitrogen was supplied as the food or the nitrogenous components being compared with the food, and the nitrogen furnished by the essential amino acids was 15% or less of the total nitrogen intake, a percentage considerably smaller than that found in any protein.

A mixture of 18 essential and nonessential amino acids simulating casein was as effective in the present investigation as the intact protein in maintaining nitrogen balance in men when the total nitrogen intake was 6.28 g daily. Rose et al. (1) fed three men intact casein or acid-hydrolyzed casein supplemented with tryptophan in amounts which furnished about 96% of the total nitrogen intake of approximately 10 g daily. When the calorie intake was 45 kcal/kg body weight, the men retained similar amounts of nitrogen from both sources; however, when the calorie intake of the subjects was 35 kcal/kg body weight, nitrogen balance was better when casein was given than when acid-hydrolyzed casein was given. In a single experiment by the same investigators, one subject given 35 kcal/kg body weight retained nitrogen when fed casein but lost almost 1.0 g daily when fed an isonitrogenous mixture containing 20 amino acids recognized as components of casein. Since it was necessary to use the racemic forms of valine, isoleucine, methionine, threonine, phenylalanine, alanine, serine, aspartic acid and citrulline, about 2.6 g of the daily nitrogen was furnished by the D-enantiomorphs. Results of several investigations

[7] See footnote 4.

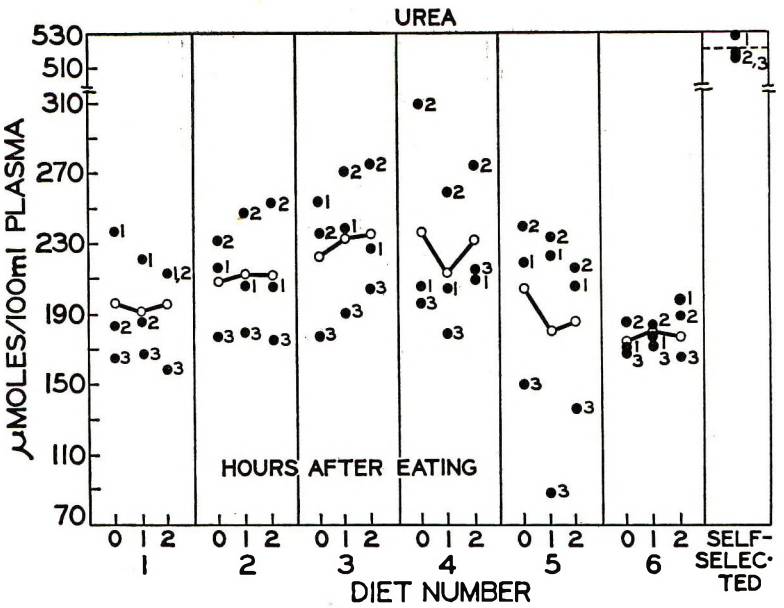

Fig. 3 Effect of diet on the concentration of plasma urea. The values were obtained from subjects fasted overnight (zero time) and at 1- and 2-hour intervals following the consumption of one-third of the daily allotment of foods. The open circles represent the mean plasma urea concentration of all subjects and the closed circles, that of the individual subjects for the last day of each of the 7-day experimental periods.

reviewed by Berg (15) show that L-amino acids are better utilized by the rat and by man than DL-amino acids.

Data from the present investigation show that casein was not significantly better than a mixture of 18 amino acids at either calorie intake, although the subjects did retain somewhat more nitrogen at both calorie intakes when casein was given. The lower calorie intake was not quite adequate for the subjects as evidenced by a slight weight loss of 0.8 kg during the 28-day period, but it was considerably higher than the 35 kcal/kg body weight given by Rose et al. (1). Recent results [8] indicate that nitrogen balances of subjects given 35 kcal/kg body weight became progressively more negative over a 15-day period. If Rose et al. fed the diets in the order in which they appeared in the tables (1), the more negative nitrogen balances obtained with the acid-hydrolyzed casein, compared with the intact casein, may have been due in part to the length of time the subjects were given the low calorie intake.

Whole egg has consistently resulted in better nitrogen retention than amino acids simulating egg protein even though the calorie intakes of the subjects exceeded 50 kcal/kg body weight [9] (3). Whole egg supplies many substances other than protein which may be responsible for the superiority of whole egg over amino acids. Results of other studies, in which only a minor portion of the dietary nitrogen was provided by an intact protein or by amino acids simulating the protein, are not directly comparable with those of the present one (6, 7).

In the present study the subjects retained significantly more nitrogen when fed casein than when fed essential amino acids and glycine and diammonium citrate, or essential amino acids and glycine, diammonium citrate and glutamic acid. Other investigators who have given the major portion of the nitrogen as either an intact protein or a mixture of essential amino acids patterned after the protein and various combinations of glycine, glutamic acid, diammonium citrate and urea have found that

[8] Zahler, L. P., E. N. Alcantara and H. Linkswiler 1968, unpublished data.
[9] See footnote 4.

subjects retained more nitrogen when fed the intact protein (17–21). A critical appraisal of these data indicates that high calorie intakes minimize the difference in nitrogen balance of subjects fed the two sources. Rose et al. (1) reported that subjects given approximately 10 g of total nitrogen and 35 kcal/kg body weight per day were in positive nitrogen balance when fed intact casein but were in negative nitrogen balance when fed the eight essential amino acids and glycine and urea. When the calorie intake was increased to 45 kcal/kg body weight, positive nitrogen balance was attained when essential amino acids supplemented with glycine and urea were fed; the subjects fed casein also showed improvement at the higher calorie intake, but the difference in nitrogen balance resulting from the two sources was less at the higher calorie intake. Watts et al. (3) fed diets containing 4 g of total nitrogen daily and more than 50 kcal/kg body weight per day and found that five of the six subjects retained an average of 0.32 g nitrogen daily when fed whole egg and five of the six subjects lost nitrogen (0.23 g/day) when given the essential amino acids and glycine and diammonium citrate.

In both the present and a previous [10] investigation from this laboratory, subjects retained more nitrogen when given nonessential amino acids than when glycine and diammonium citrate were substituted for the nonessential amino acids. This observation is compatible with that of Rose et al. (1) who reported that acid-hydrolyzed casein appeared to be superior to a mixture of eight essential amino acids and glycine and urea. Rose and co-workers reported, however, that a mixture of 20 amino acids was no better than one of the eight essential amino acids and glycine and urea; but a larger amount of nitrogen was supplied by the D-isomers when the diet contained nonessential amino acids than when it contained glycine and urea. Watts et al. (3) also reported no difference in nitrogen balance of subjects fed the two sources of nonessential nitrogen when total nitrogen intake was 4.0 g daily, but the fact that four of the six subjects were in negative nitrogen balance when fed either source of nitrogen indicates that total nitrogen was limiting and might have masked any effect of source of nonessential nitrogen. The data of Swendseid et al. (16), which indicate that a combination of glycine and diammonium citrate is as effective as a mixture of nonessential amino acids in maintaining nitrogen equilibrium, cannot be directly compared with the present data because the amino acids were not proportioned as in a protein; of the total intake of 10 g of nitrogen, 0.75 g was supplied by essential amino acids, 8.75 g by the nonessential nitrogen sources and the remainder by ordinary foods.

The present results indicate that intact casein or amino acids simulating the casein are significantly better utilized than the eight essential amino acids and glycine and diammonium citrate, or the eight essential amino acids and glycine, diammonium citrate and glutamic acid. The poorer nitrogen balance obtained when the nonspecific sources of nitrogen were given suggests that the in vivo synthesis of nonessential amino acids was delayed long enough to adversely affect nitrogen balance.

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[10] See footnote 5.

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Effect of Source of Dietary Nitrogen on Plasma Concentration and Urinary Excretion of Amino Acids of Men [1,2]

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ABSTRACT The postprandial plasma concentration of most essential and nonessential amino acids decreased or remained at fasting values when the major portion of the daily intake of 6.28 g N was provided by casein. When a mixture of 18 amino acids simulating casein was given, the postprandial plasma concentration of both essential and nonessential amino acids increased during the first hour but decreased during the second hour to values somewhat above the fasting ones. When the nonessential nitrogen was furnished by glycine and diammonium citrate or by glycine, diammonium citrate and glutamic acid, the plasma concentration of individual essential amino acids increased by the first hour and during the second hour remained elevated or continued to increase. The fasting and postprandial concentrations of histidine and proline were much lower and that of arginine somewhat lower when these amino acids were excluded from the diet. Glycine in fasting and postprandial plasma and in urine increased significantly when this amino acid furnished one-half or one-third of the nonessential nitrogen. The concentration of threonine in fasting plasma and the amounts of threonine, serine and taurine excreted were significantly increased when large amounts of glycine or diammonium citrate were given.

Although nitrogen balance has long been used to evaluate the quality and quantity of protein in the diet of man, and plasma amino acid concentrations have been suggested as criteria for judging protein nutriture, few investigators have attempted to correlate plasma free amino acid concentrations with utilization of dietary nitrogen as determined by nitrogen balance. It has been reported that diets inadequate in total protein, or in an essential amino acid, resulted in a decrease in the plasma concentration of free essential amino acids and an increase in plasma free nonessential amino acids of human subjects fasted overnight (1–4). To our knowledge, however, no information correlating postprandial changes in plasma free amino acids with nitrogen balance is available.

In a previous paper from this laboratory (5), it was reported that the nitrogen balance of subjects given casein, or a mixture of 18 essential and nonessential amino acids simulating casein, was significantly better than that obtained when glycine and diammonium citrate or glycine, diammonium citrate and glutamic acid furnished the nonessential nitrogen, when total nitrogen intake was 6.28 g daily. This paper presents the effects of the above sources and kinds of dietary nitrogen on fasting and postprandial plasma amino acid concentrations and on the urinary excretion of amino acids.

EXPERIMENTAL PROCEDURES

The effect of source of dietary nitrogen on the concentration of individual free amino acids in the plasma and urine of adult man was studied at intervals throughout a 50-day investigation which consisted of six 7-day experimental periods preceded by an 8-day adjustment period. The experimental procedure and the diets have been described in detail in the previous paper (5). A different diet was fed during each experimental period. All six diets contained 6.28 g N daily; 11% of the nitrogen was provided by a few ordinary foods which were common to all diets and the

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remainder by casein or crystalline amino acids and diammonium citrate. The eight essential amino acids, and cystine and tyrosine from the casein or the crystalline sources, furnished 35% of the dietary nitrogen, and the nonessential amino acids and diammonium citrate furnished 54%. The diets differed as to source of essential amino acids, cystine and tyrosine (intact protein versus crystalline amino acids), and as to source and type of material furnished for the nonessential nitrogen (table 1).

All six diets contained the same amount of essential amino acids, cystine and tyrosine; except for small supplements of methionine and tryptophan they were furnished by casein in diets 1 and 5 and by crystalline L-amino acids in the other diets. The nonessential nitrogen was furnished by casein (diets 1 and 5), by eight nonessential amino acids patterned as in casein with sufficient amounts of diammonium citrate to compensate for the nitrogen of casein not accounted for by the 18 L-amino acids (diets 2 and 6), by isonitrogenous amounts of glycine and diammonium citrate (diet 3), or by isonitrogenous amounts of glycine, diammonium citrate and glutamic acid (diet 4). The calorie intake of the individual subjects ranged from 35 to 52 kcal/kg body weight when diets 1 through 4 were given. Diets 5 and 6 were the same as diets 1 and 2, respectively, except they contained 500 kcal more per day; the calorie intake varied among the subjects from 41 to 60 kcal/kg body weight when diets 5 and 6 were given. The additional calories were furnished by sucrose, jelly and butterfat, and the ratio of carbohydrate to fat calories was maintained constant throughout the investigation.

Blood from the antecubital vein of subjects fasted overnight was obtained on the morning of day 1 of the experiment, as representative of subjects consuming self-selected diets, and on the last day of each of the six experimental periods. After the fasting blood was drawn, subjects were given one-third of the daily allotment of foods. They ate the breakfast within 20 minutes; 1 and 2 hours after the beginning of the meal venous blood was obtained

TABLE 1

Daily amount of amino acids and of diammonium citrate furnished by each experimental diet [1]

Amino acid	Diet no.					
	1 [2]	2	3	4	5 [2]	6
	g/subject per day					
L-Isoleucine	2.27	2.27	2.27	2.27	2.27	2.27
L-Leucine	3.48	3.48	3.48	3.48	3.48	3.48
L-Lysine	2.77	2.77 [3]	2.77 [3]	2.77 [3]	2.77	2.77 [3]
L-Methionine	2.07	2.07	2.07	2.07	2.07	2.07
L-Phenylalanine	1.86	1.86	1.86	1.86	1.86	1.86
L-Threonine	1.48	1.48	1.48	1.48	1.48	1.48
L-Tryptophan	0.50	0.50	0.50	0.50	0.50	0.50
L-Valine	2.56	2.56	2.56	2.56	2.56	2.56
L-Cystine	0.13	0.13	0.13	0.13	0.13	0.13
L-Tyrosine	2.01	2.01	2.01	2.01	2.01	2.01
L-Alanine	1.16	1.16	0.00	0.00	1.16	1.16
L-Arginine	1.41	1.41 [4]	0.00	0.00	1.41	1.41 [4]
L-Aspartic acid	2.55	2.55	0.00	0.00	2.55	2.55
L-Glutamic acid	7.97	7.97	0.00	10.06	7.97	7.97
Glycine	0.69	0.69	7.70	5.13	0.69	0.69
L-Histidine	1.04	1.04 [5]	0.00	0.00	1.04	1.04 [5]
L-Proline	4.06	4.06	0.00	0.00	4.06	4.06
L-Serine	2.30	2.30	0.00	0.00	2.30	2.30
Diammonium citrate	0.00	4.11	15.71	11.84	0.00	4.11

[1] These amounts do not include the amino acids contained in the 0.69 g N in the ordinary foods of the diet.
[2] These amounts of amino acids were calculated to be present in 38.7 g casein (6).
[3] Given as L-lysine·HCl.
[4] Given as L-arginine·HCl.
[5] Given as L-histidine·HCl·H_2O.

from three subjects. The noon and evening meals were consumed as usual. The subjects made 24-hour urine collections throughout the investigation, but only the urine obtained on the days when the blood was drawn was analyzed for amino acid content.

Subjects. Six male students at the University of Wisconsin, ranging in age from 19 to 24 years, in weight from 64 to 89 kg and in height from 173 to 191 cm, were used. Information concerning each individual has been presented by Anderson et al. (5). The subjects pursued their usual activities during the investigation.

Analytical methods. Ten milliliters of venous blood were drawn from each subject into heparinized tubes and chilled immediately in an ice bath. Plasma obtained by refrigerated centrifugation at 5000 × *g* for 15 minutes was deproteinized with sufficient 15% sulfosalicylic acid to make the resulting supernatant 3% sulfosalicylic acid. The acidified plasma was centrifuged at 28,000 × *g* for 15 mintues at 5°, and the supernatant was frozen until the analyses were made.

Plasma and urinary amino acids were determined by the method of Spackman et al. (7) using an amino acid analyzer.[4] Samples of each individual were analyzed separately. To determine the plasma concentration of basic and of acidic and neutral amino acids, a 0.5-ml aliquot of the sulfosalicylic acid supernatant was placed on the short and the long column, respectively. Before analyzing urine for basic amino acids, the quantity of ammonia was reduced by storing a 5-ml aliquot at pH 11 over concentrated sulfuric acid. When the sample was dry, it was taken up in a small amount of 0.2 N sodium citrate buffer at pH 2.2, then 2.5 ml of 0.4% ethylenediaminetetraacetic acid (EDTA) were added, the pH was adjusted to 2.0 and the sample was diluted to a volume of 10 ml; 1.0 ml of the resulting solution was placed on the short column. For the determination of acidic and neutral amino acids, 10 ml of urine containing EDTA to make the final concentration 0.1% were diluted to 25 ml at pH 2.0; 1.0 ml of the resulting solution was placed on the long column. Alpha-amino-alpha-guanidinobutyric acid was placed on the short column and norleucine was placed on the long column in 0.1-μmole quantities as internal standards. Methionine was not corrected for sulfoxides and tryptophan was not determined.

RESULTS

The method used to determine statistical significance was Duncan's new multiple range test for comparison among treatment means.

Plasma amino acids following an overnight fast. The average fasting concentration of free amino acids in plasma of the six subjects consuming the self-selected and six experimental diets is presented in tables 2 and 3. The plasma concentration

[4] Beckman model 120C, Beckman Instruments, Inc., Spinco Division, Palo Alto, Calif.

TABLE 2

Mean fasting plasma concentration of the essential amino acids, cystine and tyrosine of subjects consuming the six experimental diets and self-selected diets

Amino acid	Diet no.						Self-selected diets
	1	2	3	4	5	6	
	μmoles/100 ml						
Isoleucine	7.6 ± 2.0 [1]	7.0 ± 1.3	7.1 ± 1.2	7.6 ± 2.2	8.1 ± 1.1	7.9 ± 1.3	10.0 ± 2.2
Leucine	13.3 ± 1.3	11.8 ± 1.3	13.3 ± 1.7	14.7 ± 2.0	14.4 ± 2.3	13.2 ± 1.7	15.5 ± 3.3
Lysine	17.1 ± 2.1	16.0 ± 0.7	18.8 ± 0.8	17.5 ± 1.8	17.3 ± 2.2	17.6 ± 1.9	15.5 ± 3.2
Methionine	3.3 ± 0.2	3.5 ± 0.7	4.1 ± 0.5	3.7 ± 1.2	4.0 ± 0.3	4.0 ± 0.4	3.5 ± 0.6
Phenylalanine	5.4 ± 1.6	5.0 ± 1.2	5.6 ± 1.1	6.6 ± 1.4	5.2 ± 0.9	5.4 ± 0.5	6.4 ± 1.3
Threonine	13.3 ± 2.6	12.6 ± 1.3	16.1 ± 2.7	15.8 ± 2.0	13.3 ± 2.3	13.7 ± 1.8	13.6 ± 3.2
Valine	19.9 ± 2.3	17.1 ± 4.6	20.5 ± 2.5	20.9 ± 3.3	21.1 ± 3.1	21.1 ± 2.2	29.7 ± 6.4
Half-cystine	9.2 ± 1.4	8.1 ± 1.5	10.0 ± 3.3	10.4 ± 1.6	10.0 ± 1.2	10.5 ± 2.9	9.5 ± 1.2
Tyrosine	6.0 ± 0.6	5.6 ± 0.9	6.0 ± 1.5	6.7 ± 0.7	6.1 ± 0.9	6.2 ± 0.7	6.9 ± 2.1
Total essential	95.1 ± 7.8	86.7 ± 10.9	101.5 ± 10.7	103.9 ± 9.6	99.5 ± 3.2	99.6 ± 9.1	110.6 ± 18.0

[1] SEM.

TABLE 3

Mean fasting plasma concentration of the nonessential amino acids and taurine of subjects consuming the six experimental diets and self-selected diets

Amino acid	Diet no.						Self-selected diets
	1	2	3	4	5	6	
	μmoles/100 ml						
Alanine	46.1 ± 9.8 [1]	48.0 ± 20.1	47.3 ± 4.9	53.6 ± 19.1	59.9 ± 23.0	56.0 ± 21.5	35.8 ± 11.0
Arginine	11.6 ± 2.8	10.2 ± 1.4	9.4 ± 1.8	8.5 ± 1.8	10.9 ± 3.4	11.2 ± 1.8	9.2 ± 1.4
Glycine	26.5 ± 3.5	23.8 ± 4.0	41.8 ± 7.6	36.1 ± 7.2	26.6 ± 3.1	27.0 ± 4.8	23.8 ± 3.3
Glutamic acid	13.1 ± 2.1	11.1 ± 4.0	14.0 ± 4.9	14.5 ± 4.6	15.3 ± 3.6	16.8 ± 2.3	11.2 ± 1.9
Glutamine-asparagine	73.9 ± 21.4	82.2 ± 10.5	77.3 ± 18.3	82.4 ± 26.9	81.5 ± 14.6	79.0 ± 11.9	76.7 ± 12.8
Histidine	9.1 ± 1.3	8.8 ± 0.9	4.2 ± 1.3	3.7 ± 1.4	9.5 ± 0.9	9.7 ± 0.9	8.2 ± 0.7
Proline	18.9 ± 7.0	17.7 ± 3.3	13.3 ± 4.3	14.2 ± 3.0	22.3 ± 6.4	22.5 ± 3.0	22.0 ± 7.2
Serine	13.2 ± 2.2	12.1 ± 1.4	15.2 ± 4.6	12.7 ± 3.2	11.8 ± 2.0	12.5 ± 2.3	12.1 ± 5.8
Nonessential (total)	212.4 ± 30.4	213.9 ± 29.0	222.5 ± 25.6	225.7 ± 39.4	237.8 ± 17.2	234.7 ± 16.4	199.0 ± 20.4
Taurine	4.3 ± 0.6	4.2 ± 0.8	4.4 ± 0.9	4.5 ± 0.7	4.0 ± 0.5	3.8 ± 0.3	4.6 ± 0.7

[1] SEM.

of isoleucine, leucine and valine tended to be higher when subjects consumed self-selected diets than when they consumed the experimental diets, but plasma concentration of alanine was highest when the experimental diets were given.

The plasma concentration of most essential amino acids from fasting subjects was similar for all experimental diets except for phenylalanine, which was highest when glycine, glutamic acid and diammonium citrate provided the nonessential nitrogen (diet 4; $P < 0.05$), and for threonine, which was higher when the nonessential nitrogen was furnished by glycine and diammonium citrate or glycine, diammonium citrate and glutamic acid (diets 3 and 4; $P < 0.01$).

The plasma concentration of histidine and proline was significantly decreased by the omission of these amino acids from the diet ($P < 0.01$), that is, when sources other than preformed nonessential amino acids furnished the nonessential nitrogen. The proline concentration was significantly increased by the high calorie intake ($P < 0.01$). The plasma glycine concentration was highest when glycine provided one-half or one-third the nonessential nitrogen ($P < 0.01$).

Essential amino acids in plasma following the consumption of a meal. Alterations which occurred in plasma concentrations of the seven essential amino acids determined during the 2 hours following the consumption of one-third of the daily allotment of foods of each experimental diet are presented in figure 1. Following the ingestion of casein (diets 1 and 5), the plasma concentration of all essential amino acids with the exception of methionine, approximately one-half of which was given in crystalline form, either decreased or remained at fasting values. Although the absolute amount of leucine in the diet was higher than any of the other essential amino acids, the plasma concentration of leucine decreased most. When subjects were given a mixture of 18 amino acids simulating casein (diets 2 and 6), the concentration of all essential amino acids increased within 1 hour, but in almost all instances, by the end of the second hour the concentration decreased to a value somewhat above the fasting one. When

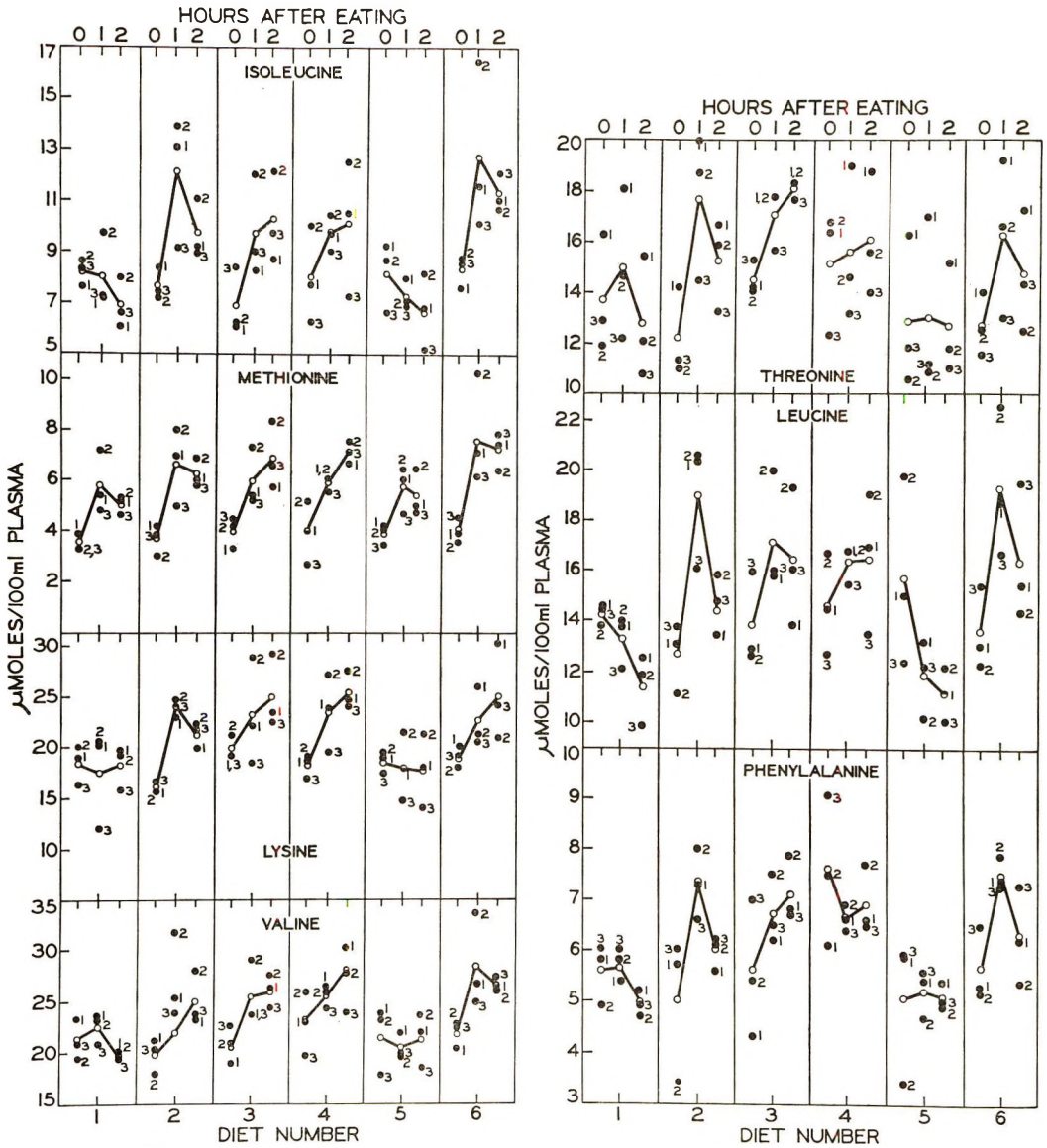

Fig. 1 Individual and mean plasma concentrations of seven essential amino acids at zero hour and at 1 and 2 hours following the ingestion of one-third of the daily allotment of foods of each of the six experimental diets. See text for composition of diets. Closed circles with adjacent numbers represent the individual values for each subject. Open circles represent the mean values of all three subjects.

glycine and diammonium citrate or glycine, diammonium citrate and glutamic acid provided the nonessential nitrogen, there was an increase in plasma concentration of all essential amino acids within 1 hour, and the concentrations remained elevated or continued to increase during the second hour, with the exception of phenylalanine, which decreased from a very high fasting level when glutamic acid furnished part of the nonessential nitrogen.

Nonessential amino acids in plasma following the consumption of a meal. The plasma concentration of six nonessential amino acids is presented in figure 2. The plasma concentrations of alanine and ser-

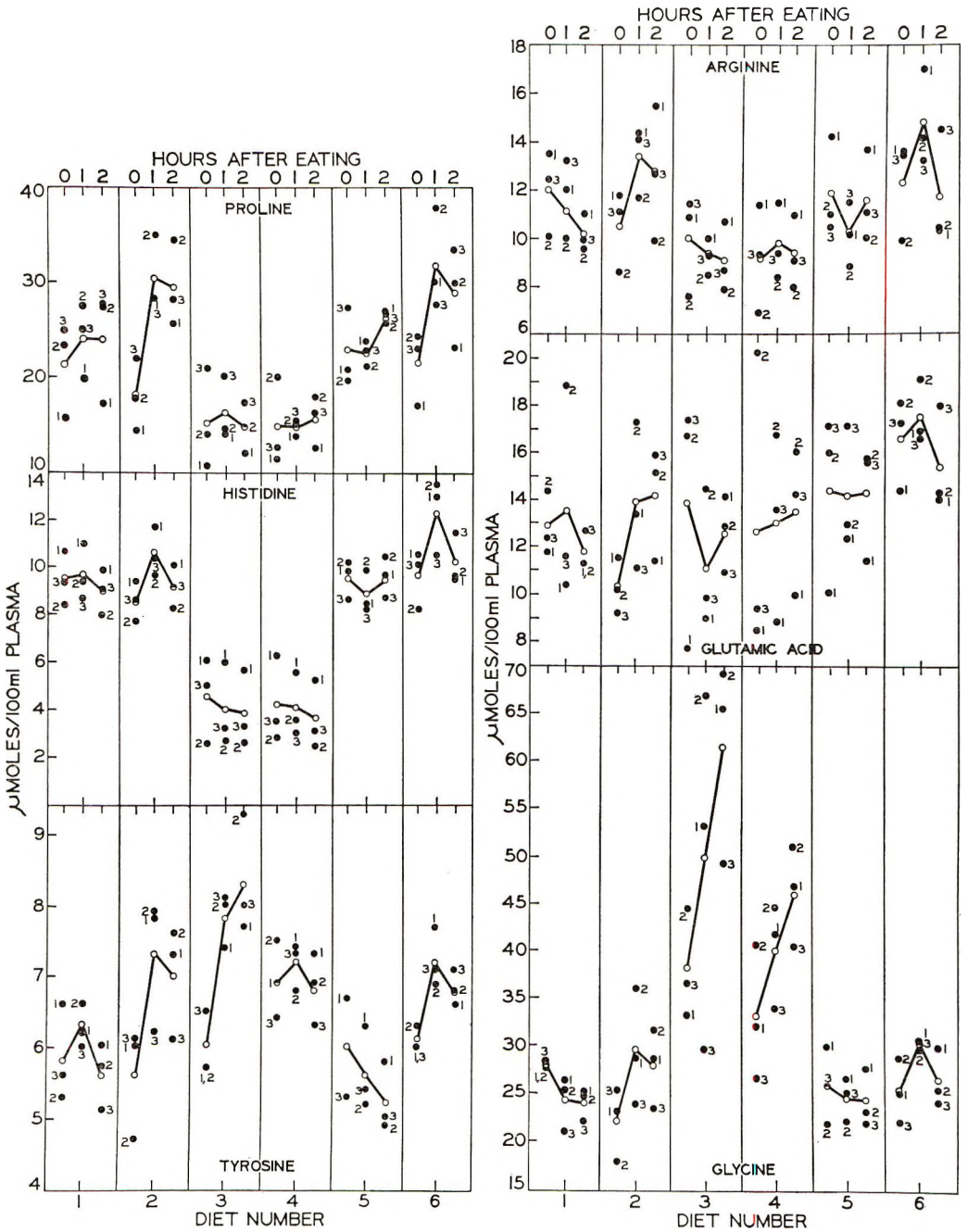

Fig. 2 Individual and mean plasma concentrations of six nonessential amino acids at zero hour and at 1 and 2 hours following the ingestion of one-third of the daily allotment of foods of each of the six experimental diets. See text for composition of the diets. Closed circles with adjacent numbers represent the individual values for each subject. Open circles represent the mean values of all three subjects.

ine will be discussed but are not shown in the figure. With the exception of proline and alanine, the concentration of the nonessential amino acids decreased slightly following consumption of the casein meal. When the mixture of 18 amino acids was given, however, the plasma concentration of all eight nonessential amino acids increased during the first hour; usually by the end of the second hour the concentration had decreased to values somewhat above the fasting ones.

The postprandial plasma concentration of histidine, proline, arginine and glycine was affected by dietary intake. When histidine was omitted from the diet, the plasma concentration decreased postprandially even though the fasting level was only one-half as great as that observed when histidine was given. When proline and arginine were not included as sources of nonessential nitrogen, the postprandial levels of these amino acids were not different from the fasting ones; however, as has already been shown, the fasting concentrations of these amino acids were depressed by lack of dietary supply. The plasma glycine concentration of the subjects increased markedly following meals in which glycine furnished one-half or one-third of the nonessential nitrogen; 2 hours postprandially the concentrations were, respectively, 62 and 46 μmoles/100 ml plasma, compared with about 25 μmoles when the other four diets were fed.

Plasma alanine increased postprandially whether it was present in the diet or not, but the greatest increase occurred when crystalline alanine was given. The glutamic acid concentration was quite variable, but it was no higher when glutamic acid furnished one-third of the nonessential nitrogen than when no glutamic acid was given. The postprandial tyrosine was highest when the nonessential nitrogen was furnished by glycine and diammonium citrate. Postprandial plasma serine was higher when glycine and diammonium citrate rather than glycine, glutamic acid and diammonium citrate supplied the nonessential nitrogen, but the overall range of plasma serine including fasting and postprandial values was only 10 to 17 μmoles/100 ml plasma.

Urinary amino acids and taurine. Mean values for urinary amino acids and taurine of subjects fed the experimental diets are presented in table 4. Significantly more of the essential amino acid, threonine, was excreted when subjects were fed the diet containing the highest amount of glycine ($P < 0.01$), causing more total essential amino acids to be excreted during this time. When subjects were given the diets containing casein, more phenylalanine ($P < 0.01$) but less threonine ($P < 0.01$) was excreted than when the other diets were given; and less total essential amino acids were excreted when casein was fed at the high calorie intake ($P < 0.01$).

The subjects excreted significantly more serine when it was given in crystalline form than when given in casein ($P < 0.01$), and significantly more serine when the nonessential nitrogen was provided by glycine and diammonium citrate rather than glycine, diammonium citrate and glutamic acid or casein ($P < 0.01$). Glycine excretion was related to dietary intake, the greatest excretion occurring when it supplied one-half of the nonessential nitrogen ($P < 0.01$). Histidine excretion was greater when this amino acid was present in the diet than when it was absent ($P < 0.01$). Taurine excretion was significantly higher when glycine and diammonium citrate or glycine, diammonium citrate and glutamic acid supplied the nonessential nitrogen ($P < 0.01$).

DISCUSSION

The observation that the plasma concentration of most amino acids decreased or remained at fasting levels during the 2-hour period following ingestion of casein meals containing 2.1 g N appears to be in opposition to those of other investigators who studied human subjects. They reported an increase in plasma concentration within 1 or 2 hours following ingestion of eggs plus milk (8); wheat gluten mixed with orange juice and corn oil (9); casein (10); fish, fish plus sucrose, fish plus potato, fish plus butter or cereal plus milk (11); or milk, cottage cheese, cream, butter, bread, orange juice and jelly (12). Since the quantity of individual amino acids in plasma following a meal is affected by both quantity of dietary nitrogen and the presence of other dietary components (11–13), comparisons among the studies cited and

TABLE 4

Mean urinary amino acid and taurine excretion of the subjects as affected by the experimental diets [1]

Amino acid	Diet no.					
	1	2	3	4	5	6
			μmoles/24 hr			
Isoleucine	64 ± 18 [2]	32 ± 11	38 ± 17	32 ± 21	30 ± 14	39 ± 12
Leucine	78 ± 23	72 ± 24	63 ± 16	79 ± 20	56 ± 10	70 ± 31
Lysine	84 ± 39	63 ± 36	79 ± 46	73 ± 26	73 ± 17	72 ± 26
Methionine	62 ± 21	61 ± 27	51 ± 29	54 ± 33	44 ± 20	54 ± 16
Phenylalanine	71 ± 26	47 ± 10	53 ± 8	52 ± 12	72 ± 14	50 ± 16
Threonine	182 ± 51	316 ± 60	362 ± 105	295 ± 62	179 ± 58	299 ± 70
Tyrosine	94 ± 58	98 ± 30	117 ± 37	101 ± 27	89 ± 30	99 ± 30
Total essential	635 ± 184	689 ± 164	763 ± 172	686 ± 223	543 ± 119	683 ± 141
Alanine	366 ± 117	420 ± 137	513 ± 170	411 ± 150	447 ± 157	501 ± 234
Glutamine-asparagine	576 ± 227	564 ± 199	742 ± 360	624 ± 201	604 ± 219	511 ± 114
Glycine	1402 ± 873	1546 ± 922	3700 ± 2449	2261 ± 1504	1449 ± 886	1681 ± 1153
Histidine	585 ± 318	666 ± 254	179 ± 102	144 ± 75	660 ± 270	769 ± 324
3-Methyl-histidine	197 ± 17	194 ± 33	177 ± 31	186 ± 64	213 ± 29	208 ± 39
Serine	330 ± 97	665 ± 131	579 ± 264	372 ± 118	318 ± 99	656 ± 134
Total nonessential	3456 ± 1435	4055 ± 1940	5890 ± 1051	3998 ± 1905	3691 ± 1543	4326 ± 1841
Taurine	569 ± 210	853 ± 381	1216 ± 209	1166 ± 237	725 ± 297	574 ± 178

[1] Valine, cystine, arginine, aspartic acid and glutamic acid were present only in trace amounts.
[2] SEM.

the present one are limited because the amount of nitrogen in the test meals varied from 2.1 g, the amount given in the present investigation, to as high as 12 g; some of the test meals contained a single food high in protein, others contained mainly protein and carbohydrate or protein and fat, while still others contained protein, carbohydrate and fat.

Differences in plasma amino acids following the meal containing intact casein and the ones containing crystalline amino acids suggest that there is a difference between the two sources in rate of availability of amino acids for absorption, or in uptake of amino acids from the blood by the tissues. It is possible that the stomach-emptying time of the subjects was different when the two sources of nitrogen were given, but data obtained from rat studies indicate that stomach-emptying time is approximately the same following ingestion of intact proteins and crystalline amino acids (14, 15). Necessity for digestion also may have delayed the absorption of the amino acids from casein. There is some evidence that amino acids appear in the plasma of the rat (16) and of man (17) more rapidly when fed in crystalline form than when fed as an intact protein, but the difference in the rate of absorption from the two sources was no greater than 10 minutes.

There is much evidence, however, which indicates that man absorbs amino acids from casein within 2 hours. Stein et al. (10) gave one human subject 50 g of casein and noted a marked increase in plasma amino acids within 2 hours; determinations were not made earlier. When other protein foods were given to men in fairly large amounts, the concentration of plasma amino acids increased within 1 or 2 hours, indicating absorption had occurred (8, 9, 11, 12). Thus, it appears reasonable to assume that in the present study amino acids from casein entered the blood plasma within 2 hours, and that the postprandial decrease was caused by a rapid removal of amino acids from plasma by tissues.

That elevated plasma amino acid concentrations may be the result of inhibition of concentrative uptake rather than superior amino acid nutrition has been sug-

gested by Christensen (18). In the present investigation, postprandial changes appeared to be related to the nutritive quality of the diets. The casein diets caused a postprandial decrease in plasma free amino acids, and nitrogen balance data presented earlier (5) show that these diets resulted in the highest nitrogen retentions, 0.44 and 0.97 g/day, respectively, at the low and high calorie intake. Thus, it is clear that a low protein diet which is adequate for the maintenance of nitrogen equilibrium in man can cause a postprandial decrease in the plasma amino acid concentrations rather than an increase as might be expected. When the diets containing the mixture of 18 amino acids were given, the uptake of amino acids by the tissues appears to have been delayed for at least 1 hour postprandially; and these diets resulted in somewhat less positive nitrogen balance than the casein diets. The diets containing glycine and diammonium citrate or glycine, diammonium citrate and glutamic acid as sources of nonessential nitrogen resulted in slightly negative nitrogen balances, and the uptake of amino acids by the tissues was delayed 2 hours or more postprandially as evidenced by the sustained plasma elevations.

The fact that the diets containing glycine and diammonium citrate or glycine, diammonium citrate and glutamic acid were less well utilized than the diets containing preformed nonessential amino acids may have been caused by the low plasma concentrations of histidine, proline and arginine. It appears these amino acids were synthesized so slowly that plasma concentrations could not be maintained during their dietary absence, and that their low plasma concentrations were responsible for the apparent delay in uptake of the other amino acids. Since histidine and arginine are required by some mammals, it is not unexpected that the plasma concentration of these two amino acids could not be maintained unless they were present in the diet. We have no explanation, however, for the fact that the plasma concentrations of proline decreased so markedly when this amino acid was excluded from the diet.

The lower fasting plasma concentrations of isoleucine, leucine, valine and alanine of subjects consuming experimental diets, compared with self-selected diets, may have been caused by differences in total nitrogen intake. Although the nitrogen content of the self-selected diet was not determined, it was known to be considerably higher than the 6.28 g given in the experimental diets. Some investigators have reported that the concentration of individual amino acids in plasma from healthy, well-nourished adults after an overnight fast are relatively constant in proportion and amount (19, 20). Swendseid et al. (1), however, reported that when the nitrogen intake of men over 55 years of age was decreased from 14 to 3.5 g/day, the plasma concentration of essential amino acids decreased throughout a 5-week period, with valine showing a significant reduction and the concentration of total nonessential amino acids showing an increase after 1 week. No significant changes in plasma amino acids were observed when the nitrogen intake was decreased from 14 to 7 g/day. Arnold et al. (3) reported a significant decrease in the plasma concentration of several essential amino acids and increases in some of the nonessential amino acids when women were fed a low protein diet for 18 days.

Although there was a tendency for total amino acid excretion to be lower when casein was given than when the other diets were given, the difference was not sufficient to account for the better nitrogen retention obtained with casein. The total excretion of amino acids ranged from 1.3% of the intake when the diets containing casein were given to 3.2% when the diet containing glycine and diammonium citrate was given.

The observation that taurine excretion was greatest when the diets contained glycine and diammonium citrate or glycine, diammonium citrate and glutamic acid as sources for the nonessential nitrogen suggests that the amount of urinary taurine was affected either by the absence of the eight preformed nonessential amino acids or by the presence of large amounts of glycine or diammonium citrate. Swan et al. (21) reported that vitamin B_6 deficiency in human subjects resulted in a decrease in taurine excretion in response to a cysteine load dose. Thus, since other factors affect taurine excretion, caution should be used

in relating urinary taurine to vitamin B_6 nutriture.

It is of interest to note that the fasting level of plasma threonine and the amount excreted was significantly increased when large amounts of glycine, or diammonium citrate, or both, were present in the diet. This observation is difficult to explain and may warrant further investigation.

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Relationships Between the Concentration of Liver Metabolites and Ketogenesis in Chickens Fed "Carbohydrate-free" Diets [1]

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ABSTRACT The effects on ketogenesis of feeding growing chickens diets in which all nonprotein calories were supplied by either soybean oil or soybean oil fatty acids were studied. A glucose reference diet was fed for comparison. Concentrations of several metabolites in liver, frozen in situ with the freeze-clamp technique, showed a general increase in those compounds associated with ketogenesis, and a general decrease in precursors of glucose, including citrate, when the "carbohydrate-free" diets were fed. Deletion of dietary carbohydrate decreased the NAD/NADH ratio in liver but oxaloacetate content was not decreased proportionately. In the absence of any significant dietary effect on the specific activity of the acetoacetate synthesis system, citrate synthase or citrate-cleavage enzyme, it is proposed that the utilization of acetyl-CoA shifts from the normal oxidative pathway to ketogenesis when the "carbohydrate-free" diets were fed, because acetyl-CoA is transported out of the mitochondria to the cytoplasm, where acetoacetate synthesis occurs in chicken liver.

Chickens are able to utilize diets in which all nonprotein calories are provided by soybean oil or soybean oil fatty acids, but feeding these "carbohydrate-free" diets, particularly the latter, causes reduced growth rate, hypoglycemia and ketonemia (1, 2). The development of these diets has provided an opportunity to study the effects of chronic carbohydrate insufficiency on liver ketogenesis and gluconeogenesis. Although these two processes are undoubtedly interrelated, only those factors associated with ketogenesis are considered here; aspects of gluconeogenesis will be discussed in a later paper.

Ketogenesis can be considered a normal physiological process whereby 2-carbon units are transferred from liver to other tissue. Synthesis of ketone bodies becomes excessive, however, under conditions which alter carbohydrate metabolism, such as diabetes, fasting, or feeding diets high in fat (3). These conditions increase liver acetyl-CoA concentration (4, 5), but from a regulatory point of view, increased ketogenesis depends upon shifting acetyl-CoA utilization from the normal oxidative pathway to acetoacetate synthesis (6). Since citrate synthase (citrate oxaloacetate-lyase (CoA-acetylating) EC.4.1.3.7) is located at the branching point of these two pathways, the relationship between the activity of this enzyme and ketogenesis has been studied extensively. Citrate synthase is inhibited by long-chain acyl-CoA (6), but the required concentration of long-chain acyl-CoA for inhibition, relative to enzyme concentration, is much greater than occurs in vivo (7). The proposal that the ATP/ADP ratio controls the activity of this enzyme (8) has also been subjected to serious question(9, 10). A more plausible explanation has been offered by Krebs (3, 11), in which it is argued that oxaloacetate concentration in mitochondria is depleted by increased gluconeogenesis and by a shift in the NAD/NADH ratio towards a more reduced state, which would decrease oxaloacetate in favor of malate. This shift in the "redox state" of liver has been shown to accompany increased utilization of fatty acids as an energy source (11, 12).

This study was conducted to determine the effect of chronic carbohydrate insufficiency on the concentration of liver metabolites and the activities of some enzymes involved in acetyl-CoA production and utilization.

METHODS

One-day-old male chickens of a meat strain were fed either a glucose reference diet or diets in which all nonprotein calo-

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ries were supplied by either soybean oil (SO) or soybean oil fatty acids [2] (SOFA), as described by Brambila and Hill (1). The calorie-protein ratio was constant on these diets. The chickens were kept in electrically heated batteries with raised wire floors and given food and water ad libitum.

The chickens were weighed at 3 weeks of age and blood samples were taken from the wing vein using heparin as an anticoagulant. Blood glucose was determined enzymatically on deproteinized whole blood (13). Blood acetoacetate was determined colorimetrically using a modification (14) of Walker's method (15) and β-hydroxybutyrate was measured enzymatically (16) in neutralized perchloric acid extracts of whole blood.

For measurement of steady-state concentrations of liver metabolites, chickens were decapitated during week 4 of the experiment; the body cavity was opened immediately and a lobe of liver was frozen in situ by clamping with tongs precooled in liquid nitrogen. The frozen liver sample was immediately pulverized with a percussion mortar cooled to liquid nitrogen temperature. A neutralized perchloric acid extract of weighed liver powder was prepared (11). The concentrations of the following compounds were determined enzymatically in the neutralized extract by the indicated methods: acetyl-CoA and CoA (17); acetoacetate and β-hydroxybutyrate (18); ADP, ATP, pyruvate, lactate, malate and oxaloacetate (18); and citrate (19). Liver free fatty acids were determined by titration of extracts of frozen liver (20).

The portion of liver, not frozen with the freeze-clamp, was used for enzyme assay. Acetoacetate synthesis activity was measured with an acetyl-CoA generating system (21) in whole homogenates, and the supernatant obtained after cell debris was removed by centrifugation at 500 × *g* for 20 minutes. Citrate synthase (22) and citrate-cleavage enzyme (EC.4.1.3.8) (23) were measured by the indicated methods. Protein was determined colorimetrically (24).

To minimize the possible influence of diurnal variation on these results, blood samples were taken and the chickens were killed between the hours of 9:00 AM and 1:00 PM in all cases. Because of the extreme lability of some liver metabolites, it was not possible to measure all of them simultaneously in the same liver extracts from chickens fed each diet. Therefore, the chickens fed each diet were divided into randomly selected groups for these measurements.

Biochemical reagents for determination of metabolites and enzyme activities were obtained commercially.[3] Phosphate acetyltransferase (acetyl - CoA - orthophosphate acetyltransferase EC.2.3.1.8) and citrate synthase also were purchased from a commercial source,[4] and aconitase (citrate (isocitrate) hydrolyase EC.4.2.1.3), used for citrate determination, was purified from pig heart (25).

RESULTS AND DISCUSSION

The effects of feeding these diets on growth rate, blood glucose concentration and blood ketone bodies (table 1) were in agreement with the results reported by Brambila and Hill using the same diets (1), including the observation that β-hydroxybutyrate was the major circulating ketone body in the chicken. The observed β-hydroxybutyrate/acetoacetate ratio was much higher than has been observed in rats (26), even when the glucose control diet was fed. Although both β-hydroxybutyrate and acetoacetate were increased in response to the "carbohydrate-free" diets, the ratio between the two increased as sources of carbohydrate were deleted from the diet, suggesting that the NAD/NADH ratio in liver, the site of ketogenesis, decreased as fatty acids became more important sources of energy.

Feeding these diets had no direct effect on the specific activity of citrate synthase as measured in vitro [5] (table 2). The activity of the rate-limiting enzyme in

[2] "Wecoline S" kindly provided by Drew Chemical Company, Inc., New York.

[3] Sigma Chemical Company, St. Louis, Mo.

[4] Boehringer-Mannheim Corporation, New York.

[5] An interesting trend was observed in relation to specific activities of the enzymes assayed in that feeding the SOFA diet tended to decrease the specific activity of cytoplasmic enzymes (acetoacetate synthesis system and citrate-cleavage enzyme) but tended to increase the specific activity of mitochondrial citrate synthase. At least part of this effect is probably due to an increased proportion of liver protein in mitochondria when the SOFA diet was fed (27). This would tend to decrease the specific activity of cytoplasmic enzymes because of dilution by mitochondrial protein, but would increase the specific activity of mitochondrial enzymes because of less dilution by cytoplasmic protein.

TABLE 1

Effect of feeding "carbohydrate-free" diets for 3 weeks on body weight, blood glucose and blood ketone bodies

	Diet		
	Glucose	SO [1]	SOFA [1]
Body weight, g	480	337	181
Blood concentration, mg/100 ml			
Glucose	189 ±4 (14) [2]	173 ±4 (15)	150 ±6 (14)
Acetoacetate	0.072±0.025(5)	0.126±0.013(6)	0.210±0.036(7)
β-Hydroxybutyrate	4.9 ±1.5 (8)	16.8 ±1.6 (8)	49.0 ±2.6 (8)

[1] SO = soybean oil; SOFA = soybean oil fatty acids.
[2] Mean ± SEM for number of chickens shown in parentheses.

TABLE 2

Specific activity of citrate synthase and acetoacetate synthesis system as a function of source of dietary energy

	Diet		
	Glucose	SO [1]	SOFA [1]
	mμmoles/min per mg protein		
Citrate synthase	44.6 ±5.3 (6) [2]	41.7 ±9.1 (6)	49.0 ±6.2 (6)
Acetoacetate synthesis			
Whole homogenate	4.29±0.62(5)	4.58±0.29(6)	3.30±0.22(6)
Cell-debris supernatant	6.80±0.58(6)	6.72±0.16(6)	5.95±0.29(6)

[1] See table 1, footnote 1.
[2] Mean ± SEM for number of chickens shown in parentheses.

acetoacetate synthesis, presumbly hydroxymethylglutaryl-CoA hydrolyase (3-hydroxy-3-methylglutaryl-CoA hydro-lyase EC.4.2.1.-18) (28), was certainly not increased and indeed may have decreased in response to the "carbohydrate-free" diets [6] (table 2). Thus, the shifting of acetyl-CoA utilization from the oxidative pathway to ketogenesis is apparently not related to changes in enzyme levels, although it should be noted that the enzyme assays used may not accurately reflect in vivo conditions since any allosteric modifiers may have been diluted or destroyed during enzyme extraction.

Data on the effect of feeding these diets on the steady-state concentration of liver metabolites (table 3) showed a general increase in the concentration of those compounds associated with fatty acid oxidation and ketogenesis as the sources of carbohydrate were deleted from the diet. The increase in liver free fatty acids was paralleled by a change in acetyl-CoA. Liver β-hydroxybutyrate followed the same pattern as blood concentrations, in that feeding the SO diet increased its concentration and feeding the SOFA diet caused a further increase. In contrast to the effects of feeding these diets on blood acetoacetate concentrations, liver acetoacetate concentration appeared to remain essentially unchanged.

The concentration in liver of several gluconeogenic precursors is also shown in table 3. As would be expected, a general decrease in the concentration of those compounds which could serve as a carbon source for glucose synthesis, including citrate, was observed when the "carbohydrate-free" diets were fed. It is significant that feeding these diets has a greater effect on the concentration of ketoacids than on their hydroxy analogues, again suggesting a change in the "redox state" of liver in response to the "carbohydrate-free" diets.

Finally, the effects of feeding these diets on the concentration in liver of free coenzyme A, ATP and ADP, all of which have been proposed to be involved in control of ketogenesis, are shown in table 3. Although

[6] See footnote 5.

TABLE 3

Concentration of liver metabolites in chickens as a function of diet

	Diet		
	Glucose	SO [1]	SOFA [1]
	μmoles/g fresh tissue		
Free fatty acids	11.81 ±0.45 (6) [2]	15.34 ±0.53 (6)	20.95 ±1.36 (7)
Acetyl-CoA	0.014±0.001(6)	0.022±0.002(6)	0.025±0.002(6)
Acetoacetate	0.015±0.006(5)	0.027±0.012(5)	0.016±0.006(5)
β-Hydroxybutyrate	0.41 ±0.16 (5)	1.44 ±0.43 (5)	2.37 ±0.16 (5)
Lactate	5.54 ±0.54 (5)	5.82 ±0.93 (5)	4.10 ±0.42 (5)
Pyruvate	0.048±0.025(5)	0.029±0.009(5)	0.010±0.003(5)
Malate	2.61 ±0.43 (5)	2.38 ±0.38 (5)	1.76 ±0.32 (5)
Oxaloacetate	0.020±0.002(5)	0.022±0.008(5)	0.012±0.003(5)
Citrate	2.43 ±0.27 (5)	2.03 ±0.42 (5)	1.76 ±0.27 (5)
Coenzyme A	0.095±0.016(6)	0.108±0.015(6)	0.114±0.016(6)
ATP	0.96 ±0.13 (4)	1.28 ±0.10 (4)	0.93 ±0.21 (4)
ADP	0.94 ±0.19 (4)	0.89 ±0.13 (4)	0.76 ±0.12 (4)

[1] See table 1, footnote 1.
[2] Mean ± SEM for number of chickens shown in parentheses.

the concentration of coenzyme A has been shown to be critical in acetoacetate biosynthesis in vitro (29), feeding these diets had only minor influence, if any, on the concentration of free coenzyme A in liver, and thus was not correlated with ketogenesis. The proposal that the ATP/ADP ratio controls citrate synthase activity (8) has been questioned as to relevance to in vivo conditions (9, 10). The effect of feeding these diets on the concentration of ATP and ADP or on their ratio are not consistent with the change in citrate content or ketogenesis, and thus, are not likely to be responsible for shifting acetyl-CoA utilization from oxidation to the synthesis of acetoacetate.

The data on substrate concentration in liver are based on extracts of whole liver and therefore do not indicate subcellular distribution, yet subcellular distribution of substrates and enzymes is undoubtedly critical to the control of ketogenesis. For example, the data in table 3 show that the concentration of oxaloacetate in liver decreased when the SOFA diet was fed, but the more fundamental question is what is the effect of feeding these diets on mitochondrial oxaloacetate. Krebs and Williamson et al. (3, 11) contend that limiting carbohydrate available to the liver decreases the mitochondrial NAD/NADH ratio, which in turn decreases mitochondrial oxaloacetate by shifting the equilibrium of the malate–oxaloacetate couple toward the more reduced state.

Unfortunately, methods are not yet available which allow a reliable, direct determination of NAD or NADH within subcellular compartments but the ratio of these two compounds can be calculated from the data in table 3 if certain assumptions are made (11). If, for example, it is assumed that β-hydroxybutyrate dehydrogenase is sufficiently active to cause equilibration of the acetoacetate-β-hydroxybutyrate couple,[7] the mitochondrial NAD/NADH ratio can be calculated. Similarly, if lactate dehydrogenase is sufficiently active to cause equilibration of the lactate-pyruvate couple, the cytoplasmic NAD/NADH ratio can be calculated. Such calculations (table 4) show that the "redox state," as measured by the NAD/NADH ratio, becomes more reduced in both cytoplasm and mitochondria as carbo-

TABLE 4

Calculation of the NAD/NADH ratio in chicken liver mitochondria and cytoplasm as a function of diet

	Diet		
	Glucose	SO [1]	SOFA [1]
Mitochondria	0.75	0.38	0.14
Cytoplasm	78.1	44.9	22.0

[1] See table 1, footnote 1.

[7] Lack of equilibration of this reaction would cause the calculated NAD/NADH ratios to be too high because acetoacetate is an obligatory precursor of β-hydroxybutyrate (30). Other assumptions such as ionic strength, pH, etc., and their effects on these calculations have been previously discussed (11).

hydrate is deleted from the diet. As might be expected from comparing the β-hydroxybutyrate acetoacetate ratio in blood of chickens (table 1) with that observed in rats (26), the calculated NAD/NADH ratio in both mitochondria and cytoplasm of chicken liver (table 4) was 10 times lower than that reported for rats (11). This difference in the "redox state" of liver of chickens and rats probably accounts for the early erroneous concept that the chicken embryo does not become ketotic, since measurement of keto compounds only was attempted (31). Recent determinations in this laboratory show that the acetoacetate concentration in pooled blood samples from 1-day-old, unfed chickens was only 0.05 mg/100 ml while β-hydroxybutyrate concentration, measured enzymatically (16), was 16 mg/100 ml.

The significance of the change in "redox state" of the liver in relation to acetyl-CoA utilization depends upon how these changes affect mitochondrial oxaloacetate. The expected oxaloacetate concentration could be calculated from the NAD/NADH ratios if the subcellular distribution of malate were known (11). Although malate is permeable to the mitochondrial membrane (32) and is, therefore, probably distributed throughout the cell, for purposes of calculation it was assumed that all the observed malate was either mitochondrial or cytoplasmic (table 5). Even though the actual distribution of malate is undoubtedly intermediate between these two extremes, calculation of the expected oxaloacetate using these extreme possibilities shows that the observed oxaloacetate concentration was at least 3 to 10 times greater than that expected, depending on diet. This means that the malate-oxaloacetate couple is not in equilibrium in either the mitochondria, the cytoplasm, or both; of more importance, it means that malate is not the only major source of oxaloacetate, since the concentration of malate and oxaloacetate are far removed from equilibrium conditions in favor of oxaloacetate, especially in the liver of chickens fed the SOFA diet. In addition to malate, the only other direct net precursors of oxaloacetate are pyruvate and aspartate. Although amino acids undoubtedly contribute carbon for gluconeogenesis when the "carbohydrate-free" diets are fed (33), for aspartate to be the other major source of oxaloacetate, it would have to contribute 10 times more carbon to the oxaloacetate pool when the SOFA diet was fed than the several amino acids that are utilized, via malate, contribute to the malate pool. Such a disproportionate contribution of gluconeogenic carbon from aspartate is unlikely. Because of the change in pyruvate concentration (table 3) and the importance of pyruvate as a glucose precursor (34), it is more probable that pyruvate is a major, direct contributor to oxaloacetate. This conclusion is significant because the direct conversion of pyruvate to oxaloacetate would provide mitochondrial oxaloacetate since pyruvic carboxylase (pyruvate–CO_2 ligase (ADP) EC.6.4.1.1) occurs in the mitochondrion in chicken liver (35). Mitochondrial oxaloacetate could condense with acetyl-CoA, produced by β-oxidation of fatty acids, to form citrate. This condensation may be decreased somewhat by feeding the "carbohydrate-free" diets, however, because of increased competition

TABLE 5

Comparison of observed oxaloacetate concentration in liver with the expected concentration calculated from the NAD/NADH ratios and the equilibrium constant (11)

	Diet		
	Glucose	SO [1]	SOFA [1]
		mμmoles/g	
Oxaloacetate observed	20 ± 2	22 ± 8	12 ± 3
Oxaloacetate expected if all malate were:			
Mitochondrial	0.055	0.025	0.008
Cytoplasmic	5.7	3.0	1.3

[1] See table 1, footnote 1.

among mitochondrial enzymes for which oxaloacetate serves as substrate. For example, phosphoenolpyruvate carboxykinase (GTP–oxaloacetate carboxy-lyase (transphosphorylating) EC.4.1.1.32) is located predominately in mitochondria in chicken liver (36). The specific activity of malate dehydrogenase (L-malate–NAD oxidoreductase EC.1.1.1.37) is at least five times greater in mitochondria than cytoplasm [8] and, with the change in the mitochondrial NAD/NADH ratio, would be expected to decrease oxaloacetate in favor of malate. Thus, feeding the "carbohydrate-free" diets would be expected to increase the synthesis of both oxaloacetate (from pyruvate) and acetyl-CoA (from fatty acids) in the mitochondrion, but would also increase the utilization of oxaloacetate. The net effect would be dependent upon how effective citrate synthase is in competing for oxaloacetate but this point can only be clarified by turnover-rate studies. Even if citrate synthesis is not decreased by feeding the "carbohydrate-free" diets, its further oxidation may be decreased since NAD-linked isocitrate dehydrogenase (L_s-isocitrate–NAD oxidoreductase (decarboxylating) EC.1.1.1.41) is inhibited by NADH (37).

With such competition for mitochondrial oxaloacetate, it is probable that much of the unexpectedly large amount of oxaloacetate observed (table 5) is present in cytoplasm. The only direct cytoplasmic precursor of oxaloacetate, however, in addition to malate and aspartate, is citrate, through the action of citrate-cleavage enzyme. Citrate-cleavage enzyme has been proposed to be involved in transfer of oxaloacetate out of the mitochondrion for gluconeogenesis (38) and of acetyl-CoA for lipogenesis (39). Although this enzyme is induced by conditions which stimulate lipogenesis (40), it was still active even when the SOFA diet was fed (table 6). The activity was somewhat reduced by feeding the "carbohydrate-free" diets but the differences were not statistically significant [9] by analysis of variance (41). Generation of cytoplasmic oxaloacetate by this route would require an initial synthesis of citrate from mitochondrial oxaloacetate and acetyl-CoA so that the net effect of such a reaction sequence would be the transport of both oxaloacetate and acetyl-CoA across the mitochondrial membrane. This possibility is particularly intriguing in view of the evidence, to be published elsewhere, that most of the activity of the acetoacetate synthesis system is located in the cytoplasm of chicken liver. Recent evidence indicates that there is also acetoacetate synthesis activity in rat liver cytoplasm (28). Diffusion of citrate out of the mitochondrion, followed by cleavage, would effectively shift the utilization of acetyl-CoA from the oxidative pathway to acetoacetate synthesis, and thus, account for increased ketogenesis in response to the "carbohydrate-free" diets even though there was no change in the activity of either citrate synthase or the acetoacetate synthesis system.

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[8] Allred, J. B., and K. L. Roehrig, unpublished observations.
[9] See footnote 5.

TABLE 6

Specific activity of citrate-cleavage enzyme as a function of dietary energy source

	Diet		
	Glucose	SO [1]	SOFA [1]
	mμmoles NADH formed/min per mg protein		
Citrate-cleavage enzyme	17.7±2.8(6) [2]	15.8±3.6(6)	11.6±1.0(6)

[1] See table 1, footnote 1.
[2] Mean ± SEM for number of chickens shown in parentheses.

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Respiration of Isolated Liver Mitochondria from Chickens Fed "Carbohydrate-free" Diets [1]

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ABSTRACT One-day-old chickens were fed either a glucose control diet or diets in which all nonprotein calories were provided by either soybean oil or soybean oil fatty acids. Since feeding these "carbohydrate-free" diets has previously been found to reduce the liver mitochondrial NAD/NADH ratio without altering the ATP/ADP ratio in liver extracts, the possibility was considered that either the rate or efficiency of oxidative phosphorylation in liver mitochondria was adversely affected by feeding these diets. The rates of oxygen uptake per milligram of mitochondrial protein (in the presence and absence of added ADP) and the P/O ratios were measured in isolated mitochondria using either succinate, malate, α-ketoglutarate or citrate as electron donors. These parameters did not change as a function of diet. There was, however, an increase in the rate of NADH oxidation in uncoupled mitochondria when the "carbohydrate-free" diets were fed, and an increase in mitochondrial protein in response to feeding the soybean oil fatty acid diet. It is concluded that the change in the NAD/NADH ratio in liver mitochondria in response to feeding the "carbohydrate-free" diets is not directly attributable to a simple mitochondrial lesion.

Conditions which stimulate gluconeogenesis and ketogenesis have been shown to cause an increase in the NAD/NADH ratio in liver mitochondria of both chickens (1) and rats (2). The control of the concentration of mitochondrial NADH is undoubtedly very complex (2), in part because there are several mechanisms whereby electrons are removed from mitochondria. Electrons can be transported to the cytoplasm by the malate-oxaloacetate (3) and the α-glycerolphosphate-dihydroxyacetone phosphate (4) shuttles, which decreases the cytoplasmic NAD/NADH ratio. Formation and subsequent diffusion of β-hydroxybutyrate into blood also removes electrons from mitochondria. Despite increased removal of electrons by these mechanisms, when "carbohydrate-free" diets were fed to chickens, the calculated NAD/NADH ratio in mitochondria still decreased (1).

From equilibrium considerations, a decreased NAD/NADH ratio would be expected to increase the ATP/ADP ratio in liver, but analysis of extracts of liver from chickens fed "carbohydrate-free" diets showed no apparent correlation between "redox state" and ATP or ADP content (1). Since diet has been shown to influence oxidative phosphorylation (5, 6), the possibility was considered that feeding "carbohydrate-free" diets adversely affected either the rate or efficiency of oxidative phosphorylation. The rates of oxidation of NADH and NADH-linked substrates and of succinate, by mitochondria isolated from liver of chickens fed either a glucose control diet or diets in which all nonprotein calories were provided by soybean oil (SO) or soybean oil fatty acids (SOFA), were studied. The results indicate that the shift in the NAD/NADH ratio cannot be attributed to mitochondrial lesion.

METHODS

Diets and treatment of chickens were previously described (1). The chickens were decapitated after they were fed the diets for 3 weeks. Liver was removed immediately and mitochondria were isolated in mannitol preparation media, pH 7.4, by the method of Ito and Johnson (7), except that the centrifugation force was 10,000 × *g* for mitochondrial precipitation. The isolated mitochondria were incubated in an isotonic reaction medium (7) to which was added 50 μmoles of either succinate, malate, α-ketoglutarate or citrate. These substrates were stored frozen at pH 7.4

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until they were used. The oxygen content of the reaction mixture was monitored continuously during incubation in a closed cell using a Clarke oxygen electrode connected to a recorder. The rate of oxygen uptake was measured in the absence and presence of added ADP (37 mμmoles, assayed by the method of Hohorst et al. (8)). The rate of oxygen uptake was also measured using a hypotonic reaction mixture (7) to uncouple oxidative phosphorylation, with 0.6 μmole NADH as the electron donor. Protein was determined colorimetrically (9).

RESULTS

The data in table 1 show, in terms of oxygen uptake per milligram of mitochondrial protein, there was no dietary effect on the rate of succinate oxidation under state 3 (ADP present) or state 4 (ADP absent) conditions. In general, the same results were obtained when the NADH-linked substrates (malate, α-ketoglutarate and citrate) served as the source of electrons, except that the rates of oxygen uptake were lower than was observed with succinate. These data indicate that feeding the "carbohydrate-free" diets did not adversely affect the rate of electron transport in isolated mitochondria. Further, the efficiency of oxidative phosphorylation as measured by the P/O ratio, was similarly unaffected by diet, regardless of the electron donor. The P/O ratio for succinate was very close to the theoretical value of 2 although the ratios for the NADH-linked substrates were somewhat below the theoretical value of 3.

Respiratory control values (rate state 3/rate state 4) were calculated as a measure of mitochondrial damage during isolation. The observed values were lower than those ordinarily reported for rats (5), but it is not clear whether this is due to inherent properties or greater fragility of chicken liver mitochondria. There was no apparent effect of diet on respiratory control values.

Although succinate and malate were readily oxidized, α-ketoglutarate and citrate served as electron donors only when catalytic amounts of NADH were added to the reaction mixture. Even then, the latter two substrates were poorly utilized, especially by mitochondria from liver of

TABLE 1

Effect of diet on rate and efficiency of oxidative phosphorylation in chicken liver mitochondria

Diet	No. of chickens	Oxygen uptake		P/O ratio	Respiratory control
		mμmoles O_2/min per mg protein			
		State 3 [1]	State 4 [2]		
		substrate – succinate			
Glucose	4	19.82 ± 3.49 [3]	6.74 ± 1.1	1.93 ± 0.05	2.94
SO [4]	5	19.09 ± 2.95	8.05 ± 0.87	1.92 ± 0.14	2.35
SOFA [4]	5	19.13 ± 1.29	6.96 ± 0.54	1.92 ± 0.13	2.87
		substrate – malate			
Glucose	4	11.54 ± 1.96	5.00 ± 0.72	2.23 ± 0.11	2.30
SO	5	11.78 ± 1.82	5.17 ± 0.59	2.38 ± 0.16	2.34
SOFA	5	11.64 ± 1.92	4.34 ± 0.60	2.25 ± 0.17	2.68
		substrate – α-ketoglutarate			
Glucose	3	11.10 ± 1.70	6.25 ± 1.67	1.92 ± 0.62	1.97
SO	2	18.88	7.38	2.46	2.53
SOFA	5	12.50 ± 1.55	5.71 ± 0.94	2.01 ± 0.21	2.23
		substrate – citrate			
Glucose	3	8.34 ± 1.22	4.72 ± 1.04	2.09 ± 0.19	1.86
SO	1	11.89	5.66	2.60	2.10
SOFA	3	10.39 ± 1.54	6.64 ± 2.53	2.33 ± 0.45	1.99

[1] ADP present.
[2] ADP absent.
[3] Mean ± SEM.
[4] SO = soybean oil; SOFA = soybean oil fatty acid.

chickens fed the SO diet; these substrates, however, were utilized as well by mitochondria from liver of chickens fed the SOFA diet as they were by mitochondria from liver of chickens fed the glucose control diet.

The rate of oxygen uptake in uncoupled mitochondria when NADH served as the source of electrons was actually increased when the SO or SOFA diets were fed (table 2). This unexpected result is certainly not consistent with an adverse effect of the "carbohydrate-free" diets on rates of electron transport.

Analysis of variance (10) indicated that feeding the SOFA diet significantly ($P < 0.05$) increased mitochondrial protein, but did not change total protein (table 3). In contrast, feeding the SO diet apparently increased total protein (although not significantly) without altering mitochondrial protein. These interesting effects of diet on liver protein are not correlated with changes in the "redox state" of liver (1). Further, the increased mitochondrial protein in response to feeding the SOFA diet would mean that rates of oxidation of the substrates tested were increased by feeding this diet when calculated in terms of oxygen uptake per gram of liver.

TABLE 2

Rates of oxygen uptake in uncoupled mitochondria with NADH as electron donor

Diet	
	mμmoles O_2/min per mg protein
Glucose	3.17 ± 0.7 (5) [1]
SO	4.25 ± 0.2 (5)
SOFA	5.12 ± 0.55(5)

[1] Mean ± SEM for number of chickens shown in parentheses.

DISCUSSION

The decrease in the mitochondrial NAD/NADH ratio that occurred when chickens were fed "carbohydrate-free" diets (1) cannot be attributed to a decrease in either the rate or the efficiency of oxidative phosphorylation. The only data that could be interpreted as indicative of some type of mitochondrial lesion was the increase in mitochondrial protein when the SOFA diet was fed (table 3). A similar increase in mitochondrial protein (as well as in number of mitochondria) has been observed in response to riboflavin deficiency (6). This effect of riboflavin deficiency, however, is accompanied by a decreased rate of oxygen uptake and a decreased P/O ratio, neither of which were observed when the "carbohydrate-free" diets were fed. Further, the ATP content of liver from riboflavin-deficient animals was markedly reduced,[2] again in contrast to the effect of "carbohydrate-free" diets on liver ATP content (1).

The increase in the rate of NADH oxidation in uncoupled mitochondria (table 2) may also indicate some type of mitochondrial lesion, but the effect is the wrong direction to account for a decreased NAD/NADH ratio. It is of interest that feeding diets low in casein has been reported to increase the rate of succinate oxidation under state 3 (but not state 4) conditions (5). This effect, which increases respiratory control values, was reported to be correlated with mitochondrial swelling as indicated by electron microscopy, but is apparently not analogous to the effect of feeding the SO or SOFA diets.

Although the rates of oxidation of succinate and of NADH-linked substrates in vitro were not adversely affected by feed-

[2] Burch, H. B., M. E. Brodley and P. Mox 1967 Metabolic intermediates in riboflavin-deficient rat liver. Federation Proc., 26: 863 (abstract).

TABLE 3

Total and mitochondrial protein of liver as a function of diet

	Diet		
	Glucose	SO [1]	SOFA [1]
		mg/g liver	
Mitochondrial protein	24.6 ± 4.5(4) [2]	24.6 ± 1.9(5)	33.8 ± 1.5(5)
Homogenate protein	248 ±15 (6)	292 ±11 (6)	261 ±16 (6)

[1] See table 1, footnote 4.
[2] Mean ± SEM for number of chickens shown in parentheses.

ing the "carbohydrate-free" diets, the apparent increase in the NADH content of mitochondria from liver of chickens fed these diets could be due to compartmentalization of either NADH or ADP in vivo. The available evidence indicates, however, that there is only one NAD-NADH pool within the mitochondrion which is in contact with enzymes that are located in the cristae and matrix (2). On the other hand, no evidence is yet available on the subcellular distribution of ATP or ADP. Although there does not appear to be a direct correlation between the concentration of ATP in liver extracts and "redox state," there may be localized effects of these diets on the ATP/ADP ratio within the mitochondrion.

Another plausible explanation for the decrease in the NAD/NADH ratio without a concomitant rise in the ATP/ADP ratio is that the rate of production of NADH exceeded the rate of its utilization, even when the glucose diet was fed. Thus, increasing the production of NADH would not increase the rate of electron flow through oxidative phosphorylation. The specific activity of mitochondrial malate dehydrogenase is 6.5 μmoles of NADH formed per minute per milligram of mitochondrial protein.[3] This rate far exceeds the rate of oxygen uptake when malate served as substrate (table 1). This comparison adds validity to the contention that the rate-limiting step in energy production may be in oxidative phosphorylation rather than NADH formation, but the comparison is based upon data obtained in vitro under optimum assay conditions and therefore may not be completely valid in vivo.

Increased utilization of fatty acids as an energy source could obviously account for a greater production of electrons within the mitochondrion. It is clear from the data presented here that the lack of a more rapid utilization of these electrons is not explicable in simple terms of a decreased rate or efficiency of mitochondrial oxidative phosphorylation. A deeper understanding as to why these diets influence the "redox state" of liver without a concomitant alteration in the ATP/ADP ratio must await further elucidation of the complex control mechanisms involved in oxidative phosphorylation.

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[3] Allred, J. B., and K. L. Roehrig, unpublished observations.

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The words "et al.," "per se" and "ad libitum" are not underscored. In footnotes giving details of composition of diets, the repeated use of a unit may be avoided by stating the common unit used, as "the diet consisted of the following: (in grams) sucrose, 50; casein, 130; . . ." or "8, 12 and 14%." The expression "fed a diet" is preferred to "on a diet" or "given a diet." Certain other preferred usage is described in the *Style Manual for Biological Journals*.[1] Avoid the use of laboratory jargon and such phrases as "it can be seen that," "it is interesting that," and "it can be noted that."

Numbers: Use arabic numerals, not Roman or ordinal numerals, throughout, including those in tables and figures (e.g., group 3, not the third group or group III; table 1, not table I). If possible, avoid beginning a sentence with a numeral or a symbol.

Statistical methods: Tests of statistical significance should be identified, and references used should be cited. Statements that results are statistically significant should be accompanied (in text, tables, and figure legends) by indications of the level of significance (as P, the probability of the event's being due to chance alone, a decimal quantity; or as α, the level of significance, a percentage).

Units of measure, abbreviations, symbols, and nomenclature: The metric system is used for all units, and temperature is expressed in the centigrade scale (degree sign without C). Letters in abbreviations such as NAD and IU are not spaced; periods are omitted except when the abbreviation might be read as another word. *Do not* add "s" when the plural is meant. Following is a list of the more common abbreviations and symbols used in the Journal:

average	avg (*tables only*)
calorie ("small," gram calorie)	cal
Calorie ("large," kilogram calorie)	*not used; use* kcal
centimeter	cm
counts per minute	cpm
cubic millimeter	mm^3
degree	° (*omit* C)
degrees of freedom	df (*tables only*)
gram	g
hour	hr (*tables only*)
international unit	IU
kilocalorie	kcal
kilogram	kg
liter	(*spell out*)
meter	m
microcurie	μCi
microgram	μg (not γ)
microliter	μl (not λ)
micromicrogram	picogram, pg (preferred to μμg)
micron (10^{-6} meter)	μ
micromolar (concentration)	μM
micromole (mass)	μmole (never μM)
millicurie	mCi
milligram	mg
milligrams %	(*never use; use* mg/100 mg, mg/100 ml, *or* mg/100 g, *as appropriate*)
milliliter	ml (*not* cm^3 *or* cc)
millimeter	mm
millimicrogram	nanogram, ng (preferred to mμg)

[1] Conference of Biological Editors, Committee on Form and Style 1964 Style Manual for Biological Journals, ed. 2. American Institute of Biological Sciences, Washington, D.C.

millimicron (10^{-9} meter)	mμ
millimole	mmole
minute	min (*tables only*)
molar (moles per liter)	M
mole	mole (*never* M)
nanogram (10^{-9} g)	ng
parts per million	ppm
percent	%
picogram (10^{-12} g)	pg
probability (statistics)	*P*
second	sec (*tables only*)
square centimeter	cm^2
square meter	m^2
square millimeter	mm^2
standard deviation	SD
standard error	SE
standard error of the mean	SEM
t (statistics)	*t*
weight	wt (*tables only*)
year	yr (*tables only*)

Other commonly accepted abbreviations may be found in the *Style Manual for Biological Journals*. In general, chemical and biochemical terms and abbreviations should conform with the usage recommended by the International Union of Pure and Applied Chemistry (IUPAC) and its committees on nomenclature. Names of chemicals are written out in the text; formulas may be used in tables or figures, or both. When appropriate, identify enzymes with their EC number and systematic name.[2]

Isotope specification should conform to the IUPAC system, with the mass number placed as a superscript preceding the chemical symbol, as ^{14}C, *not* C^{14}.

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Follow the style in recent issues of the Journal for references. Do not underscore titles of journals or books; do underscore volume numbers of journals. All references should be double-spaced. Names of journals should be abbreviated in accordance with the list of abbreviations in the *Style Manual for Biological Journals*.

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6. Source of materials, feeds, instruments, etc. (name, model number, manufacturer's name and location (city and state)).
7. Source of laboratory animals (same information).
8. Composition of diets, or vitamin or mineral mixtures, if not presented in a table.
9. References to abstracts, verbal reports or other unedited material.
10. Personal communications and unpublished experiments, including names of information sources and year.

[2] The International Union of Biochemistry Commission of Editors of Biochemical Journals 1965 Enzyme Nomenclature. Elsevier Publishing Company, Amsterdam, London and New York.

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