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FREDRICK ACCUM

From an engraving by J. Thomson, after a painting by S. Drummond, published in the European Magazine for July, 1820.

Fredrick Accum
— A Biographical Sketch

(March 29, 1769 — June 28, 1838)

The name of Fredrick Accum is very well known to readers who know the pleasure to be derived from the leisurely examination of old books on chemistry. From 1803 to 1821, this self-employed "operating chemist," as he called himself, was the author of 20 books, most of which dealt with applications of chemistry to manufacturing and the arts. Included among these early and often the first books in the modern manner were treatises on brewing, bread production, wine making, and the chemistry of cooking. Among his works also were a treatise on crystallography, a two-volume system of chemistry, a monograph on chemical reagents and tests, and a little book on chemical amusements which was regarded as a treasure by amateur scientists for generations. Probably his most important book was his volume on food adulteration, although another, a treatise on the production of coal gas and its applications to the lighting of buildings and streets, is a classic in the history of chemical technology. Some of his works went through several editions, in America as well as England where he lived, and some were translated into German, French and Italian. In addition to the recognition which he gained from these well-planned and splendidly written treatises, Accum acquired a reputation as a teacher, analyst, and a popularizer of science. He was instrumental in the establishment of a new profession, that of the consulting chemist who applies the discoveries of the laboratory to industry and the common purposes of human life. Though he wrote a number of papers on original research, it is for his writings and educational activities that his name is best remembered.

Accum lived in an age which was graced by many illustrious scientists, but it is said that he became better known than most of his contemporaries. Each year from about 1803 to 1821 seemed to add new luster to his name and probably some new book to his list of publications. He was described as a "pet chemist" of London. His activities, particularly those to expose the adulteration of foods and drugs, caused the *European Magazine* to run a laudatory article about him in their issue of July, 1820. It is from this article that the illustration of the present sketch has been taken. Then, at the peak of his career, when he was 52 years old, he became involved in one of those situations which can only bring dismay to a man's friends—a petty affair which will be described later—from which he extricated himself by fleeing England in disgrace. This was early in 1821. The remainder of his life was spent in Germany, where he became within a year associated with two technological institutions in Berlin, and where he seems to have been highly regarded. In 1826 he published in German one outstanding book in connection with his new teaching duties, a book on the chemistry and physics of building materials. He also wrote from time to time various articles, but it is said that he published them anonymously or under the name Mucca, which is Accum spelled backwards. His life of extraordinary activity and influence was essentially at an end when he made the decision to leave England.

The circumstances of Accum's life as well as his services to chemistry have long attracted the attention of historically minded chemists. Information about him was fragmentary or scattered, because after his downfall there was a tendency to try to forget his very existence. The publisher of his books even left his name off the title page of a new printing of one of his works. The late Dr. Charles A. Browne, however, a number of years ago gathered together the available informa-

tion on Accum, obtained much new material, and wrote a sympathetic account of his life and contributions. It is now possible to summarize briefly the essential facts of Accum's life, and to emphasize the significance of his contributions to the broad field of nutrition, more informatively than otherwise would have been possible.

The subject of the present sketch was born in Bückeberg, a small town near Hanover, Germany, on March 29, 1769. He was baptized Friedrick Christian Accum, though later he dropped the middle name and anglicized the spelling of the first. His father was a Jew, named Herz Marcus, who embraced Christianity, changed his name to Christian Accum (the name Accum is said to mean Gentile) and married a girl who was a French Huguenot. She assumed the care of the family when her husband died in 1772, when Fredrick, one of seven children of whom but three lived to maturity, was three years old. The family was engaged in the manufacture of soap. The boy Christian was schooled at the local gymnasium, which appears to have been staffed by unusually competent instructors who taught the classical studies of the times. While still a youth, he was apprenticed to the pharmaceutical firm of Brande in nearby Hanover. This firm served as royal apothecaries to George III, King of England and Hanover, and they operated establishments in both Hanover and London. In 1793, young Fredrick went to London, where he was employed as an assistant and later as a chemist in the Brande organization. He learned to write fluent English, and he continued his studies of chemistry. He made the acquaintance of William Nicholson and Anthony Carlisle, two scientists of distinction who were his friends during his formative years and later. It was Sir Anthony Carlisle who came to his defense when he became involved in the difficulties that clouded his name—but this unhappy event was then far in the future. In 1798 he married, and struck out on his own as an independent consulting chemist in London.

Before 1800, young Accum had contributed three little articles to Nicholson's *Journal*, one of these being on methods for the chemical testing of the purity of drugs. This subject attracted his attention throughout his London career. He had a laboratory in his home, and he continued work there even though, in 1801, he accepted an appointment as an assistant at the newly founded Royal Institution in London. Here he remained three years, assisting Humphry Davy, who was several years his junior. While still employed by the Royal Institution, Accum wrote his first book. It was called "A System of Theoretical and Practical Chemistry," and it is remarkable because it is the first textbook on chemistry which was arranged to help the student trying to learn the subject. Accum, being then relatively unknown, secured subscribers and published the book himself. He dedicated it to the managers of the Royal Institution. Because of the circumstances of his later disgrace, it is of interest to note that when he left the Royal Institution, the managers expressed their appreciation of his services in their minutes.

From 1803 to early 1821, as has been mentioned, Accum was extraordinarily busy. At his home on Old Compton Street in the Soho section of London, he not only operated a laboratory but also conducted a school for the training of students in both theoretical and practical phases of chemistry. In addition to his writing and his consultation work, he conducted a business of supplying chemicals and apparatus. Beginning in 1809 and for the duration of his London life, he was a lecturer at another technological institution in London. It was there that he delivered his public lectures on applied chemistry before large audiences. For their benefit, he wrote "Outlines of a Course of Lectures on Chemistry Applied to the Arts and Manufactures," which contained blank pages interleaved on which the students could write their notes. This book also carried advertising pages which mentioned that complete portable laboratories, the contents of which would not spill or break if overturned in transport, could be purchased from the author.

To understand a man it is necessary to consider the times in which he lived. Accum's span of years, from 1769 to 1838, was a period marked by a succession of wars and revolutionary social changes. During his lifetime, America won its independence, the French revolution occurred, and Napoleon rose to military heights,

shook all Europe to its political foundations, and died in exile. In the very year in which Accum was born, James Watt had perfected the steam engine which made possible the subsequent industrial revolution. All during Accum's productive years scientific discoveries were in the news, and there was tremendous public interest in each new report. There were also expressions of hope for the prompt application of each new development to human affairs. Yet practical instruction in the experimental sciences was not given by the universities. Medical schools did offer their students lectures in chemistry, but almost no laboratory work. The only way for a young man to gain any training in the practical operations of the laboratory was to try to do experiments himself, or to become apprenticed to a pharmacist. It is not surprising that Count Rumford was able to find support for the creation in 1800 of the Royal Institution, which was planned to fill a need for both research and instruction in science and technology. It was Rumford himself who hired Accum as well as Humphry Davy. In a short time Davy was lecturing to audiences—paying audiences —of a thousand persons, many of whom were socially prominent Londoners; and, assisting Davy in the demonstrations, there was Fredrick Accum.

Now the Royal Institution had been conceived by Rumford on a scale which, if fully developed as someone has said, could have been financed only by an interested government with considerable funds. But it was a private institution, and the demand then as it always has been, was for something practical. Davy, with the vision of the genius which he was, and with the audacity of youth, had demanded that his time in research be largely devoted to fundamental problems. When requested he did engage in studies on tanning, and on problems of agriculture, and afterwards in still other applied subjects, but his interests were in science for its own sake. It was not long before the objectives of the Royal Institution had to be limited; this was at about the time when Rumford went to France and Accum left the organization. It would appear from his life's work that Accum decided to devote his attention to some of the applications of science which the Royal Institution in large measure had been obliged to abandon. For him this was a wise decision. To a large extent modern food technology can point to Accum as one of the first of those who introduced scientific methods in the operations of the food and beverage industries.

Of course the writings of Accum on subjects of food technology are now primarily of historical interest. There are, however, tidbits of interesting information to be gleaned from an examination of his books on brewing, wine making, and the production of bread. For example, the latter volume contains these statements:

> The discovery of the application of yeast, to improve the panification of bread flour, was made and first secretly adopted by the bakers of Paris, but when the practice was discovered, the College of Physicians there, in 1688, declared it prejudicial to health, and it was not till after a long time that the bakers succeeded in convincing the people, that bread made with yeast was superior to bread made with sour dough or leaven.

There is evidence in this book that Accum did considerable experimental work on baking. He pointed out that flour which was aged absorbed more water in making a dough, and produced better bread. He mentioned admiringly the skill of a team of five men, working with military precision—a moulder, a marker, a splitter, a chucker, and a depositor—who made biscuits at the rate of 70 a minute.

Looking at Accum's numerous books today, one may come across some quotable statement almost anywhere.

From his book on a system of chemistry:

> . . . mere facts do not constitute a science.

From his book on culinary chemistry:

> Cookery, or the art of preparing good and wholesome food, and preserving all sorts of alimentary substances in a state fit for human sustenance, of rendering that agreeable to the taste which is essential to the support of life, and of pleasing the palate without injury to the systems, is, strictly speaking, a branch of chemistry . . .

From his book on adulterations:

> Children are apt to put every thing, especially what gives them pleasure, into their mouths; the painting of toys with colouring substances that are poisonous,

> ought therefore to be abolished; a practice which lies the more open to censure, as it is of no real utility (p. 344).

The book on adulterations contains many facts of interest concerning the possible contamination of foods with lead. It is difficult to think of the following observations as having been made nearly 150 years ago:

> The baking of fruit tarts in cream-coloured earthenware, and the salting and preserving of meat in leaden pans, are no less objectionable. All kinds of food which contain free vegetable acids, or saline preparations, attack utensils covered with a glaze, in the composition of which lead enters as a component part. The leaden beds of presses for sqeezing the fruit in cider countries, have produced incalculable mischief (p. 343).

In describing the possible contamination of soda water, he wrote:

> . . . the great excess of carbonic acid which the water contains, particularly enables it to act strongly on the metallic substances of the apparatus; a truth, of which the reader will find no difficulty in convincing himself, by suffering a stream of sulphuretted hydrogen gas to pass through the water (p. 308).

The full title of Accum's classic in food adulteration was "A Treatise on Adulterations of Food, and Culinary Poisons." Like most of his books, it bore a descriptive subtitle, which read: "exhibiting the fraudulent sophistications of bread, beer, wine, spirituous liquors, tea, coffee, cream, confectionery, vinegar, mustard, pepper, cheese, olive oil, pickles, and other articles employed in domestic economy, and methods of detecting them." Actually, the book contained sections on the adulteration of pigments and of drugs as well. The author was described on the title page as "Fredrick Accum, operative chemist, lecturer on practical chemistry, mineralogy, and on chemistry applied to the Arts and Manufactures; Member of the Royal Irish Academy; Fellow of the Linnaean Society; Member of the Royal Academy of Sciences, and of the Royal Society of Arts of Berlin, &c, &c." Illustrating the title page was a drawing. It was a figure of an urn on which rested a skull, partly covered by a cloth. The urn bore this inscription: "There is Death in the Pot. 2 Kings, C. IV, V. 40." Two snakes and a branch of a vine were in the foreground and, in the background, there was a portion of a tombstone.

Perhaps the startling appearance of the book was needed at the time to call attention to a misuse of the growing knowledge of chemistry. Accum was one of the first to employ chemical methods to expose undesirable practices. It is interesting to note that he does not appear to have employed the microscope in his work; yet his catalog of supplies listed "solutions for the microscope" at 12 s. per dozen. After mentioning that Peruvian bark was often ground and mixed with sawdust from the bark of trees which did not contain quinine, Accum wrote that "there is no ready test for detecting the fraud." On the whole his book was factual. He distinguished between practices which harm the pocketbook and those which may affect the health. He pointed out that in some cases manufacturers unwittingly used undesirable materials in processing. Brewers in an attempt to improve the flavor of their beer might use a material offered to them as "black extract." Accum showed that it was made from *Cocculus indicus* and contained a poisonous material; it is picrotoxin, which is described in current books on toxicology as a convulsant poison. Accum asserted that few processed foods of the time could be relied upon to be free of possible harmful ingredients. He was in a position to know. There is no doubt that his book attracted attention—Browne stated that it was probably the most extensively reviewed book in the history of chemistry—and that it made him many enemies.

It is certain that soon after the publication of this book, which went through several editions in a short time, Accum brought about the downfall which some of his enemies may have wished for, as the result of the petty incident which has been mentioned, and which now must be described.

It happened at the Royal Institution, of which Accum was a subscriber, on a night in December, 1820. The librarian of the Institution had been plagued by the loss of volumes, and by pages being torn from current unbound journals. Somehow Accum was suspect. It is known that he was careless in his housekeeping habits, that he would ruthlessly tear equipment apart to set up some new experiment, and that he

used books and journals like expendable pads of paper. Did he follow the same practice with publications that did not belong to him? On this particular night the librarian secreted himself in a closet, in the door of which he previously had drilled a peephole. He claimed to have observed Accum removing pages from one of the library's journals. Later, armed with a warrant and accompanied by two officers, he visited Accum at his home. The following account of the results of this visit appeared in the *Gentleman's Magazine* for January, 1821:

> At Bow-Street, Mr. Fredrick Accum, the well known lecturer on practical chemistry, was brought to the office by Bishop and Nicholls, the officers, from his house in Compton-street, Soho, where he has resided for about thirty years, charged with robbing the Royal Institution in Albemarle-street; to which the prisoner was a subscriber, and had been so for a number of years. The Magistrate, after hearing the whole of the case, observed that, however valuable the books might be from which the leaves found in the prisoner's house had been taken, yet the leaves separated from them were only waste paper. If they had weighed a pound, he would have committed him for the value of a pound of waste paper, but as that was not the case, he discharged him.

The managers of the Royal Institution were not satisfied; they brought charges of malicious destruction of property, and Accum was arraigned on this new charge and released on bond. Trial was set for a date in April, 1821. Friends of Accum attempted to intercede, hoping that the matter might be settled privately. Accum himself appears not to have known what to do. He stopped all work and became very depressed. He was subjected to considerable abuse in the magazines and newspapers of the day. One magazine with a reputation for scurrilous articles, *John Bull,* published some verses under the title, "Death in the Pot." They read: "What is his crime? A trick at most/ A thing not worth debating./ 'Tis only what the *Morning Post/* Would call *Accum*-ulating."

Accum was living as a widower alone with one daughter. He and his wife had had eight children, of whom six were stillborn or died in infancy. Seeing his world collapse around him, he suddenly fled England, with his daughter, before the trial was held. He never returned. Before leaving, a friend had prevailed upon him to complete his almost finished book on culinary chemistry. This friend also arranged for its publication, because Accum's regular publisher refused to handle it. It was the last book which Accum wrote in English. It was popular for many years both in England and in America.

The remainder of Accum's life, until his death in Germany on June 28, 1838, was active and useful, and he became financially well off. But the remarkable pace of his former activities was not continued, and probably could not be.

No man ever devoted his waking hours so completely to chemistry as did Accum in his London years. His home was given over to apparatus and supplies. He slaked lime in the cellar and crystallized citric acid in the garret. He had rooms set up as laboratories where research was done and where individual instruction was offered students on a fee basis. Among his students were men who were or became prominent in British affairs, and a number of distinguished Americans. Among them was Benjamin Silliman, the elder, who had been appointed the first professor of chemistry at Yale College, and who went to Philadelphia to study under Robert Hare. He came to Accum in 1805, and found the additional training that he wanted there. Later came William Peck of Harvard and James Freeman Dana of Dartmouth. Silliman wrote many years afterwards of his experiences in Accum's laboratory. He mentioned how the two would spend the morning analyzing minerals together, and he mentioned the obliging, kind-hearted nature of Accum as well as his manipulative skill. He also stated that Accum refused all payment for his time, reagents, or services, adding that Accum may have felt recompensed because of the considerable order for apparatus and supplies which Silliman placed with him for outfitting the laboratory to be established at Yale. Professor Peck also stated, in his report to the President of the Board of Visitors of Harvard College in 1808, that Accum would not accept any compensation from him, though pressed to do so.

In his biography of Accum, Doctor Browne has presented at some length his

opinion that the sudden eclipse of Accum's reputation probably served to delay for a long time the enactment of suitable laws in Great Britain to control adulteration of foods. It was not until nine years after the studies initiated by the *Lancet*, under the direction of Dr. A. H. Hassall in 1851, that this was done. The Act of Parliament of 1860 to accomplish this purpose has served in a sense as the basis for all subsequent legislation to control the safety of foods and drugs. Realizing as we must that time is required for communities and nations to develop suitable programs for the conduct of their citizens in their relationships with one another, and that the combined efforts of many persons are necessary in order to effect desired social changes or health measures, we can be sure that there was no undue delay. Accum, who integrated all his activities into a career devoted wholly to the applications of chemistry to the welfare of man, did his work well. There can be no question about the influence of what he tried to do. To an important degree, he is part of the history of food technology, culminating in the safe and nutritious processed foods we know today. We do not need now to pass judgment on the man himself. He administered his own punishment, after that thoughtless act in the library of the Royal Institution, one memorable night in December, so long ago.

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ADDENDUM

I have tried to omit from this sketch any conjectures about Accum's guilt or innocence, although he himself maintained that the "tear sheets" found in his home came from his own copy of the journal, to which he subscribed. Why then did he forfeit his bond and, after a delay of several months during which the matter was not pressed, suddenly go to Germany? My own guess is that he left in dismay, his ebullience crushed, because of the attitude of the friends who tried to help him. They freely admitted that he could have mutilated the journal in the library. They sought to have the charges dropped on the basis of what they called his unfortunate background, which caused him to be unaware of the obligations of an English gentleman and the seriousness of the offence. Incidentally, Silliman, Accum's most distinguished pupil, and a contemporary of Robert Hare rather than a student under him while in Philadelphia, ventured no firm opinion on the matter that I can find.

F. C. B.

Minimum Dietary Essential Amino Acid-to-total Nitrogen Ratio for Whole Egg Protein Fed to Young Men [1,2]

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ABSTRACT Daily urinary nitrogen excretion was measured for 11 young men fed constant nitrogen intakes, equivalent to 0.36 to 0.54 g protein/kg body wt, which approximated their minimum daily requirement. Whole egg furnished 78.6 to 90% of the nitrogen of the basal diet, and oatmeal and tomato juice the remainder. When the ntirogen of the basal diet was replaced isonitrogenously by a mixture of glycine and diammonium citrate, with each compound supplying equal amounts of nitrogen, a 30% replacement did not significantly alter urinary nitrogen excretion. In 4 subjects, a 40% replacement had no significant effect on urinary nitrogen excretion after initial adaptation. A 50% replacement caused no apparent decrease in dietary nitrogen utilization in one subject. A 30% replacement gave an essential amino acid-to-total nitrogen ratio (E/T_N) of 2.16 corresponding to 25% of the total nitrogen furnished by essential amino acids. A 40% replacement gave an E/T_N ratio of 1.85 and 21% of the nitrogen from essential amino acids.

The recent report of the FAO/WHO Expert Group on Protein Requirements (1) for the first time separates clearly the protein needs of man into 2 components: 1) the amounts and proportions of essential amino acids, and 2) the total available nitrogen required. For reference purposes the group adopted the essential amino acid pattern of whole egg expressed in terms of the relationship of each amino acid to the total essential amino acids, in contrast with the 1957 FAO reference pattern (2) which was in terms of grams of essential amino acids per gram of total nitrogen or per 100 grams of conventional protein (N × 6.25). Tyrosine and cystine were included with the essential amino acids because they replace part of the requirements for phenylalanine and methionine, respectively. The report points out that expression of the pattern in this way allows separate investigation of changes in the ratio of the essential amino acids to one another and also of changes in the relationship between the essential and nonessential amino acid components of the protein requirement.

Significantly, the 1957 FAO reference protein contained a much lower proportion of essential amino acids to total nitrogen than exists in egg. Nevertheless, protein scores based on this reference protein agreed reasonably well with biological assays (1, 3–5). In adopting the amino acid pattern of whole egg for scoring purposes, Block and Mitchell (6) calculated a regression equation which empirically corrected for the relatively high concentration of essential amino acids in whole egg, thereby giving good agreement between the chemical score based on the amino acid pattern of egg and the protein quality as measured in animal experiments. Similarly, the work of Snyderman et al. (7) showed that cow's milk contains a relative excess of essential amino acid nitrogen even for the needs of young infants.

The proportion of essential to nonessential amino acids required in experimental diets of animals has been studied by Stucki and Harper (8, 9) who reported the chick more sensitive than the rat to lowered

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[2] This manuscript is contribution no. 757 from the Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts.

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ratios. Despite several pertinent studies (10, 11) the proportion of grams of essential amino acids to total nitrogen (E/T_N ratio) in human nutrition is yet to be defined clearly. The data of Tuttle et al. (11), for example, cannot be used for estimating the minimum E/T_N ratio for the egg pattern because they were derived from dietary nitrogen levels well above the probable minimum requirement of the subjects studied. A valid estimate of the minimum E/T_N ratio permitting maximal utilization of essential amino acids for protein synthesis can be obtained only when the nitrogen intake is at or below the subject's minimal maintenance requirement. Above this level amino acids are converted and stored as fat or carbohydrate or used to meet caloric needs, and distort the apparent required E/T_N ratio. The data of Rose and Wixom (12) permit an approximation of the amount of essential amino acid nitrogen required at a minimal nitrogen intake. They fed the 8 essential amino acids to 2 subjects at the *safe* level as defined in their previous studies and found that to maintain nitrogen balance, from 14 to 23% of the total nitrogen intake was required as essential amino acid nitrogen. Interpretation of their results is complicated by the uncertain extent to which excess quantities of the essential amino acid nitrogen were diverted to synthesis of dietary nonessential amino acids.

These studies and the recommendations of the recent FAO/WHO report emphasize the need for reliable measurements of minimum E/T_N ratios in human nutrition. The present paper, the first of a series designed to determine the minimum proportion of essential amino acids to total nitrogen in human nutrition, reports results obtained for young adults consuming a diet approximating the essential amino acid pattern of whole egg. The work will be extended to the minimum E/T_N ratios for other age groups, for other physiologic and pathologic states, and to other protein sources.

EXPERIMENTAL

Subjects. Groups of 3 and 8 healthy, male students 17 to 22 years old were the experimental subjects (table 1). With one possible exception all were in good health as determined by medical history and examination. Subject RW had a history of adrenal hyperplasia since infancy, and his urinary 17-hydroxycorticosteroid excretion was elevated. Subjects in the first experiment, except MK, continued their normal activities and were not confined to the campus. Subject MK and the subjects in the second experiment were restricted to the MIT Clinical Research Center and required to adhere to a regular and strictly monitored pattern of activities.

Diet. A liquid formula supplied the dietary protein in both experiments (13). In experiment 1 dried whole egg[4] supplied

[4] Spray-dried, pasteurized, whole egg powder. Supplied through the courtesy of the British Egg Marketing Board, London.

TABLE 1

Age, weight and daily intake of calories and protein of young men studied for metabolic effects of dietary protein dilution with glycine and diammonium citrate

Subject	Age	Weight		Caloric intake	Protein intake
		Initial	Final		
	years	*kg*	*kg*	*kcal*	*g/kg*
Experiment 1					
KP	22	71.4	70.9	3380	0.36
FW	21	67.0	67.3	3030	0.43
MK	19	63.0	63.0	3880	0.54
Experiment 2					
DG	19	65.1	65.6	2930	0.41
DP	18	68.6	67.8	2880	0.37
BR	19	67.4	67.4	2897	0.37
IJ	22	81.3	80.0	2962	0.39
MR	18	75.1	75.0	2650	0.40
JS	18	68.2	67.5	2742	0.40
RW	18	70.5	69.4	2349	0.38
NM	17	73.2	72.0	2725	0.41

78.6% of the nitrogen, oatmeal[5] 16.3% and tomato juice 5.1%. The tomato juice was the vehicle for part of the diammonium citrate and glycine. Contributions from the 3 protein sources were kept proportional so that the pattern of the essential amino acids was the same at different levels of dilution. The dietary regimen was similar in experiment 2 except that whole egg supplied 90% of the nitrogen, with oatmeal and tomato juice each supplying 5%. Again the contributions from the 3 protein sources were kept proportional, except those of subject NM who was studied during changes of the egg protein component alone.

Additional calories were supplied by cornstarch cookies and soft drinks, as well as by cornstarch pudding which also acted as a carrier for some of the glycine and diammonium citrate. Previous caloric intakes were estimated from individual dietary histories and empirically adjusted to maintain body weight by varying the nonprotein calorie supplements. Throughout the calorie adjustment period the formula diet supplied 0.8 to 1.0 g protein/kg/day. With this procedure no further changes in caloric intake were necessary during the experiments. Caloric intakes do not correspond precisely to variations in body weight because of individual differences in body composition and energy expenditure.

"Dilution" of dietary protein. Various sources of nonessential amino acid nitrogen have been used in determining essential amino acid requirements (14). Both human and animal experiments suggest that mixtures containing the essential amino acids with one or more simple nitrogen-containing compounds, such as glutamic acid or glycine plus diammonium citrate, can be as effective in maintaining nitrogen balance as mixtures of a number of nonessential amino acids (15). In the two present studies the protein nitrogen in the formula diet was "diluted" by substituting part of the nitrogen with a mixture of glycine and diammonium citrate, in which each compound supplied equal amounts of nitrogen. Table 2 summarizes the E/T_N ratios and percentages of total nitrogen contributed by the essential amino acids in experimental diets, whole egg and the 1957 FAO reference protein.

TABLE 2

Proportions of essential amino acids to total nitrogen in experimental diets, whole egg and the 1957 FAO reference protein

Food	E/T_N[1]	E_N/T_N[2]
		%
Whole egg[3]	3.22	37.6
Experiment 1		
Basal diet[4]	2.97[5]	34.4
14% Dilution[6]	2.55	29.6
18% Dilution	2.44	28.2
22% Dilution	2.32	26.8
28% Dilution	2.14	24.8
33% Dilution	1.96	22.9
Experiment 2		
Basal diet[7]	3.08[5]	35.7
20% Dilution	2.46	28.6
30% Dilution	2.16	25.0
40% Dilution	1.85	21.4
50% Dilution	1.54	17.8
1957 FAO reference protein	2.02	23.6

[1] Grams essential amino acids including cystine and tyrosine/g total nitrogen.
[2] Grams essential amino acid nitrogen/g total nitrogen × 100.
[3] 1965 FAO/WHO Protein Requirements report (1).
[4] Nitrogen from whole egg (78.6%), oatmeal (16.3%), tomato juice (5.1%) by analysis.
[5] Calculated from Orr, M. L., and B. K. Watt 1957 Amino acid content of foods. Home Econ. Res. Rept. no. 4, U. S. Department of Agriculture, Washington, D. C.
[6] Percentage of total nitrogen of basal diet replaced by glycine and diammonium citrate.
[7] Nitrogen from whole egg (90%), oatmeal (5%), tomato juice (5%) by analysis.

Experimental sequence. Experiment 1. Following an adjustment period of 8 days, during which time caloric intake was fixed at amounts sufficient to maintain body weight, the subjects were fed protein in a proportion of 0.42 g/kg/day for 13 days. Thereafter, the levels of protein were increased or decreased by 0.06 g/kg/day for periods of 5 days to find an intake close to minimal requirements.

The FAO/WHO Expert Group on Protein Requirements (1) suggested that 20 mg nitrogen/kg be allowed for endogenous fecal loss and 20 mg nitrogen/kg for dermal loss. Since the aim in the present experiments was to maintain subjects at or below minimal nitrogen requirement, only 20 mg nitrogen/kg were allowed for fecal and dermal losses combined. By this standard the subjects were in slightly negative

[5] Buckeye Rolled Oats, homogenized. Specially prepared in Standardized form through the courtesy of the Quaker Oats Company, Barrington, Illinois.

balance during the baseline and dilution periods. After 5 days at the minimal dietary protein level, first 0.06 and then 0.12 g protein/kg were replaced by isonitrogenous amounts of glycine-diammonium citrate mixture for 5-day periods. Subjects KP and FW in this experiment were then allowed to eat a self-selected diet for one week, after which they again received the minimal nitrogen intake for 12 days and the dilution steps were repeated.

Experiment 2. Minimal nitrogen requirement was calculated from the endogenous urinary and metabolic fecal nitrogen loss at the end of a 7- or 10-day protein-free period. An allowance of 15 mg nitrogen/kg for integumental and sweat loss was used in calculating the minimal requirement. The protein intake of each subject is shown in table 1. The subjects were fed the estimated minimal nitrogen requirement for 7 to 15 days depending upon the time required to reach a relatively steady rate of urinary nitrogen excretion. The sequence in which dilutions of the nitrogen of the basal diet were made and the length of each dilution phase are given with the results.

Handling of samples and chemical analysis. Complete 24-hour specimens of urine were collected throughout the studies and analysis for total nitrogen made on fresh urine by the Kjeldahl method as modified by Scales and Harrison (16). Nitrogen in food samples was analyzed by the same method. Urinary creatinine was determined on fresh urine by the method of Pino et al. (17) and urea nitrogen (18) on aliquots of previously frozen urine.

Preprandial morning blood samples were taken when weight stabilization was established, during adjustment to minimal protein intake, and at the end of each dilution phase. Plasma was separated from red cells, centrifuged at 4°, and frozen until it was analyzed for free amino acids. Quantitative analysis for free amino acids was made by column chromatography using the method of Spackman et al. (19), and a Technicon amino acid analyzer.

Statistical analyses. Total urinary nitrogen excretion was used as the principal indicator of utilization of dietary nitrogen. Since no significant differences in deviations within the individuals were found by Bartlett's test for homogeneity, Student's *t* test was used to determine whether urinary nitrogen excretion during a dilution period differed significantly from that for the corresponding baseline period. The baseline period represents the period after a relatively steady-state urinary nitrogen excretion was achieved by subjects fed the basal or undiluted diet.

The precision in detecting changes in dietary nitrogen utilization depends upon the daily variation in urinary nitrogen excretion and is the limiting factor in our determinations of minimum E/T_N ratios. In the present experiments the baseline coefficient of variation in nitrogen excretion averaged 7.6%, excluding subject KP who showed an average coefficient of variation of 14.5% during the baseline periods.

TABLE 3

Effects of dilution of protein[1] with glycine and diammonium citrate on urinary nitrogen excretion[2] of young men (exp. 1)

Subject	Dietary intake	Dilution of dietary protein, %					
		0	10–14	16–18	22	28	33
	g N/day	*g urinary N/day*		*g urinary N/day*		*g urinary N/day*	
KP:							
a[3]	4.11	3.42 ± 0.65		3.54 ± 0.44			4.16 ± 0.64
b	4.11	3.17 ± 0.32		3.07 ± 0.30			4.12 ± 0.59
FW:							
a	4.61	3.51 ± 0.16	4.30 ± 0.28 *			4.30 ± 0.22 *	
b	4.61	4.19 ± 0.33	4.46 ± 0.84			4.02 ± 0.48	
MK:	5.44	4.88 ± 0.46	4.18 ± 0.23		4.17 ± 0.26		

[1] Nitrogen from whole egg (78.6%), oatmeal (16.3%), tomato juice (5.1%).
[2] Mean ± SD for periods of 5 days.
[3] a, first replication; b, second replication.
* Difference significant from baseline (0% dilution) at the 0.05 level.

RESULTS

Experiment 1

Table 3 summarizes the daily urinary nitrogen excretion during experimental periods. Since the dilution of dietary protein was carried out in steps of 0.06 and 0.12 g protein/kg the actual percentage of protein replaced varied among subjects. Subject FW showed a significantly increased rate of urinary nitrogen excretion during the first dilution phases. This appears to have been the result of an abnormally low baseline level since the second replication indicated a higher rate of urinary nitrogen excretion during a basal period and agrees with the overall experimental findings. Subject KP showed an increase in urinary nitrogen excretion following a 33% dilution. Although this was just short of statistical significance, at the 5% level, because of his large daily variation in nitrogen excretion, it is probable that 33% dilution was too much for this subject. Subject MK showed a lower rate of urinary nitrogen excretion during the dilution phases when compared to the baseline period.

Experiment 2

Urinary nitrogen. The data from subject DG, the one studied longest, are shown in figure 1 to illustrate the method of daily evaluation. As indicated in table 4, which

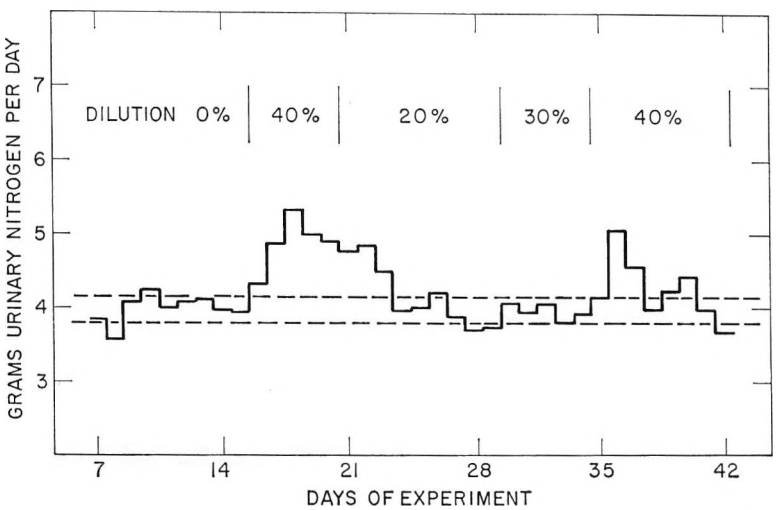

Fig. 1 Urinary nitrogen excretion per day in subject DG fed the basal diet with zero, 40, 20, 30 and 40% replacement of nitrogen with glycine and diammonium citrate. Broken lines represent mean baseline nitrogen excretion ± one standard deviation.

TABLE 4

Effects of dilution of protein [1] with glycine and diammonium citrate on urinary nitrogen excretion [2] of young men (exp. 2)

Subject	Dietary intake	Dilution of dietary protein, %				
		0	20	30	40	50
	g N/day	*g urinary N/day*		*g urinary N/day*		*g urinary N/day*
DG	4.32	3.99 ± 0.19	3.93 ± 0.18	4.01 ± 0.13	4.09 ± 0.28 [3]	
DP	3.94	3.36 ± 0.18			4.02 ± 0.20 *	
BR	4.02	3.69 ± 0.33			3.68 ± 0.38	
IJ	4.97	4.48 ± 0.41		5.08 ± 0.23	5.78 ± 0.29 *	
MR	4.88	4.42 ± 0.41			4.83 ± 0.61	
JS	4.31	4.43 ± 0.47		4.38 ± 0.41	5.18 ± 0.54 *	
RW	4.31	4.70 ± 0.33			4.68 ± 0.44	4.27 ± 0.26
NM	4.84	4.47 ± 0.31	5.22 ± 0.19 *		5.09 ± 0.22 *	

[1] Nitrogen from whole egg (90%), oatmeal (5%), tomato juice (5%).
[2] Mean ± SD for periods varying from 4 to 12 days.
[3] Result from second period of 40% dilution. Mean value for first period was 4.89 ± 0.36 which was significantly different ($P < 0.05$) from baseline.
* Difference from baseline (0% dilution) significant at 0.05 level.

summarizes the data for each subject, the within-period standard deviations were larger for most of the other subjects.

Subject DG: Urinary nitrogen stabilized at an average of 3.99 g/day during the last 8 days of a 16-day period with a basal diet of 0.41 g protein/kg. Diluting the nitrogen of the basal diet by 40% caused a sharp increase in urinary nitrogen excretion; and upon return to a 20% dilution, urinary nitrogen excretion decreased to 3.93 g/day. A 30% dilution had no further effect, and a subsequent 40% dilution resulted in a rapid initial increase in urinary nitrogen excretion which returned to the basal level during the final 5 days under this regimen. It was concluded that the subject could adapt to a 40% dilution of the nitrogen of the basal diet with glycine and diammonium citrate.

Subject DP: Urinary nitrogen averaged 3.36 g/day during the last 7 days of a 15-day period with a basal diet of 0.37 g protein/kg. A 40% dilution was judged excessive since it caused a sharp increase in urinary nitrogen excretion which continued significantly above the basal level during the later 10-day period at this level of dilution.

Subject BR: Urinary nitrogen excretion averaged 3.69 g/day during the last 7 days of a 15-day period with a basal diet of 0.37 g protein/kg. A 40% dilution for 10 days did not result in an increased rate of urinary nitrogen excretion, and it was concluded that the subject could utilize the 40% diluted diet and the basal diet equally well.

Subject IJ: Urinary nitrogen excretion stabilized at an average of 4.48 g/day during the last 8 days of a 12-day period with a basal diet of 0.39 g protein/kg. A 40% dilution for 9 days caused a significant increase in urinary nitrogen excretion. Upon return to a 30% dilution urinary nitrogen excretion decreased toward the basal level and reached a mean of 5.08 g/day during the last 4 days of a 7-day period at this dilution. This was not significantly different from the basal level. It was concluded that the subject could adapt to a 30% dilution.

Subject MR: Urinary nitrogen excretion during the last 5 days of a 7-day period was 4.42 g/day. Diluting the nitrogen of the basal diet by 40% during an 8-day period did not result in a statistically significant increase in urinary nitrogen excretion. It was concluded that the subject could tolerate a 40% dilution.

Subject JS: Urinary nitrogen excretion during the 8 days of a 12-day period with a basal diet of 0.40 g protein/kg averaged 4.43 g/day. A 40% dilution for 9 days caused an increase in urinary nitrogen excretion to 5.18 g/day. Upon return to a 30% dilution urinary nitrogen excretion decreased toward the basal level and reached a mean of 4.38 g/day during the last 4 days of a 7-day period on this regimen. It was concluded that the subject could adapt to a 30% dilution.

Subject RW: Urinary nitrogen excretion during the last 7 days of a 12-day basal period was 4.70 g/day. Diluting the nitrogen of the basal diet by 40% for 12 days and 50% for 4 days did not result in an increased rate of urinary nitrogen excretion.

Subject NM: Urinary nitrogen excretion during the 11-day period did not reach a constant level and was increasing when the first dilution was made. Average urinary nitrogen excretion during the last 5 days of the 11-day period with a basal diet of 0.41 g protein/kg was 4.47 g/day. A 20% dilution caused an apparent increase in urinary nitrogen excretion to 5.22 g/day with no further increase during the 12-day period at a 40% dilution. In view of the failure to reach a constant excretion rate during the basal period, comparison of the results of the dilution periods with the basal period may be misleading. Since there was no change between the 20 and 40% dilution periods, it is likely that this subject tolerated a 40% dilution.

Urinary creatinine and urea. The results for urinary creatinine and urea nitrogen excretion are shown in table 5. Urea nitrogen excretion tended to parallel changes in total urinary nitrogen except that the former was more variable within each period.

Plasma amino acids. The total amounts and molar ratios of essential and nonessential plasma amino acids for subjects in experiment 2 are summarized in table 6. The molar ratio of essential to nonessential amino acid concentration (E_m/N_m) decreased when the subjects were supplied with minimal intakes because of a decrease

TABLE 5

Urinary creatinine and urea nitrogen excretion of young men fed dilutions of dietary protein with glycine and diammonium citrate (exp. 2)

Subject	Dilution of dietary protein	Creatinine	Urea nitrogen
	%	g/day [1]	g/day [1]
DG	0	1.83 ± 0.14	2.38 ± 0.20
	20	1.70 ± 0.03	2.47 ± 0.15
	30	1.71 ± 0.06	2.66 ± 0.10
	40(1st dil.)	1.66 ± 0.06	3.22 ± 0.45 *
	40(2nd dil.)	1.71 ± 0.04	2.57 ± 0.38
DP	0	1.72 ± 0.03	2.15 ± 0.29
	40	1.70 ± 0.03	2.75 ± 0.47 *
BR	0	1.53 ± 0.04	2.51 ± 0.40
	40	1.49 ± 0.03	2.59 ± 0.36
IJ	0	1.98 ± 0.12	3.25 ± 0.42
	30	1.99 ± 0.16	4.16 ± 0.32 *
	40	1.96 ± 0.20	4.80 ± 0.33 *
MR	0	1.78 ± 0.17	3.04 ± 0.49
	40	1.74 ± 0.18	3.97 ± 0.56
JS	0	1.65 ± 0.20	3.49 ± 0.44
	30	1.56 ± 0.12	3.23 ± 0.15
	40	1.59 ± 0.19	4.01 ± 0.57
RW	0	1.60 ± 0.05	3.61 ± 0.28
	40	1.54 ± 0.08	3.72 ± 0.41
	50	1.52 ± 0.06	3.15 ± 0.14
NM	0	1.64 ± 0.05	2.45 ± 0.68
	20	1.65 ± 0.06	4.13 ± 2.25
	40	1.65 ± 0.11	3.29 ± 0.95

[1] Mean ± SD for periods varying from 4 to 12 days.
* Difference from baseline period (0%) significant at 0.05 level.

in the concentration of essential amino acids with or without change in the nonessential amino acids. Further decreases occurred in the E_m/N_m ratio during the dilution phases.

DISCUSSION

The results reported in this paper support the conclusions of the FAO/WHO Expert Group on Protein Requirements (1) that 60 to 70 mg tryptophan and 190 to 220 mg methionine and cystine/g nitrogen intake are necessary for adequate human nutrition. The diet diluted to 30% supplied 69.4 mg tryptophan and 224 mg methionine plus cystine, whereas the 40% diluted diet supplied 59.5 and 192.4 mg/g nitrogen intake, respectively.

Allison (20) stated: "It may be possible to reduce the amount of egg protein below so-called maintenance requirements if some form of nonessential nitrogen is added to supply the deficit in nitrogen." The present results suggest that the nitrogen contributed by the essential amino acids of whole egg protein may be replaced isonitrogenously with a mixture of glycine and diammonium citrate to the extent of at least 30% without influencing the nutritive value of this protein in most young adult males fed at minimal nitrogen intakes for maintenance.

In a diet where whole egg supplies 90% of the total protein the minimal E/T_N ratio is likely to be within the range of 1.85 to 2.16 g essential amino acid/g total nitrogen (table 2). Thus, for a diet supplying the essential amino acids, including cystine and tyrosine, in the proportions found in whole egg protein, 21 to 25% of the total nitrogen would be supplied by essential amino acids. It is of interest that the 1957 FAO reference protein contained 23.6% of the total nitrogen as essential amino acid nitrogen and that the results are in approximate agreement with those of Rose and Wixom (12). Since it may have been possible, however, to dilute further the diets of the subjects who were able to tolerate a 40% dilution, the normal range may extend below 1.85. The only subject studied, RW, tolerated a 50% dilution, indicating

TABLE 6

Total amounts and molar ratios of essential and nonessential amino acids in blood plasma of young men fed dilutions of dietary protein with glycine and diammonium citrate (exp. 2)

Subject	Protein intake	Dilution of dietary protein	Total essential amino acids (E_m)	Total nonessential amino acids (N_m)	E_m/N_m
	g/kg/day	*%*	*μmoles/liter*	*μmoles/liter*	*μmoles/liter*
DG	0.80	0	1599	2658	0.60
	0.41	0	1043	2239	0.46
	0.41	30	965	2524	0.38
	0.41	40 [1]	820	2251	0.36
DP	0.80	0	1606	2340	0.69
	0.37	0	1108	2256	0.49
	0.37	40	1109	2618	0.42
BR	0.80	0	1710	2251	0.76
	0.37	0	892	1713	0.52
	0.37	40	1123	2507	0.45
IJ	0.80	0	1181	1569	0.75
	0.39	0	1107	2039	0.54
	0.39	30	1036	2198	0.47
	0.39	40	1091	2368	0.46
MR	0.80	0	1090	1484	0.73
	0.40	0	924	1344	0.69
	0.40	40	879	1941	0.45
JS	0.80	0	843	1458	0.58
	0.40	0	923	1929	0.48
	0.40	30	1007	2134	0.47
	0.40	40	814	2238	0.36
RW	0.80	0	1136	1521	0.75
	0.38	0	1100	1610	0.68
	0.38	40	1029	1912	0.54
	0.38	50	861	1762	0.49
NM	0.80	0	1523	2217	0.69
	0.41	0	1448	2875	0.50
	0.41	20	974	1810	0.54
	0.41	40	798	1650	0.48

[1] First of the 2 dilutions at 40%.

the possible extent of variation of the ratio of essential amino acid to total nitrogen required between individual subjects when the diet supplies essential amino acids approximating the pattern in egg protein. It is noteworthy that Rose and several subsequent workers have reported a twofold variation in the requirement of human subjects for certain of the essential amino acids (21).

Kirk et al. (22) and Swendseid et al. (23, 24) compared the metabolic response to the amino acid pattern of the 1957 FAO reference protein with that of the proteins of whole egg, milk and peanut. The nitrogen balance maintained by their subjects at such low E/T_N ratios as 0.54 was the result of an intake of total nitrogen between 9 and 10 g/day, an amount far in excess of minimum requirements (1). However, they never intended their data to be used to calculate minimum E/T_N ratios.

The low E/T_N ratios in the recent study by Tuttle et al. (11) also were due to intakes of nitrogen in excess of minimum requirements. Assuming a minimum protein requirement of 0.43 g protein/kg/day (1) interpolation of their data suggests that a 30% reduction of essential amino acids would not have affected the nitrogen utilization of their purified amino acid diet which contained amino acids proportioned as in whole egg protein. These observations are of special interest because elderly subjects have been reported to require higher amounts of essential amino acids than young adults (11, 25, 26). Determination of minimum E/T_N ratios in elderly subjects at nitrogen intakes at or below minimum requirements is needed.

Kies and Linkswiler (10) recently measured the nitrogen retention of 6 young adult males fed varying intakes of essential amino acids but at a constant 5.8 g total nitrogen/day. Nitrogen balance was achieved when the diet contained essential amino acids equivalent to those in 40 to 60 g of whole egg protein. Their results indicate that E/T_N ratios between 3.5 and 5.2 are necessary to achieve balance at this nitrogen intake. Such ratios supply 40 to 60% of total nitrogen as essential amino acids, amounts in excess of those provided either by diets of the present studies or by whole egg (table 2). We are unable to reconcile these results with our own or those of others.

Recently, Kofrányi and Jekat (27) described experiments seemingly comparable to ours. They observed no difference in nitrogen balance when as much as 67% of egg protein was replaced with ammonium citrate, glutamate or aspartate as sources of nonessential amino nitrogen. From our results this dilution of egg protein would have markedly increased urinary nitrogen loss. At a 67% dilution the essential amino acid nitrogen would amount to only 12.4% of total nitrogen. They also reported that milk protein could be diluted only 10 to 15% and tuna fish protein not at all. The fish protein used in this study may, however, have undergone damage during processing or storage.

The reduced molar ratios of essential to nonessential amino acids (E_m/N_m ratios) in plasma occurring when subjects were fed at basal levels of protein intake confirm the observations of Swendseid et al. (28) in man and parallel those in rats (29). A decreased ratio of essential to nonessential amino acids was observed in rats when an 18% casein diet was supplemented with 7.5% glycine but not when 14% glutamic acid was added. The decrease in E_m/N_m ratios noted in our subjects following dilutions of egg protein may have occurred partially because glycine formed part of the nitrogen supplement, a possibility which will be explored further. The E_m/N_m ratios found in our experiments are higher than those reported by Swendseid et al. (28) for elderly subjects.

The results have practical implications. Proteins less expensive than whole egg and more readily available in areas of the world where protein malnutrition is important may also contain a relative excess of essential amino acids. An important saving and a more efficient use of existing foods could then be effected by adding relatively inexpensive nonessential amino acids or simple nitrogenous compounds such as ammonium citrate. For example, the E/T_N ratios of corn meal, fish, rice, and soya flour are 2.78, 2.66, 2.61, and 2.58, respectively, and might be reduced to some extent without affecting their nutritive value. We plan to measure this.

From the data presented the minimum E/T_N ratios for diets approximating the pattern of amino acids in whole egg appears to lie between 1.85 and 2.16, at which essential amino acids account for 21 to 25% of the total dietary nitrogen. These data apply only to young men not experiencing unusual stress. The possibility that infection, trauma, and even fear or anxiety may produce changes requiring a higher proportion of essential amino acids should be examined experimentally. Furthermore, minimum E/T_N ratios for infants and possibly also for elderly persons are likely to be higher than for young adults. Finally, the present experiments are relatively short-term ones; long-term studies will be required before minimum E/T_N ratios can be recommended as satisfactory for the continued well-being of population groups.

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Vitamin B_6 Depletion in Man: Blood Vitamin B_6, Plasma Pyridoxal-phosphate, Serum Cholesterol, Serum Transaminases and Urinary Vitamin B_6 and 4-Pyridoxic Acid [1]

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ABSTRACT The effect of vitamin B_6 depletion in man on several biochemical parameters was studied. Feeding a partially purified diet containing 0.16 mg vitamin B_6 resulted in significant decreases in excretion of vitamin B_6 and 4-pyridoxic acid, blood vitamin B_6, plasma pyridoxal-phosphate and serum transaminase activities. After 5 days of depletion, blood vitamin B_6 values were approximately 20% of pre-depletion values, and after 25 days of depletion there was a disappearance of blood vitamin B_6 and of urinary 4-pyridoxic acid. Serum glutamic-pyruvic transaminase appeared to be affected more than serum glutamic-oxalacetic transaminase. Supplementation with 0.6 or 0.9 mg pyridoxine caused significant increases in most of the parameters which were affected by vitamin B_6 depletion. Serum cholesterol concentration was not affected by the vitamin B_6 intake.

Several different biochemical parameters were studied in 6 normal men fed a partially purified diet deficient in vitamin B_6.

Previously reported are the effects of vitamin B_6 depletion on urinary taurine and sulfate excretion and nitrogen balance (1), the urinary excretion of tryptophan metabolites (2) and the urinary excretion of quinolinic acid and niacin metabolites (3).

The present paper gives the effects of vitamin B_6 depletion on the urinary excretion of vitamin B_6 and 4-pyridoxic acid; the concentration of blood vitamin B_6, plasma pyridoxal-phosphate and serum cholesterol; and the activities of serum glutamic-oxalacetic and glutamic-pyruvic transaminases.

EXPERIMENTAL

Six normal male students consumed a vitamin B_6-deficient diet for 50 days following a 5-day preliminary period with self-chosen foods. The composition of the diet, the experimental design and pertinent data concerning the subjects have been given (1). The daily diet contained 0.16 mg of vitamin B_6, 100 g protein and a mixture of animal and vegetable fats which contributed 40% of the caloric intake. When the subjects became deficient in vitamin B_6 as judged by a definite disturbance in tryptophan metabolism (2), a daily supplement of either 0.6 or 0.9 mg of pyridoxine was given. The subjects did not become deficient at the same rate; thus, all were not given the vitamin supplement at the same time. On the last 3 days of the study all subjects were given 50 mg pyridoxine daily.

The 24-hour excretion of vitamin B_6 and 4-pyridoxic acid in urine, the concentration of blood vitamin B_6, plasma pyridoxal-phosphate, serum cholesterol and the activities of serum glutamic-oxalacetic transaminase (SGO-T) and serum glutamic-pyruvic transaminase (SGP-T) were determined on day 5 which was the last day of the preliminary period and on certain selected days thereafter. The analyses were made according to the following methods; urinary and blood vitamin B_6 by the microbiological method of Atkin et al. (4) using *Saccharomyces carlsbergensis* 9080 ATCC as the test organism; plasma pyridoxal-phosphate by the manometric method of Umbreit et al. (5) as modified

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by Boxer et al. (6) and Wachstein et al. (7); urinary 4-pyridoxic acid by the fluorometric method of Huff and Perlzweig (8) as revised by Reddy et al. (9); transaminase activities by a simplified colorimetric method[4]; and total and free serum cholesterol by the method of Sperry and Webb (10) as modified by Galloway et al. (11) for micro determinations. Probability values were calculated using Student's *t* test for paired observation. Transaminase values were first transformed to square root because of the heterogeneity of the values.

RESULTS

With the exception of serum cholesterol all biochemical parameters measured were affected when the subjects were fed the diet deficient in vitamin B_6. The 24-hour excretion of vitamin B_6 and 4-pyridoxic acid decreased rapidly (figs. 1 and 2). Just prior to pyridoxine supplementation (after 27 days of depletion for subjects 4 and 5, thirty-six days for subjects 2 and 6 and forty days for subjects 1 and 3) urinary vitamin B_6 ranged from 0.1 to 0.2 μmoles per 24 hours, amounts significantly less ($P < 0.001$) than the pre-depletion values which ranged from 0.5 to 0.8. μmoles. No 4-pyridoxic acid was detected after 25 days of depletion. Supplementation with 0.6 or 0.9 mg of pyridoxine daily resulted in significant increases in urinary vitamin B_6 ($P < 0.01$) and in 4-pyridoxic acid ($P < 0.01$).

The vitamin B_6 concentration of the blood of subjects depleted for 5 days was approximately 20% that of pre-depletion values (fig. 3). No vitamin B_6 was found in the blood of subjects depleted for 25 days. Marginal supplementation with pyridoxine resulted in small but clearly detectable increases in blood vitamin B_6 in four of the subjects, but in subjects 4 and 5, there was no response after 20 days to daily supplements of 0.6 mg pyridoxine.

Plasma pyridoxal-phosphate appeared to decrease during vitamin B_6 depletion (fig. 4). There was no response to marginal vitamin supplementation. However, when 50 mg pyridoxine were given daily for 3 days, the plasma pyridoxal-phosphate concentrations were higher than pre-depletion values.

TABLE 1

Serum cholesterol

Periods	Subjects (paired) [1]		
	4–5	2–6	1–3
Pre-depletion [2]	184	186	176
Height of depletion [3]	142	172	158
End of repletion [4]	138	168	161

[1] Subjects who were depleted for the same number of days are paired.

[2] Averages of values obtained on days 1 and 5 of study.

[3] Subjects 4 and 5 were depleted for 27 days, subjects 2 and 6 for 36 days, and subjects 1 and 3 for 40 days.

[4] Subjects 4 and 5 were administered 0.6 mg of pyridoxine supplement on days 33 through 52, subjects 2 and 6 were given 0.9 mg of pyridoxine on days 42 through 52, and subjects 1 and 3 received 0.6 mg of pyridoxine on days 46 through 52. All subjects received 50 mg pyridoxine for 2 days prior to this sampling (day 55).

The activities of both transaminase enzymes in serum decreased during depletion of vitamin B_6 (fig. 5 and 6). Values at the height of depletion were significantly lower than pre-depletion values ($P < 0.05$ for SGO-T; $P < 0.01$ for SGP-T). Supplementation with 0.6 or 0.9 mg pyridoxine caused slight increases in the activities of both enzymes. Supplementation with 50 mg pyridoxine daily for 3 days restored the SGO-T activity to pre-depletion values in most subjects, but the SGP-T activity did not reach original levels.

Serum cholesterol did not appear to be affected by vitamin B_6 depletion (table 1).

[4] Sigma Technical Bulletin no. 505. A simplified method for colorimetric determination of glutamic-oxalacetic and glutamic-pyruvate transaminases at approximately 505 mμ. Sigma Chemical Company, St. Louis.

Figs. 1–6 Effect of vitamin B_6 intake on the urinary excretion of vitamin B_6 and 4-pyridoxic acid, the blood concentration of vitamin B_6 and plasma pyridoxal-phosphate, the activities of serum glutamic oxalacetic transaminase and serum glutamic-pyruvic transaminase. Values given for day 5 represent those of the pre-depletion period (day 1–5) during which time subjects consumed self-selected diets. Each line in each figure represents the average value for the 2 subjects who were depleted in vitamin B_6 for equal lengths of time: open circle for subjects 4 and 5, triangle for subjects 2 and 6, closed circle for subjects 1 and 3. The arrow indicates the day on which pyridoxine supplementation was begun and the value above the arrow shows the amount of supplement in milligrams.

Transaminase activity is reported in Sigma-Frankel units. One theoretical Sigma-Frankel unit of transaminase will form 4.82×10^{-4} μM of glutamate/minute at pH 7.5 at 25°.

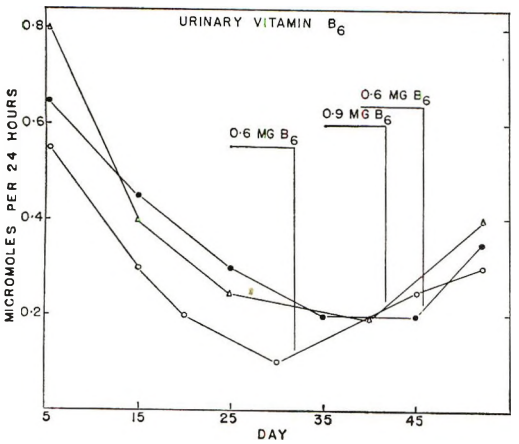


Figure 1

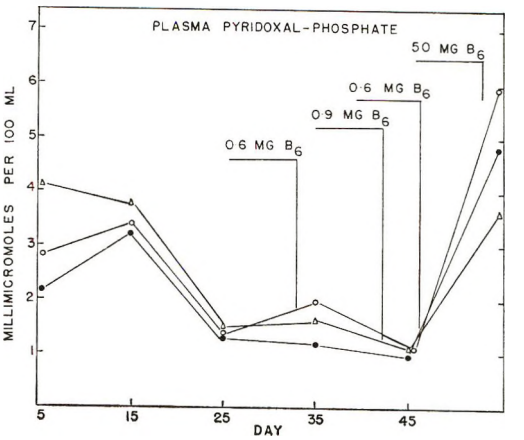


Figure 4

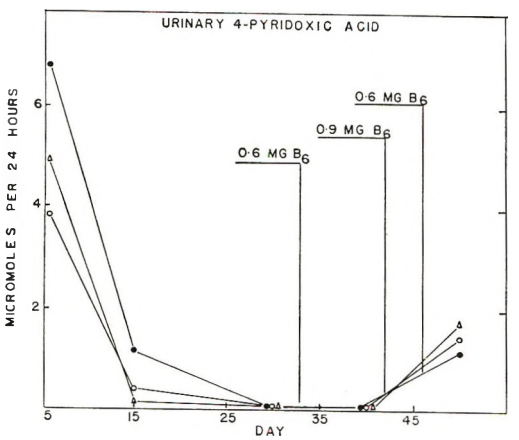
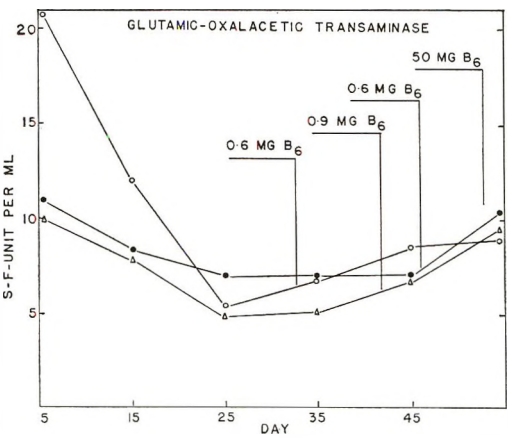


Figure 2



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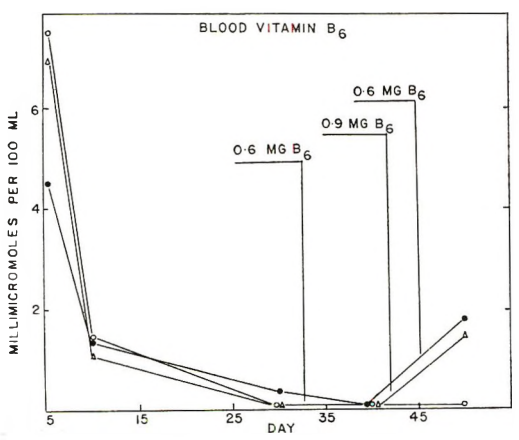

Figure 3

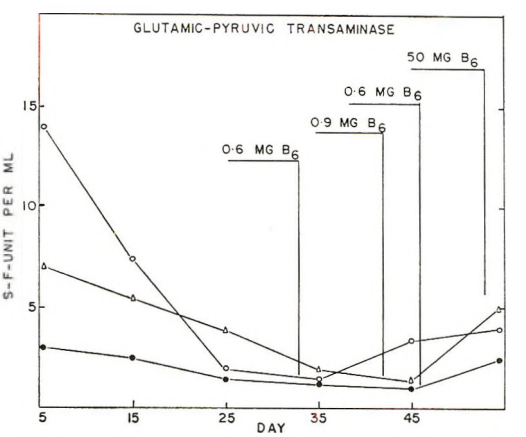

Figure 6

The slight decrease which occurred was not significant. No response to pyridoxine supplementation occurred.

DISCUSSION

Vitamin B_6 depletion occurred rapidly in adult men fed a partially purified diet containing 0.16 mg vitamin B_6 daily. After 5 days of depletion there was a marked decrease in the blood vitamin B_6 concentration of all subjects and somewhat less marked decreases in urinary vitamin B_6 and 4-pyridoxic acid. These parameters continued to decrease as the period of depletion progressed, and at the height of depletion little, if any, urinary 4-pyridoxic acid or blood vitamin B_6 was detected.

In general the decrease in these constituents paralleled the increase in excretion of abnormal tryptophan metabolites following a 2-g loading dose of L-tryptophan (2); however, the decrease in blood vitamin B_6 seemed to occur before the other biochemical parameters changed. After 5 days of depletion when blood vitamin B_6 concentration was approximately 20% of pre-depletion values, only 2 subjects exhibited significant abnormalities in tryptophan metabolism. Marginal supplementation with 0.6 or 0.9 mg pyridoxine restored tryptophan metabolism to nearly normal (2); but urinary vitamin B_6 and 4-pyridoxic acid and blood vitamin B_6 increased only slightly and remained far below predepletion values.

Others have reported decreased excretion of vitamin B_6 and 4-pyridoxic acid during vitamin B_6 deficiency. Baker et al. (12) reported that men fed a diet containing 0.06 mg vitamin B_6 and 100 g protein daily excreted approximately 0.1 μmole of the vitamin daily after 5 weeks of depletion, and Snyderman et al. (13) observed that infants fed vitamin B_6-deficient diets excreted no 4-pyridoxic acid and very little vitamin B_6 after 15 to 25 days of depletion.

Although there was a gradual decrease in the activities of both transaminase enzymes, SGP-T appeared to be more severely affected than SGO-T. These results are in agreement with those of Caldwell and McHenry (14) and Brin et al. (15) who reported that SGP-T activity is depressed more than SGO-T activity in vitamin B_6-deficient rats. Furthermore, the results of the present study indicate that restoration of SGP-T activity occurs more slowly than SGO-T. This observation appears to support the suggestion of Brin et al. (15) that the SGO-T apoenzyme may have greater affinity for the vitamin B_6 coenzyme than does SGP-T and would exhibit priority over the SGP-T upon marginal pyridoxine supplementation.

Even though the activities of both transaminase enzymes decreased significantly in serum during depletion, the wide range of transaminase activities observed normally in human subjects makes these criteria of little use in assessing vitamin B_6 nutriture. Others (16, 17) have reported that SGO-T activity in itself cannot be used to detect vitamin B_6 deficiency in man.

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Effect of Dietary Amino Acid Source on the Zinc-deficiency Syndrome in the Chick [1,2,3]

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ABSTRACT The objective of these studies was to compare isolated soybean protein, casein hydrolysate and dried egg white as amino acid sources in chick diets varying in zinc content. Compared with isolated soybean protein in low zinc diets for chicks, casein hydrolysate and dried egg white produced severe zinc deficiency, but the chicks did not develop leg abnormalities, and tibias and femurs of the chicks had higher concentrations of zinc, ash and fat and less water. The length-to-width ratios of femurs from zinc-deficient chicks fed casein hydrolysate or egg white were much greater than for chicks fed zinc-deficient soy protein diets. Dietary amino acid source had no effect on the zinc content of heart, liver or muscle. Supplements of Mn, inorganic P, vitamin D_3, cod liver oil, or a mixture of 13 additional trace elements had no effect on the zinc-deficiency syndrome in chicks fed soy protein. Autoclaving the soy protein or adding 80 ppm Cu had slight beneficial effects, whereas doubling of the mineral mix had slight detrimental effects on the zinc-deficient chick fed soy protein. HEDTA in a soy protein diet containing 12 ppm zinc overcame all zinc-deficiency symptoms. Addition of phytic acid in amounts comparable to those in soy protein to egg white and casein hydrolysate diets caused little or no depression in zinc availability and caused no leg abnormalities. It appears that soy protein contains a complicating factor other than phytic acid which affects zinc metabolism, especially in bone.

It is well established that the source of protein or amino acids in a zinc-deficient diet for chicks or poults affects zinc availability (1–3). In particular, protein sources such as isolated soybean protein and sesame meal adversely influence zinc availability. The main criteria used to establish zinc requirements in most studies using different protein sources have been growth and severity of zinc-deficiency symptoms. Limited attention has been given to the effect of dietary amino acid sources on zinc content of body tissues, on the chemical composition of bone, or on the relative severities of the different symptoms of zinc deficiency.

The effect of several other dietary variables on zinc availaibility for the chick have also been studied. O'Dell and Savage (3) and Savage et al. (4) reported that phytic acid, which occurs in isolated soy protein, decreased the availability of zinc from either a casein or soy protein diet. Likuski and Forbes (5) obtained evidence that phytic acid also decreased zinc availability from diets containing free amino acids but no protein.

Other workers have studied the interrelationships between zinc and other dietary components. These include calcium, phosphorus, copper, cadmium, vitamin D, molybdenum and various chelating agents such as ethylenediaminetetraacetic acid (EDTA). A recent discussion of several of these interrelationships has been published (6).

The studies to be reported were conducted with chicks to determine whether dietary amino acid source affected the nature of the zinc-deficiency syndrome and the distribution of zinc within the body. The effects of several other dietary variables, including minerals, phytic acid, vitamin D and a chelating agent, on the amino acid-to-zinc relationship were also ascertained.

MATERIALS AND METHODS

Three experiments were conducted with day-old New Hampshire × Single Comb

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[3] Part of this paper was based on a thesis submitted by the senior author to the University of Wisconsin in partial fulfillment of the requirements for the B.S. degree, and part was based on a thesis submitted by the senior author to the Graduate School, University of Wisconsin, in partial fulfillment of the requirements for the M.S. degree.

TABLE 1

Composition of the basal diets

	Soy protein	Casein hydrolysate	Egg white
	g	*g*	*g*
Glucose monohydrate [1]	652.44	605.24	613.19
Casein hydrolysate (acid, salt-free) [2]	—	250.00	—
Soybean protein [3]	225.00	—	—
Sprayed dried egg white [2]	—	—	267.00
Corn oil	50.00	50.00	50.00
Choline chloride	2.00	2.00	2.00
Mineral mix [4]	59.96	59.96	59.96
Tryptophan	—	2.20	—
Glycine	2.00	15.00	3.00
Arginine	—	6.00	0.25
Methionine	5.00	6.00	—
Vitamin A mix [5]	1.00	1.00	1.00
Biotin mix [6]	—	—	1.00
α-Tocopheryl acetate	0.10	0.10	0.10
Vitamin mix [7]	2.50	2.50	2.50
Total	1000.00	1000.00	1000.00

[1] Cerelose, Corn Products Company, New York.
[2] General Biochemicals, Chagrin Falls, Ohio.
[3] ADM C-1 Assay Protein, Archer-Daniels-Midland, Cincinnati.
[4] The mineral mix contained: (in grams) $CaCO_3$, 15; K_2HPO_4, 9; Na_2HPO_4, 7.3; $Ca_3(PO_4)_2$, 14; $MgSO_4$, 2.44; NaCl, 8.9; $Fe_2(SO_4)_3 \cdot 6H_2O$, 0.28; $MnSO_4 \cdot H_2O$, 0.42; KI, 0.04; $CuSO_4 \cdot 5H_2O$, 0.02; glucose, 2.56.
[5] The vitamin A mix contained vitamin A conc (250,000 units/g, Chas. Pfizer and Company, New York), 1.0 g, and sucrose, 24.0 g to provide 10,000 IU/kg of diet.
[6] The biotin mix contained biotin, 0.1 g and glucose monohydrate, 99.9 g.
[7] The vitamin mix contained: (in mg) thiamine·HCl, 100; niacin, 100; riboflavin, 16; Ca pantothenate, 20; vitamin B_{12} (0.001% triturate), 20; pyridoxine·HCl, 6; biotin, 0.6; folic acid, 0.4; inositol, 100; menadione, 5; and vitamin D_3 concentrate (Vita Plus Corporation, Madison, Wisconsin), 1 g (1500 ICU); and glucose monohydrate, 1.12 g.

White Leghorn chicks without segregation according to sex. The birds were allotted at random, each group containing 11 or 12 in experiment 1, and 10 or 11 in experiments 2 and 3, and placed in a stainless steel battery at 37° to 40°. Feed and distilled water were provided ad libitum in aluminum troughs.

The compositions of the basal isolated soybean protein (soy protein), egg white, and casein hydrolysate diets are shown in table 1. Feed was mixed every 2 weeks and stored in a refrigerator until fed.

In experiment 1 (11 groups), 5 groups of chicks were fed the casein hydrolysate diet with zinc supplementation of zero, 5, 10, 20 and 40 ppm. Five groups were fed the soy protein diet with zinc supplementation of zero, 5, 10, 20 and 40 ppm. All zinc was added in the form of zinc oxide. One group of chicks was fed a practical-type corn-oats-soybean meal diet.[4] The basal soy protein diet contained by analysis 6.3 ppm zinc, and the basal casein hydrolysate diet, 1.8 ppm zinc on an air-dried basis.

In experiment 2 (20 groups), 3 groups of chicks were fed the casein hydrolysate diet with zinc supplementation of zero, 5 and 20 ppm. Four groups were fed the soy protein diet with zinc supplementation of zero, 5, 40 and 80 ppm zinc. Eight additional groups were fed the soy protein diet containing 5 ppm supplemental zinc and an additional supplement to provide, respectively: 5 ppm Cu ($CuSO_4 \cdot 5H_2O$), 80 ppm Cu ($CuSO_4 \cdot 5H_2O$), 136 ppm Mn ($MnSO_4 \cdot H_2O$), 3000 ICU vitamin D_3/kg, cod liver oil (3000 ICU vitamin D_3/kg), 500 ppm hydroxyethylethylenediaminetriacetic acid (HEDTA), 59.96 g additional mineral mix/kg (table 1), and a trace mineral mixture containing: (in ppm) Mo ($(NH_4)_6Mo_7O_{24} \cdot 4H_2O$), 3; F (NaF), 2; Br (KBr), 10; W ($Na_2WO_4 \cdot 2H_2O$), 3; Se ($Na_2SeO_3 \cdot 5H_2O$), 2; Ba ($BaCO_3$), 50;

[4] Composition of the practical-type diet (in per cent): ground corn, 42; ground oats, 10; wheat middlings, 10; alfalfa meal, 5; meat scraps, 5; soybean oil meal, 20; iodized salt, 0.5; fish meal, 5; chick size oyster shells, 1; granite grit, 1; and supplements providing vitamin D (600 ICU/kg diet), vitamin A (1600 IU/kg diet), riboflavin (4.4 mg/kg diet), vitamin B_{12} (6.5 μg/kg diet), penicillin (3.2 mg/kg diet), and $MnSO_4$ (0.22 g/kg diet), 0.5.

B (H_3BO_3), 10; Cd ($CdCl_2 \cdot 2\frac{1}{2}H_2O$), 1; Cr ($K_2CrO_4$), 2; Co ($2CoCO_3 \cdot 3Co(OH)_2$, 2; As ($Na_2HAsO_4 \cdot 7H_2O$), 1; Sr ($SrCO_3$), 50 and V ($VSO_4 \cdot 7H_2O$), 3. Two groups were fed the casein hydrolysate diet with 0.5% phytic acid/kg (1.25% commercial phytic acid),[5] the same amount present in isolated soybean protein (3), and with zinc supplementation of 5 and 20 ppm. The commercial phytic acid was analyzed by the method of Holt (7), and was found to be 40% phytic acid. Two groups of chicks were fed the soy protein diet with zinc supplementation of zero, and 5 ppm, and in which the soy protein had been autoclaved for 15 minutes at 120°. The last group of chicks was fed a corn-oats-soybean meal practical-type diet.[6] In this experiment, the basal soy protein diet analyzed 7.3 ppm and the casein hydrolysate diet analyzed 1.2 ppm zinc on an air-dried basis.

In experiment 3 (10 groups), 2 groups of chicks were fed the soy protein diet with zinc supplementation of 5 and 40 ppm. Three groups were fed the egg white diet with zinc supplementation of zero, 5 and 20 ppm and 3 groups were fed the egg white diet with 0.4% phytic acid (1.0% commercial phytic acid) and with zinc supplementation of zero, 5 and 20 ppm. Two groups of chicks were fed the soy protein diet containing 5 ppm supplemental zinc and an additional 0.5% inorganic phosphorus and 15,000 ICU vitamin D_3/kg, respectively. The basal egg white diet analyzed 2.1 ppm zinc and the soy protein diet with 5 ppm supplemental zinc analyzed 12.4 ppm zinc on an air-dried basis.

During the course of the experiment, the chicks were weighed weekly and observed for abnormalities. When the chicks were 4 to 5 weeks of age, leg scores and body weights were taken. The chicks were then immobilized with ether and decapitated. In experiment 1, blood serum, liver, femur, heart, wing feathers, and skin from the back of each chick were removed for analysis. In experiment 2 and 3, only the femurs and tibias were removed for analysis. All tissues were stored at −8° until they were analyzed.

Leg scores were assessed at the time the chicks were killed (1 indicated normal and 5 indicated very abnormal legs with extremely shortened and thickened bones, and with hocks twisted and swollen so that the chick could hardly walk).

Diameter and length measurements of femurs and tibias were made by removing the flesh from the bone by rubbing with cheesecloth and measuring the smallest diameter and the largest length with micrometers. Since differences due to treatment on the tibia and femur data were similar, only the femur data are presented in this report.

In preparing the samples for zinc analysis, flesh was removed from the bone by rubbing with cheesecloth. In experiment 3, the bone ends were analyzed separately from the shaft of the bone. One end was defined as one-sixth of the length of the whole bone. Liver, muscle, skin and heart were analyzed as obtained from the chick. The feathers were washed in deionized water before zinc analysis to remove adhering foreign material.

After drying at 110° and ashing at 600°, each sample was dissolved in redistilled 2 N HCl and diluted to a known volume with deionized water. The zinc content of each sample was obtained by using an atomic absorption spectrophotometer.[7] A standard curve, plotting parts per million of zinc as the abcissa and the percentage absorption as the ordinate was used to get the zinc content of each sample. The results obtained compared well with those obtained by the method of Vallee and Gibson (8). Statistical analysis was by Duncan's multiple range test for unequal replication (9).

RESULTS AND DISCUSSION

Comparison of soy protein, casein hydrolysate and egg white as amino acid sources. Each of the diets, unless supplemented with zinc, caused severe zinc deficiency as assessed by growth rate (table 2). However, maximal growth was greater with the soy protein diet than with the casein hydrolysate or egg white diets.

Although it was expected that the symptoms of zinc deficiency in chicks fed amino acids from different sources would be alike, this was not the case (see fig. 1). Zinc-deficient chicks fed the soy protein diet had

[5] General Biochemicals, Chagrin Falls, Ohio.
[6] See footnote 4.
[7] Model 214, Atomic Absorption Spectrophotometer, Perkin-Elmer Corporation, Norwalk, Connecticut.

TABLE 2

Body weights, leg scores and femur length-to-width ratios of chicks fed different amino acid sources at several levels of zinc

Amino acid source	Dietary zinc	Body weight at 5 weeks			Leg scores		Femur L/W ratio		
		Exp. 1	Exp. 2	Exp. 3 [1]	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
	ppm.	*g*	*g*	*g*					
Casein hydrolysate	2 [2]	92 [a 3] (27) [4]	113 [a] (75)	—	1.4 [a 5]	—	12.5 [d]	12.1 [e]	—
	7	175 [bc] (20)	139 [a] (50)	—	1.5 [a]	—	12.2 [cd]	11.9 [e]	—
	12	214 [cd] (9)	—	—	—	—	12.7 [d]	—	—
	22	236 [de] (9)	151 [a] (8)	—	1.0 [a]	—	—	13.3 [d]	—
	42	230 [cde] (9)	—	—	—	—	—	—	—
Soy protein	7 [2]	142 [ab] (0)	136 [a] (8)	—	4.2 [b]	—	9.0 [ab]	10.3 [b]	—
	12	203 [cd] (11)	221 [b] (0)	141 [a] (0)	4.2 [b]	4.5 [b 5]	8.4 [a]	8.7 [a]	9.1 [a]
	17	281 [e] (0)	—	—	—	—	8.6 [a]	—	—
	27	342 [f] (10)	—	—	—	—	10.6 [bc]	—	—
	47	391 [fg] (20)	403 [d] (0)	284 [d] (0)	1.0 [a]	1.2 [a]	11.4 [cd]	11.4 [c]	11.6 [b]
	87	—	384 [cd] (0)	—	1.0 [a]	—	—	11.0 [bc]	—
Egg white	2 [2]	—	—	55 [a] (80)	—	1.0 [a]	—	—	11.5 [b]
	7	—	—	91 [a] (10)	—	1.4 [a]	—	—	11.0 [b]
	22	—	—	208 [c] (10)	—	1.2 [a]	—	—	11.8 [b]
Practical-type diet [6]	40 [2]	420 [g] (20)	364 [c] (0)	—	1.0 [a]	—	11.2 [cd]	11.6 [c]	—

[1] Body weights were taken at 4 weeks instead of 5 weeks.
[2] Zinc content by analysis of diet with no supplemental zinc.
[3] Values followed by the same letters within a given experiment are not significantly different ($P > 0.05$) from each other.
[4] Mortality rate in per cent.
[5] 1 = normal; 5 = abnormal.
[6] See footnote 4 in the text for composition of the practical-type diet.

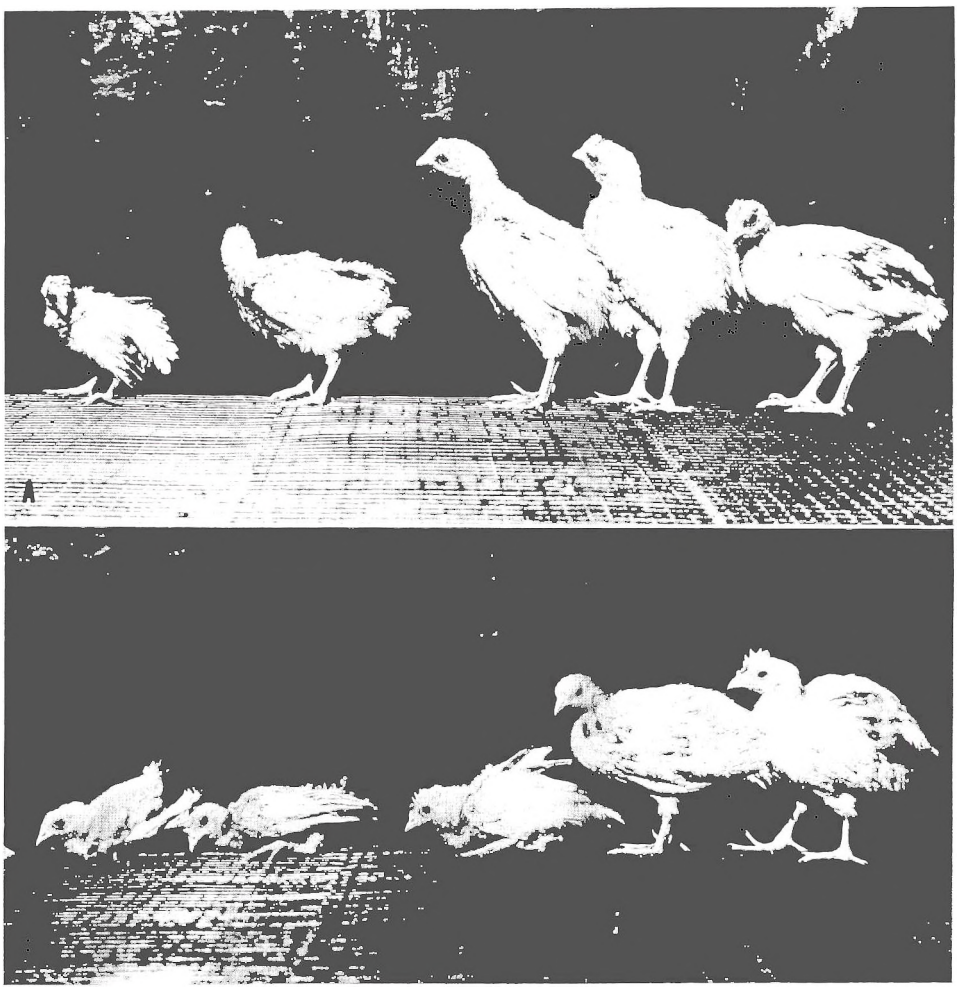

Fig. 1 The effect of isolated soy protein versus casein hydrolysate on the zinc requirement of chicks. *1A*. A representative chick from each group fed the casein hydrolysate diet with increasing levels of zinc from left to right (2, 7, 12, 22 and 42 ppm). Note that all chicks stand. *1B*. A representative chick from each group fed the soy protein diet with increasing levels of zinc from left to right (7, 12, 17, 27 and 47 ppm). Note that chicks fed the lowest 3 levels of zinc chose not to stand.

the symptoms described by O'Dell et al. (10); however, the chicks fed casein hydrolysate or egg white diets differed noticeably in 3 respects. These chicks had more severe keratosis with open sores and bleeding. Also, there was a higher mortality rate with losses as high as 80% (table 2). The most striking difference was that the chicks fed the low zinc casein hydrolysate or egg white diets did not show leg abnormalities (see figs. 1 and 2). The chicks walked normally, and the leg bones were not shortened nor thickened. There was no swelling of the hock. Length-to-width ratios of femurs and leg scores (table 2) and X-ray radiographs verified the difference in gross bone structure. Compared with chicks fed zinc-deficient soy protein diets, chicks fed the zinc-deficient casein hydrolysate or egg white diets had significantly lower leg scores and significantly higher femur length-to-width ratios ($P < 0.05$).

From these observations, it appears that isolated soybean protein contributes a complicating factor which, in addition to de-

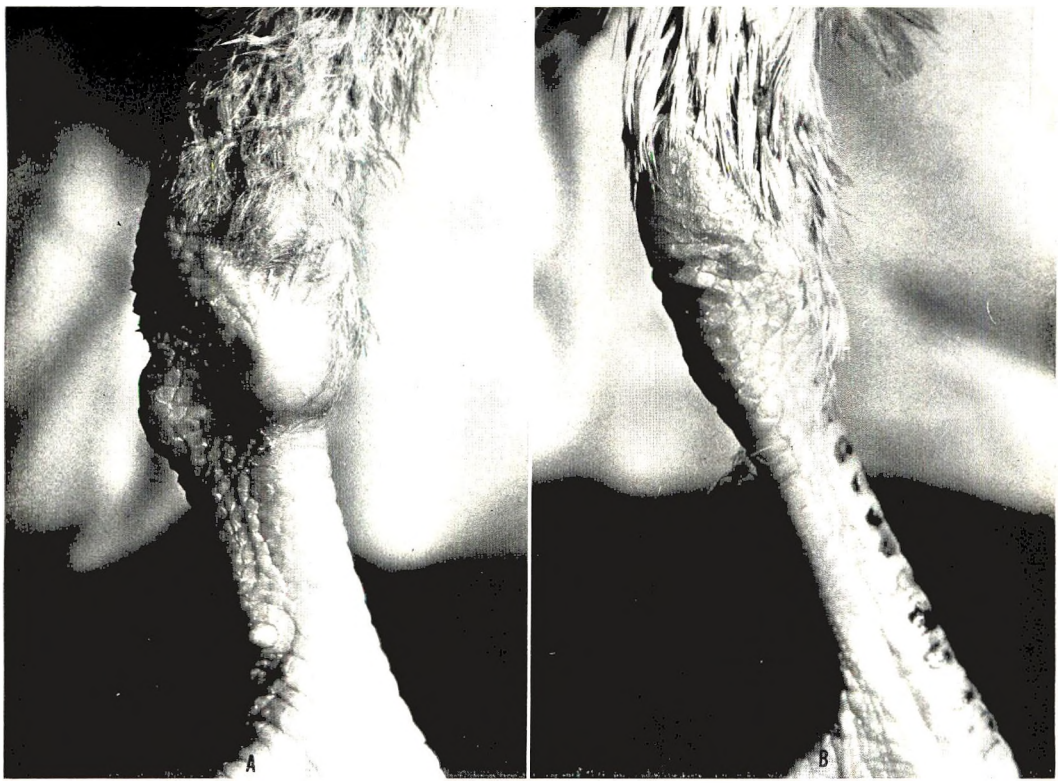

Fig. 2 Effect of isolated soy protein versus casein hydrolysate on the leg development of zinc-deficient chicks. 2A. The shorter and thicker leg bones, and the swollen hocks of a zinc-deficient chick fed the soy protein diet with 12 ppm zinc. 2B. A normal leg of a chick fed the casein hydrolysate diet with 12 ppm zinc. Both chicks were selected to be the same size to show the difference in bone length and width. The right picture, although not one of a zinc-deficient chick, is a good example of the appearance of the legs of chicks fed the zinc-deficient casein hydrolysate diet.

creasing availability of dietary zinc, inhibits the utilization of zinc in bone formation, for in all other symptoms, chicks fed the casein hydrolysate or egg white diets showed as severe or more severe symptoms of zinc deficiency.

Analyses of femurs or tibias also demonstrated the marked effect of dietary amino acid source on bone formation. The concentration of zinc in bones (table 3) was much less at every level of zinc supplementation in the chicks fed soy protein. Even at the 40-ppm supplemental zinc level, the chicks fed the soy protein diet had much lower zinc concentrations in their femurs or tibias than did the chicks fed the casein hydrolysate diets or egg white diets.

Data of table 3 indicate that to reach a plateau in zinc concentration in the tibias or femurs of the chick, about 10 ppm supplemental zinc (12 ppm total zinc) was needed in the casein hydrolysate diet and 40 ppm supplemental zinc (47 ppm total zinc) in the soy protein diet.

The amino acid source also affected the gross composition of bone (table 3). The chicks fed casein hydrolysate had a higher percentage of ash (fat-free, dry basis) and of fat (fresh basis) and a lower percentage of water in their femurs than did the chicks fed the soy protein diet at every level of zinc supplementation. These differences, especially the percentage of water, were probably partially the result of the abnormal swollen hocks. From radiographs, it appeared that the ends of the tibias from chicks fed the soy protein diet were less calcified when compared with those from chicks fed the casein hydrolysate diet. With both diets, increments of dietary zinc

TABLE 3

Concentration of zinc and gross chemical composition of femurs and tibias from chicks fed amino acids from different sources and several levels of zinc

Amino acid source	Dietary zinc	Zinc				H_2O	Fat [1]	Ash [2]	
		Exp. 1	Exp. 2	Exp. 3		Exp. 1	Exp. 1	Exp. 1	Exp. 2
		Femurs	Tibias	Tibia ends	Tibia shafts	Femurs	Femurs	Femurs	Tibias
	ppm	*ppm* [3]	*ppm* [3]	*ppm* [3]	*ppm* [3]	%	%	%	%
Casein hydrolysate	2 [4]	46 [a 5]	60 [b]	—	—	25.6 [a]	13.4 [bc]	39.2 [bcde]	44.5 [cde]
	7	88 [b]	64 [b]	—	—	27.6 [ab]	18.9 [d]	43.8 [fg]	45.1 [de]
	12	156 [de]	—	—	—	32.6 [b]	14.1 [cd]	44.4 [g]	—
	22	187 [f]	195 [e]	—	—	42.2 [c]	10.1 [abc]	42.1 [defg]	46.6 [e]
	42	176 [ef]	—	—	—	45.2 [c]	9.6 [abc]	40.2 [cdef]	—
Soy protein	7 [4]	29 [a]	28 [a]	—	—	46.9 [cd]	10.7 [abc]	34.1 [a]	41.9 [bc]
	12	42 [a]	32 [a]	33 [a]	45 [a]	48.0 [cd]	14.9 [cd]	37.8 [abcd]	43.4 [bcd]
	17	39 [a]	—	—	—	52.6 [de]	10.4 [abc]	33.8 [a]	—
	27	86 [b]	—	—	—	54.6 [de]	8.0 [ab]	35.8 [abc]	—
	47	139 [cd]	113 [d]	97 [b]	186 [c]	56.8 [e]	4.6 [a]	35.3 [ab]	42.8 [bcd]
	87	—	113 [d]	—	—	—	—	—	38.9 [a]
Egg white	2 [4]	—	—	40 [a]	103 [b]	—	—	—	—
	7	—	—	36 [a]	56 [a]	—	—	—	—
	22	—	—	142 [c]	268 [d]	—	—	—	—
Practical-type diet [6]	40 [5]	115 [bc]	100 [c]	—	—	48.2 [cd]	7.8 [ab]	43.4 [efg]	41.4 [b]

[1] Per cent fat on the fresh basis.
[2] Per cent ash on the fat-free, dry basis.
[3] Fat-free, dry basis.
[4] Zinc content by analysis of diet with no supplemental zinc.
[5] Values followed by the same letters within the same column are not significantly different ($P > 0.05$) from each other.
[6] See footnote 4 in the text for composition of the practical-type diet.

TABLE 4

Zinc content of the liver, heart, muscle, skin, feathers and blood serum from chicks fed amino acids from different sources and several levels of zinc

Amino acid source	Dietary zinc	Liver	Muscle	Heart	Skin	Feather		Serum
						Outer	Inner	
	ppm	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*μg/ml*
Casein hydrolysate	2 [1]	64 [ab 2,3]	57 [a 3]	103 [b 3]	74 [b 3]	146 [a 3]	75 [a 3]	0.54
	7	72 [abcd]	—	—	—	125 [a]	106 [bcd]	0.58
	12	80 [cde]	—	—	—	151 [a]	132 [de]	1.07
	22	75 [abcde]	—	—	—	142 [a]	141 [e]	1.72
	42	89 [e]	47 [a]	76 [a]	36 [a]	145 [a]	141 [e]	1.43
Soy protein	7 [1]	67 [abc]	55 [a]	95 [b]	42 [a]	165 [a]	66 [a]	0.49
	12	65 [ab]	—	—	—	155 [a]	83 [ab]	0.53
	17	65 [ab]	—	—	—	144 [a]	92 [abc]	0.46
	27	78 [bcde]	—	—	—	154 [a]	122 [de]	1.25
	47	83 [de]	38 [a]	72 [a]	30 [a]	189 [a]	126 [de]	—
Practical-type diet [4]	40 [1]	87 [e]	53 [a]	67 [a]	33 [a]	134 [a]	116 [cde]	1.58

[1] Zinc content by analysis of diet with no supplemental zinc.
[2] Values followed by the same letters within the same column are not significantly different ($P > 0.05$) from each other.
[3] Dry-weight basis.
[4] See footnote 4 in the text for composition of the practical-type diet.

increased bone moisture, generally decreased bone fat, but had no consistent effect on bone ash (fat-free, dry basis).

The zinc content of the livers (table 4) increased somewhat with increased dietary zinc but was not consistently affected by dietary amino acid source.

Dietary amino acid source and amount of dietary zinc had little or no effect on the zinc content of muscle, heart, skin, or outer portion of the wing feather (table 4). One exception was that the skin from chicks fed the unsupplemented casein hydrolysate diet was actually significantly higher ($P < 0.05$) in zinc than that for all other groups tested. The zinc content of the inner portion of the wing feather (the portion which developed after the chick was fed its respective diet) was less in the chicks fed the soy protein diet at every level of zinc supplementation, and was substantially increased by increments of dietary zinc. Results on blood serum zinc could not be subjected to statistical analysis as composite samples were analyzed. It appears that, in general, zinc content of blood serum was higher for casein hydrolysate than for soy protein at comparable dietary zinc levels. The amount of zinc in blood serum increased with dietary zinc additions with an abrupt increase apparently occurring at about the minimal level of dietary zinc resulting in nearly maximal growth.

At comparable dietary zinc levels, the zinc content of bone, and to some extent, the part of the feather formed after the chick was fed its respective diet, depended upon whether the dietary amino acid source was isolated soybean protein, casein hydrolysate, or egg white. Dietary amino acid source had little effect on the zinc content of the soft tissues of the zinc-deficient chick, or on the nature of many of the zinc-deficiency symptoms. Because soy protein in the low zinc diets resulted in chicks with abnormally formed bones with smaller length-to-width ratios, lower zinc and ash content, and higher water content, it is concluded that soy protein adds a complicating factor in zinc deficiency which adversely affects bone formation.

Effect of certain dietary supplements on the relationship between dietary amino acid source and zinc-deficiency symptoms. In an attempt to assess the nature of the complicating factor in soy protein which adversely affected bone formation in the zinc-deficient chick, a number of supplements were added to diets containing different amino acid sources, and the effects on the leg bones were observed. Of the supplements tested, the following had no effect on the zinc-deficient chick in the criteria tested (i.e., body weight, leg scores, femur length-to-width ratio, zinc content of tibias, table 5): 5 ppm copper, 136 ppm manganese, 3000 ICU vitamin D_3/kg, 15,000 ICU vitamin D_3/kg, cod liver oil (3000 ICU vitamin D_3/kg), the trace mineral mix, and 0.5% inorganic phosphorus.

TABLE 5

Body weights, leg scores, femur length-to-width ratios, and concentration of zinc in tibias of chicks fed various dietary supplements in zinc-deficient diets

Amino acid source	Dietary zinc	Supplement	Final body wt	Leg score	Femur L/W ratio	Zinc in tibias		
							Ends	Shafts
	ppm		*g*			*ppm* [1]	*ppm*	*ppm*
			Experiment 2, 5 weeks					
Soy protein	7 [2]	—	136 [a] [3] (8) [4]	4.2 [bcd]	10.3 [bc]	28 [a]		
	12 [5]	—	221 [c] (0)	4.2 [bcd]	8.7 [a]	32 [a]		
	47	—	403 [d] (0)	1.0 [a]	11.4 [d]	113 [c]		
	12	5 ppm Cu	224 [c] (0)	4.3 [cd]	9.0 [a]	31 [a]		
	12	80 ppm Cu	208 [c] (0)	3.8 [b]	9.8 [ab]	35 [a]		
	12	136 ppm Mn	233 [c] (0)	4.6 [de]	8.6 [a]	45 [a]		
	12	3000 ICU vitamin D_3/kg	228 [c] (0)	4.2 [bcd]	8.7 [a]	—		
	12	cod liver oil (3000 ICU vitamin D_3/kg)	230 [c] (0)	4.2 [bcd]	8.4 [a]	34 [a]		
	12	500 ppm HEDTA	422 [d] (0)	1.0 [a]	11.2 [cd]	69 [b]		
	12	double mineral mix	205 [c] (9)	4.8 [e]	8.7 [a]	30 [a]		
	12	trace mineral mix	224 [c] (0)	3.8 [bc]	8.9 [a]	34 [a]		
Soy (autoclaved)	7 [2]	—	152 [ab] (17)	4.6 [de]	8.9 [a]	30 [a]		
Soy (autoclaved)	12	—	236 [c] (0)	4.5 [de]	8.3 [a]	39 [a]		
Casein hydrolysate	7 [5]	0.5% phytic acid	130 [a] (0)	1.3 [a]	12.8 [ef]	76 [b]		
	22	0.5% phytic acid	171 [b] (0)	1.0 [a]	12.1 [de]	195 [d]		
	7	—	139 [ab] (50)	1.5 [a]	11.9 [de]	64 [b]		
	22	—	151 [ab] (8)	1.0 [a]	13.3 [f]	195 [d]		
			Experiment 3, 4 weeks					
Soy protein	12 [5]	—	141 [b] (0)	4.5 [b]	9.1 [a]		33 [a]	45 [a]
	47	—	284 [d] (10)	1.2 [a]	11.6 [b]		97 [c]	186 [c]
	12	15,000 ICU vitamin D_3/kg	142 [b] (0)	4.3 [b]	9.6 [a]		37 [a]	46 [a]
	12	0.5% inorganic P	139 [b] (0)	4.0 [b]	9.2 [a]		39 [a]	49 [a]
Egg white	2 [2]	0.4% phytic acid	46 [a] (90)	1.0 [a]	11.6 [b]		54 [b]	114 [b]
	7	0.4% phytic acid	75 [a] (10)	1.9 [a]	10.9 [b]		37 [a]	60 [a]
	22	0.4% phytic acid	239 [c] (20)	1.5 [a]	11.6 [b]		107 [d]	211 [d]
	2	—	55 [a] (80)	1.0 [a]	11.5 [b]		40 [a]	103 [b]
	7	—	91 [a] (10)	1.4 [a]	11.0 [b]		36 [a]	56 [a]
	22	—	208 [c] (10)	1.2 [a]	11.8 [b]		142 [e]	268 [e]

[1] Fat-free, dry basis.
[2] Zinc content by analysis of diet with no supplemental zinc.
[3] Within an experiment values followed by the same letters within the same column are not significantly different ($P > 0.05$) from each other.
[4] Mortality rate in per cent.
[5] Zinc content by analysis of diet with 5 ppm supplemental zinc.

Eighty parts per million of copper added to the soy protein diet appeared to alleviate slightly the bone disorder in the zinc-deficient chick. Although body growth (table 5) due to the 80-ppm copper treatment was not significantly different than that of the chicks fed the control diet (soy protein diet containing 12 ppm zinc), there was a significant increase ($P < 0.05$) in the length-to-width ratio of the tibias (data not presented). Moreover, data of table 5 show that leg score and the length-to-width ratio of the femur were improved by copper (3.8 compared with 4.2 and 9.8 compared with 8.7, respectively). However, these differences were not significant. Perhaps copper eliminated some of the zinc-complicating factor present in the soy protein diet by reacting with the factor itself, thus making the factor unavailable to react with zinc.

Doubling the mineral mix in the soy protein diet containing 12 ppm zinc resulted in a significant increase ($P < 0.05$) in the leg score of chicks (table 5). Although not significant, average body weight and zinc content of the tibias were less than those of chicks fed the control diet. This increase in the severity of zinc-deficiency symptoms was probably due to the additional calcium in the diet (6).

Adding HEDTA to the soy protein diet resulted in an expected (11) beneficial effect. Body growth, length-to-width ratio of the femur, leg score, and zinc content of the tibias were all significantly improved ($P < 0.05$) in chicks fed HEDTA when compared with the chicks fed the control diet (table 5). Although the bones from chicks fed HEDTA appeared normal, the zinc content of the tibias was significantly lower ($P < 0.05$) than that of the tibias from the chicks fed the soy protein diet with 47 ppm zinc. The beneficial effect of HEDTA was most likely due to the increased availability of zinc present in the diet as postulated by Vohra and Kratzer (11). A chelating agent, such as HEDTA, which has a proper zinc stability constant can presumably remove zinc from the unavailable zinc complex with soy protein and form a complex from which the chick can readily obtain zinc. The beneficial effect is the result of the increased absorption of zinc. However, because the zinc content of the tibias from the chicks fed HEDTA was much less than that of the tibias from chicks fed the soy protein diet with 47 ppm zinc, it is possible that HEDTA may also improve zinc metabolism in some way during bone formation.

Using autoclaved soy protein had no significant effect upon the zinc-deficient chick (table 5). However, there was a tendency for improvement in the average weight of the chick, and an increase in zinc content of tibias. Kratzer et al. (12) observed that autoclaving soy protein significantly increased the availability of zinc for the turkey poult. However, they autoclaved soy protein at 120° for 30 minutes instead of the 15 minutes used in this investigation. It appears that 15 minutes of autoclaving did not destroy the complicating factor present in the soy protein. Lease et al. (13) reported that autoclaving for 30 minutes did not always destroy the zinc-complicating factor present in sesame meal.

The addition of 0.5% phytic acid to the casein hydrolysate diet, and the addition of 0.4% phytic acid to the egg white diet which provided a concentration of phytin phosphorus of approximately 0.14% and 0.11%, respectively, had little effect upon the chick whether it was zinc-deficient or not (table 5). In external appearances, including legs, there was little difference between the chicks fed the phytic acid and those that were not. Chicks fed phytic acid tended to weigh less at the 7-ppm zinc level with the casein hydrolysate diet, and less at the 2 and 7 ppm zinc level with the egg white diet, but the differences were not significant. With the exception of the length-to-width ratio of femurs from chicks fed the casein hydrolysate diet with 22 ppm zinc and supplemental phytic acid, which was significantly less ($P < 0.05$) than that of the chicks fed the casein hydrolysate diet with no additional phytic acid and with 22 ppm zinc, there was no effect of phytic acid on the length-to-width ratios of the femurs, or on the leg scores. There was no significant effect upon the zinc content of the tibias when chicks were fed phytic acid in a casein hydrolysate diet. Adding phytic acid to the egg white diets resulted in a significant decrease ($P < 0.05$) in the zinc content of both the ends and the shafts of

the tibias of chicks fed 22 ppm zinc, but not of chicks fed 2 or 7 ppm zinc.

These results, especially the growth data, do not agree well with the results of Likuski and Forbes (5), or of O'Dell and Savage (3). These workers reported a significant decrease in the weight of chicks when fed phytic acid in an amino acid or casein diet, and thus concluded that zinc was less available for the chick when phytic acid was present in the diet. There were indications in the data of this experiment that phytic acid did decrease the availability of zinc, but it was not to any great extent. Perhaps not enough phytic acid was added to the diets, because, in reviewing the literature, no evidence was found that phytic acid, added to an animal protein or amino acid diet at the level found in isolated soy protein diets, had as great an effect on zinc availability as did the complicating factor in soy protein. In addition, no report was found in which it was shown that phytic acid added in any amount to an amino acid or casein diet resulted in chicks with the bone disorder present in chicks fed a zinc-deficient isolated soy protein diet. Much evidence indicates that phytic acid does reduce the availability of zinc for chicks, but it appears that soy protein contains a complicating factor in addition to phytic acid, which affects zinc metabolism in some way, especially in bone formation. Effects of this factor can be eliminated by the addition of enough zinc or a chelating agent such as HEDTA, and perhaps can be alleviated by the addition of large amounts of copper to the diet.

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Effect of Some Dietary Synthetic and Natural Chelating Agents on the Zinc-deficiency Syndrome in the Chick [1,2]

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ABSTRACT The objective of these studies was to assess the effects of some synthetic and natural zinc-binding agents on the zinc-deficiency syndrome in chicks fed diets containing isolated soybean protein. Synthetic chelating agents EDDA (zinc stability constant 11.10), HEDTA (14.50), EDTA (16.50), and DTPA (18.14), at 1.8 mmoles/kg diet, and natural chelating agent cysteine (18.20) at 0.5% of the diet, alleviated all zinc-deficiency symptoms, which included poor growth, poor feathering, low length-to-width ratios of femurs, low zinc content of tibias and leg abnormalities. HEIDA (8.57) slightly improved the growth, feather score and leg score. Supplemental histidine (12.88) at 0.5% of the diet alleviated the bone disorder; however, it did not improve growth or increase the zinc content of bone. CDTA (18.67) substantially depressed growth of zinc-deficient chicks, but the effect was not statistically significant. Synthetic chelating agents DHEG (5.36), IDA (7.02) and EBONTA (11.00), and natural chelating agents xanthurenic acid, kynurenic acid and anthranilic acid (20.93), at 1.8 mmoles/kg diet, and glutamic acid (9.46), cystine and tryptophan (9.30) at 0.5% of the diet had no effect on the zinc-deficiency syndrome. All chelating agents with stability constants for zinc between 11.10 and 18.20, except histidine, overcame all zinc-deficiency symptoms, whereas histidine alleviated only bone and, to some extent, feather defects.

Kratzer et al. (1) demonstrated that the addition of ethylenediaminetetraacetic acid (EDTA) to a diet containing isolated soybean protein decreased the zinc requirement of turkey poults. O'Dell and Savage (2) reported that phytic acid decreased the availability of zinc in casein diets for chicks and later they (3) showed that EDTA added with phytic acid counteracted the detrimental effect of phytic acid. Vohra and Kratzer (4) tested the influence of various chelating agents on the availability of zinc for turkey poults. Chelates with zinc stability constants ranging from 5.3 to 18.8 were tested for growth-promoting activity in poults fed a zinc-deficient diet containing isolated soybean protein. These investigators reported that, generally, chelating agents with stability constants between 13 and 17 were most satisfactory for improving the growth rate, with the optimum being approximately 14.5.

Although effects of many synthetic chelating agents on mineral deficiencies have been studied, little is known of the effects of natural chelating agents such as histidine and cysteine. These should be studied because they are efficient binders of zinc. Weinberg (5) has postulated that natural products will be found which are active in a wide variety of biosynthetic processes primarily because of their ability to combine with a specific metal at a critical intracellular site. Another indication that zinc-histidine and zinc-cysteine complexes may play an important role in the animal body is that Weitzel et al. (6, 7) showed that the tapetum lucidum from the eyes of the dog, fox, and seal contained large amounts of zinc which was in a zinc-cysteine hydrate form. Weitzel and co-workers (8, 9) have also demonstrated the preparation of zinc·histidyl peptides and zinc complexes with histidine and histidinamide. They noted that many of the zinc·histidyl peptides were readily soluble in water and resistant to hydrolysis in solution.

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[2] This work is part of a M.S. thesis prepared by the senior author.

TABLE 1

Composition of basal diet

	g
Glucose monohydrate [1]	652.44
Soybean protein [2]	225.00
Corn oil	50.00
Choline chloride	2.00
Mineral mix [3]	59.96
Glycine	2.00
Methionine	5.00
Vitamin A mix [4]	1.00
α-Tocopheryl acetate	0.10
Vitamin mix [5]	2.50
Total	1000.00

[1] Cerelose, Corn Products Company, New York.
[2] ADM C-1 Assay Protein, Archer-Daniels-Midland, Cincinnati.
[3] The mineral mix contained: (in g) $CaCO_3$, 15; K_2HPO_4, 9; Na_2HPO_4, 7.3; $Ca_3(PO_4)_2$, 14; $MgSO_4$, 2.44; NaCl, 8.9; $Fe_2(SO_4)_3 \cdot 6H_2O$, 0.28; $MnSO_4 \cdot H_2O$, 0.42; KI, 0.04; $CuSO_4 \cdot 5H_2O$, 0.02; and glucose, 2.56.
[4] The vitamin A mix contained vitamin A conc (250,000 units/g, Chas. Pfizer and Company, New York), 1.0 g, and glucose monohydrate, 24.0 g to provide 10,000 IU/kg diet.
[5] The vitamin mix contained: (in mg) thiamine·HCl,100; niacin, 100; riboflavin, 16; Ca pantothenate, 20; vitamin B_{12} (0.001% triturate), 20; pyridoxine·HCl, 6; biotin, 0.6; folic acid, 0.4; inositol, 100; menadione, 5; and (in g) vitamin D_3 conc (Vita Plus Corporation, Madison, Wisconsin), 1 g (1500 ICU); and glucose monohydrate, 1.12 g.

The objective of the present investigation was to compare the effect of various natural and synthetic chelating agents on the zinc-deficiency syndrome in chicks fed an isolated soybean protein diet.

MATERIALS AND METHODS

Two experiments were conducted with day-old New Hampshire × Single Comb White Leghorn chicks without segregation according to sex. The birds were distributed at random into groups of 10 each and placed in a stainless steel battery at 37° to 40°. Feed and distilled water were provided ad libitum in aluminum troughs. Feed was mixed every 2 weeks and stored in a refrigerator until it was fed. The composition of the basal isolated soybean protein diet (soy protein) is shown in table 1. The basal diet with 5 ppm supplemental zinc (control diet) analyzed 12.4 ppm zinc on an air-dried basis. In experiment 1, 1.8 mmoles of the following chelating agents[3] were each added to a kilogram of diet (symbol and zinc stability constant (4)), N,N-di[2-hydroxyethyl] glycine, Na salt (DHEG, 5.36); iminodiacetic acid salt monohydrate (IDA, 7.02); [2-hydroxyethylimino] diacetic acid (HEIDA, 8.57); ethylenediamine [di(*o*-hydroxyphenylacetic acid)] (EDDHA, 9.26); [ethylenebis(oxyethylenenitrilo)] tetraacetic acid (EBONTA, 11.00); ethylene-N-N′-diacetic acid (EDDA, 11.10); hydroxyethylethylenediaminetriacetic acid (HEDTA, 14.50); ethylenediaminetetraacetic acid (EDTA, 16.50), diethylenetriaminepentaacetic acid (DTPA, 18.14), *trans*-1,2, diaminocyclohexane, N,N,N′,N′-tetraacetic acid (CDTA, 18.67). For comparison purposes, one group of chicks was fed the soy protein diet which had 40 ppm supplemental zinc (total 47 ppm zinc).

In experiment 2, the following chelating agents were each added to 2 diets—one diet was the basal diet plus 5 ppm supplemental zinc, the other, basal diet plus 80 ppm supplemental zinc; (symbol and stability constant if known (10, 11)) 1.8 mmoles of anthranilic acid (AA, 20.93); xanthurenic acid (XA); kynurenic acid (KA); and hydroxyethylethyenediaminetriacetic acid (HEDTA, 14.50); or, 5.0 g/kg of diet of tryptophan (Try, 9.30); cysteine (CySH, 18.20); cystine (Cys); histidine (His, 12.88); and glutamic acid (Glu, 9.46). For controls, one group of chicks was fed the basal diet plus 5 ppm supplemental zinc; another, basal diet plus 80 ppm supplemental zinc.

During the course of the experiments, the chicks were weighed weekly and observed for abnormalities. When the chicks were 4 weeks of age, leg and feather scores, and body weights were taken. Then the chicks were decapitated after immobilization with ether. Both tibias and femurs were removed for analysis and stored at −8° until they were analyzed. Legs were scored using a scale of 1 to 5 as described previously (12). Feathers were also scored using a scale of 1 to 5 in which one indicated normal feathers and five indicated very abnormal feathers with brittle and broken rachises, and with barbules or barbicels which did not function properly so that the feathers had a very ragged appearance.

[3] The chelating agents used in experiments 1 and 2 were obtained from the following sources: DHEG, EDDHA and EDDA, K and K Laboratory, Inc., Jamaica 33, New York; IDA, HEIDA, EBONTA, and EDTA, Eastman Organic Chemicals, Rochester, New York; HEDTA, DTPA, CDTA, XA and KA, Aldrich Chemical Company, Inc., Milwaukee, Wisconsin; AA, Try, CySH, Cys, His and Glu, General Biochemicals, Chagrin Falls, Ohio.

Bone measurements were made by removing the flesh from the tibias and femurs by rubbing with cheesecloth and measuring with micrometers the widest and narrowest dimensions of the bone shaft and bone length. Since other ratios obtained showed similar effects of the various diets, only the length-to-narrowest width ratios of the femurs are presented.

Bone samples were prepared and analyzed for zinc as described previously (12). Statistical analysis was by Duncan's multiple range test for unequal replication (13).

RESULTS AND DISCUSSION

Experiment 1. Effect of various synthetic chelating agents. The data in table

TABLE 2

Body weights, feather scores, leg scores, length-to-narrow width ratios (L/NW) of femurs, and zinc content of tibias at 4 weeks of chicks fed a soy protein diet with synthetic and natural chelating agents

Chelating agent	Zinc supplemented	Body weight	Feather score	Leg score	L/NW femur ratio	Zinc in tibias	
						Ends	Shafts
	ppm	*g*				*ppm* [1]	*ppm* [1]
			Experiment 1				
None	5 [2]	141(0) [3] ab [4]	4.3 a [5]	4.5 a [5]	9.1 ab	33 a	45 a
None	40	284(10) d	1.2 c	1.2 c	11.6 cd	97 e	186 d
DHEG(5.36) [6,7]	5	143(10) ab	3.9 a	4.1 ab	9.4 ab	41 a	60 a
IDA(7.02) [7]	5	139(0) ab	4.3 a	4.0 ab	9.1 ab	35 a	45 a
HEIDA(8.57) [7]	5	191(0) c	2.8 b	3.5 b	9.5 ab	36 a	51 a
EDDHA(9.26) [7]	5	156(70) abc	3.0 ab	2.3 c	11.2 cd	42 ab	66 a
EBONTA(11.00) [7]	5	167(10) bc	4.1 a	4.3 a	8.6 a	37 a	50 a
EDDA(11.10) [7]	5	256(30) d	1.7 c	1.4 c	11.8 d	55 bc	97 b
HEDTA(14.50) [7]	5	291(0) d	1.5 c	1.3 c	10.8 cd	70 d	132 c
EDTA(16.50) [7]	5	280(30) d	1.6 c	1.3 c	11.3 cd	74 d	131 c
DTPA(18.14) [7]	5	283(30) d	1.6 c	1.1 c	11.3 cd	58 c	100 b
CDTA(18.67) [7]	5	102(40) a	4.8 a	4.3 ab	10.2 bc	36 a	54 a
			Experiment 2				
None	5	137(10) ab	4.0 a	4.2 ab	9.2 abc	32 a	
None	80	278(0) de	1.5 c	1.3 e	11.7 gh	142 e	
Try(9.30) [8]	5	141(0) ab	4.5 a	4.9 a	8.6 a	32 a	
Try(9.30) [8]	80	292(0) ef	1.5 c	1.3 e	11.5 gh	148 ef	
AA(20.93) [7]	5	170(0) b	4.1 a	4.3 ab	8.9 ab	30 a	
AA(20.93) [7]	80	318(0) fg	1.4 c	1.1 e	11.6 gh	137 e	
XA [7]	5	137(0) ab	4.3 a	4.2 b	9.4 bcd	33 a	
XA [7]	80	292(0) ef	1.3 c	1.4 e	11.9 h	142 e	
KA [7]	5	149(0) ab	4.5 a	4.6 ab	8.8 ab	30 a	
KA [7]	80	298(0) efg	1.8 c	1.5 de	11.0 fg	141 e	
CySH(18.20) [8]	5	228(0) c	1.7 c	2.3 c	10.1 de	45 b	
CySH(18.20) [8]	80	328(10) c	1.6 c	1.2 e	11.8 gh	186 g	
Cys [8]	5	146(0) ab	4.1 a	4.1 b	9.3 abc	31 a	
Cys [8]	80	305(0) efg	1.4 c	1.2 e	11.2 gh	158 f	
His(12.88) [8]	5	156(0) ab	2.9 b	2.1 cd	10.4 ef	35 ab	
His(12.88) [8]	80	315(0) fg	1.4 c	1.3 e	11.5 gh	179 g	
HEDTA(14.50) [7]	5	259(0) d	1.4 c	1.2 e	11.7 gh	95 c	
HEDTA(14.50) [7]	80	305(0) efg	1.6 c	1.1 e	11.4 gh	157 f	
Glu(9.46) [8]	5	136(0) a	4.5 a	4.2 b	9.7 cde	32 a	
Glu(9.46) [8]	80	325(0) g	1.4 c	1.3 e	11.4 gh	125 d	

[1] Fat-free, dry basis.
[2] Diet contained a total of 12 ppm zinc by analysis.
[3] Mortality rate in per cent.
[4] For each experiment values within a column followed by the same letters are not significantly different ($P < 0.05$) from each other.
[5] 1 = normal; 5 = very abnormal.
[6] Stability constant for zinc, if known, indicated in parenthesis. Meanings of abbreviations: DHEG, N,N-di[2-hydroxyethyl]glycine, Na salt; IDA, iminodiacetic acid salt monohydrate; HEIDA, [2-hydroxyethylimino]-diacetic acid; EDDHA, ethylenediamine (di(o-hydroxyphenylacetic acid)); EBONTA, [ethylenebis (oxyethylene-nitrilo)]tetraacetic acid; EDDA, ethylene-N,N'-diacetic acid; HEDTA, hydroxyethylethylenediaminetriacetic acid; EDTA, ethylenediaminetetraacetic acid; DTPA, diethylenetriaminepentaacetic acid; CDTA, *trans*-1,2-diaminocyclohexane,N,N,N',N'-tetraacetic acid; Try, tryptophan; AA, anthranilic acid; XA, xanthurenic acid; KA, kynurenic acid; CySH, cysteine; Cys, cystine; His, histidine; Glu, glutamic acid.
[7] 1.8 mmoles added/kg of diet.
[8] Five grams added/kg of diet.

2 indicate that chelating agents DHEG (5.36), IDA (7.02) and EBONTA (11.00) had no significant effect upon the growth of zinc-deficient chicks fed the soy protein diet. HEIDA (8.57) significantly increased ($P < 0.05$) growth; however, the final average weight was significantly less ($P < 0.05$) than that of chicks fed the diet with 40 ppm supplemental zinc. Addition of EDDA (11.10), HEDTA (14.50), EDTA (16.50) or DTPA (18.14) to the diet had a pronounced effect upon the growth of the zinc-deficient chicks (growth not significantly different from that of chicks fed 40 ppm supplemental zinc). CDTA (18.67) depressed growth of zinc-deficient chicks substantially but the difference was not statistically significant according to the analysis used. High mortality was observed in chicks fed EDDHA (9.36) and thus, it was difficult to interpret results obtained with these chicks. Some of these results differed from those obtained by Vohra and Kratzer (4) with turkey poults. These workers reported: (a) a slight growth response to EBONTA (no significant response was obtained in this investigation); (b) that DTPA gave an inferior growth response in comparison with EDTA (in this experiment, the growth response was comparable); (c) that a definite peak growth response was obtained with chelating agents with a stability constant of approximately 14.50 (the response appeared to be the same over a range of stability constants from 11.10 to 18.17 in this experiment with a slight, but nonsignificant peak at 14.50; and (d) an "effective" range of stability constants of 13 to 17 (this experiment indicates that with chicks, 11.10 to 18.14 is an "effective" range). Some of the differences may be attributed to Vohra and Kratzer having used turkey poults, whereas in the present investigation, chicks were used.

The effect of these various chelating agents, except that of CDTA, on feather and leg scores, and length-to-width ratios of femurs corresponded with the effect on growth (table 2). CDTA (18.67) added to the low zinc diet did not depress the leg score, nor the length-to-width ratio of the femurs as it apparently did growth.

Stability constant was correlated with the effect a chelator had on the zinc content of the ends and shafts of the tibias. As shown in table 2, chelating agents DHEG, IDA, HEIDA, EDDHA and EBONTA with stability constants ranging from 5.38 to 11.00 and CDTA with a constant of 18.67 had no significant effect upon the zinc content of the tibias. In the case of HEIDA, this is in contrast with the beneficial effect it had on growth. EDDA (11.10) or DTPA (18.14) in a zinc-deficient diet for chicks resulted in tibias which were significantly lower ($P < 0.05$) in zinc content than those fed HEDTA (14.50) or EDTA (16.50). However, chicks fed any chelating agent in the zinc-deficient diet (total 12 ppm zinc) had significantly less ($P < 0.05$) zinc in the tibia ends and shafts than did chicks fed 40 ppm supplemental zinc (total 47 ppm zinc). While a chelating agent with a stability constant of approximately 14.50 to 16.50 was apparently needed for maximal concentration of zinc in bone, in all other criteria tested a chelating agent with a stability constant from 11.10 to 18.14 would suffice.

Nielsen et al. (12) have shown that in order to largely overcome the bone disorder in chicks fed a zinc-deficient isolated soybean protein diet, at least 20 ppm supplemental zinc had to be added to the diet to make a final concentration of approximately 27 ppm zinc. This resulted in a bone zinc concentration of 86.4 ppm on the fat-free, dry basis, which is approximately equal to the whole tibia zinc concentration of the chicks fed DTPA (18.14) or EDDA (11.10) in this experiment. Apparently the bone disorder present in zinc-deficient chicks is overcome by the chelating agent making enough zinc available for the bone so that proper metabolic processes can take place even in the presence of the soy protein "complicating factor" described by Nielsen et al. (12). This "complicating factor" caused a bone disorder characterized by a low concentration of bone zinc in chicks fed soy protein diets, but not in chicks fed casein hydrolysate or egg white diets. A final zinc concentration in fat-free, dry bone of at least 70 to 80 ppm was necessary to prevent the bone disorder. The increased availability of zinc due to chelating agents is perhaps the result of increased intestinal absorption of zinc as described by Vohra and Kratzer (4). However, this does not eliminate the possibility that the chelating

agents are improving the utilization of zinc in the metabolic process of bone formation by interfering in some way with the "complicating factor."

Experiment 2. Effect of various natural chelating agents. Tryptophan, anthranilic acid, xanthurenic acid, kynurenic acid, glutamic acid, and cystine in the amounts tested had no effect upon leg and feather scores, growth, length-to-width ratios of the femurs, or zinc content of the tibias in chicks fed the zinc-deficient soy protein diet (table 2).

Both cysteine and histidine added at 0.5% of the diet had pronounced effects on the zinc-deficient chick (chemical analysis showed that both supplements were essentially devoid of zinc). Cysteine significantly increased ($P < 0.05$) growth and zinc content of the tibias. However, final body weight and bone zinc content was significantly less ($P < 0.05$) than that of chicks fed the basal diet supplemented with 80 ppm zinc or with 80 ppm zinc and 0.5% cysteine.

The addition of cysteine improved feather formation of chicks fed the zinc-deficient diet so that the feather score was not significantly different from that of chicks fed the basal diet supplemented with 80 ppm zinc or 80 ppm zinc plus cysteine.

Cysteine also definitely alleviated the bone disorder present in zinc-deficient chicks fed the isolated soybean protein diet. Cysteine significantly decreased the leg score, and increased the length-to-narrow width ratio of the femurs ($P < 0.05$). However, the length-to-narrow width ratio of the femurs was significantly less, and the leg score was significantly greater ($P < 0.05$) than those of the chicks fed 80 ppm supplemental zinc with or without supplemental cysteine.

Zinc-deficiency symptoms were alleviated, but not completely overcome by the cysteine supplement. This was in contrast with the effects of some of the synthetic chelating agents. Possibly, despite the precautions taken, cysteine was partially converted during storage of the diets to cystine which had no effect upon the zinc-deficient chick (fig. 1), and therefore, insufficient cysteine was present to completely overcome zinc-deficiency symptoms.

Compared with cysteine, histidine had a different effect upon the zinc-deficient chick. Histidine did not significantly improve growth (fig. 2 and table 2) of chicks fed the zinc-deficient soy protein diet, but did improve somewhat ($P < 0.05$) the feather score (it was still significantly higher ($P < 0.05$) than that of chicks fed 80 ppm supplemental zinc with or without additional histidine). Histidine also had a definite beneficial effect upon the bone disorder observed in chicks fed the zinc-deficient soy protein diet. In external appearance, 8 out of 10 chicks walked with a normal gait. They had no swollen hocks, and the shortening and thickening of the leg bones were alleviated (fig. 3). Leg scores were significantly decreased by histidine ($P < 0.05$); however, they were significantly higher ($P < 0.05$) than those of chicks fed 80 ppm supplemental zinc or 80 ppm supplemental zinc and 0.5% supplemental histidine. Histidine in the low zinc diet also significantly increased ($P < 0.05$) the length-to-narrow width ratio of the femurs, but the ratio was significantly less ($P < 0.05$) than that of chicks fed 80 ppm supplemental zinc with or without additional histidine. The growth stimulation resulting from supplemental histidine in chicks fed 80 ppm supplemental zinc ($P < 0.05$, table 2) suggests that this amino acid may, for some reason, be limiting in the soy protein diet.[4]

Unexpectedly, although histidine largely overcame the bone disorder, it had little or no effect on tibia zinc content (table 2). With such a pronounced improvement in the legs of the zinc-deficient chicks in the presence of such a small increase in bone zinc content, it appears that histidine or a histidine metabolite in some way counteracts the "complicating factor" which causes the bone disorder in chicks fed zinc-deficient soy protein diets. The soy protein diet used apparently met the NRC (14) requirement of 0.30% histidine for starting chicks as it contained 0.52% histidine (calculated). This is comparable to casein hydrolysate and egg white diets described previously (12) which contained 0.78% and 0.50%

[4] While the effects of histidine on the bone abnormalities of chicks fed soy protein diets have been confirmed in subsequent experiments, the apparent growth stimulation due to histidine has not been confirmed.

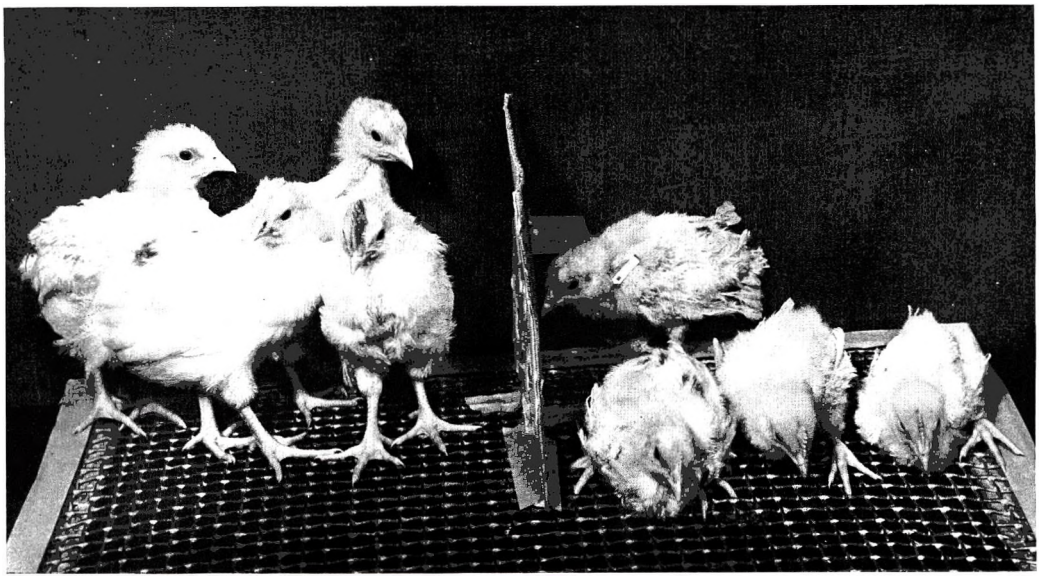

Fig. 1 The effect of cysteine and cystine on zinc-deficient chicks. The chicks on the left were fed 0.5% supplemental cysteine with 5 ppm supplemental zinc. The chicks on the right were fed 0.5% supplemental cystine with 5 ppm supplemental zinc. The chicks are representative of larger groups of 10 chicks each. Note the marked improvement in growth and leg development of chicks fed cysteine.

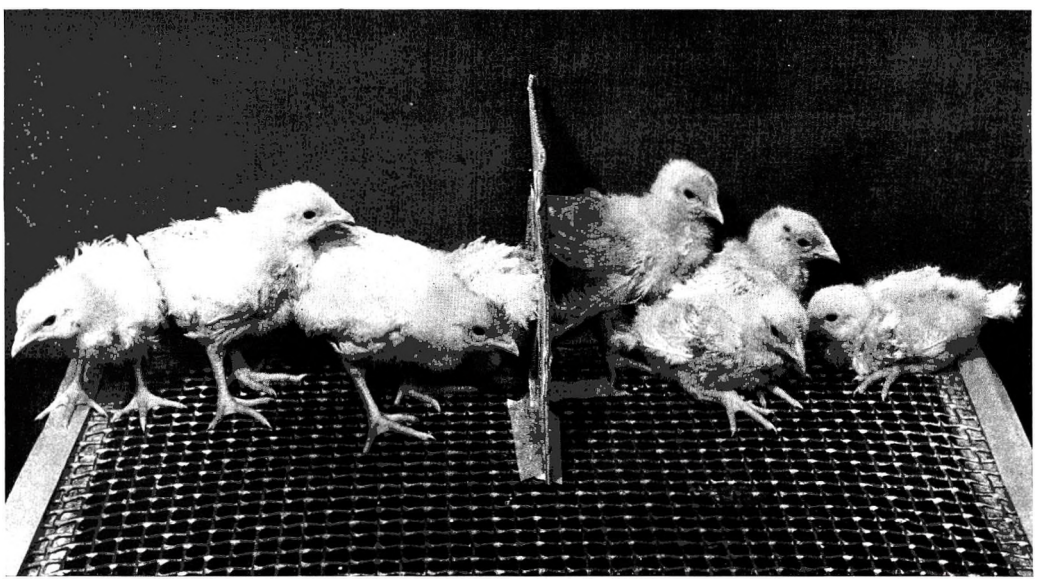

Fig. 2 Effect of histidine on zinc-deficient chicks fed a soy protein diet. The chicks on the left were fed 0.5% histidine with 5 ppm supplemental zinc in a soy protein diet. The chicks on the right were fed the soy protein diet with 5 ppm supplemental zinc, but without supplemental histidine. Notice that the chicks fed histidine had essentially normal legs. The chicks fed no additional histidine showed severe leg abnormality (swollen hocks and shortened and thickened leg bones) and chose not to stand. Also notice that there is no difference in the size of the chicks. The chicks are representative of larger groups of 10 chicks each.

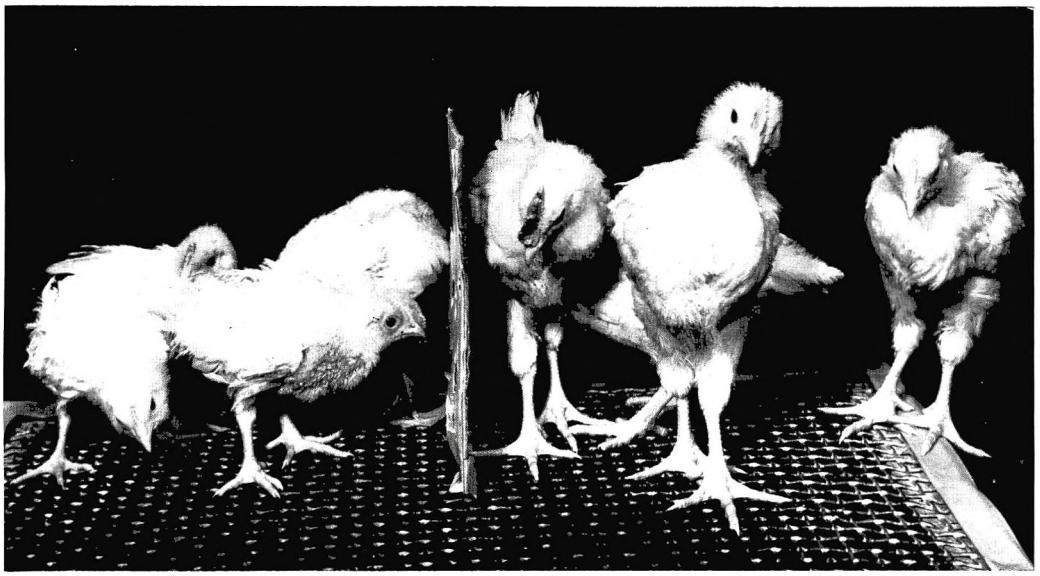

Fig. 3 Effect of zinc on zinc-deficient chicks fed a soy protein diet with supplemental histidine. The chicks on the left were fed 0.5% histidine with 5 ppm supplemental zinc in a soy protein diet. The chicks on the right were fed 0.5% histidine with 80 ppm supplemental zinc in a soy protein diet. Note the growth stimulation from zinc, and that neither group shows abnormal legs. The chicks are representative of larger groups of 10 chicks each.

histidine (calculated), respectively. Therefore, histidine content might possibly explain the effect of the casein hydrolysate diet (12) but such an explanation would not appear to apply to the egg white diet. It is presently not understood why histidine, although it apparently has a zinc stability constant in the beneficial range, does not alleviate the growth defect of zinc deficiency in chicks. Possibly the way in which amino acids such as histidine and cysteine are bound in the protein has important effects on mineral availability and metabolism. Investigations are underway to further characterize the effect of histidine on bone formation in chicks fed low zinc soy protein diets.

HEDTA again produced the expected results. HEDTA significantly increased ($P < 0.05$) growth, length-to-width ratios of the femurs, and zinc content of the tibias and decreased feather and leg scores of chicks fed the zinc-deficient soy protein diet (table 2). However, HEDTA-fed chicks had significantly less ($P < 0.05$) zinc in the tibias and significantly lighter final body weight than chicks fed the diet with 80 ppm supplemental zinc with or without additional HEDTA. The higher amount of zinc in tibias from chicks fed 80 ppm supplemental zinc was expected as Nielsen et al. (12) have shown that the zinc content in tibias increases with the increase of available dietary zinc up to a certain level. However, no explanation for the lower growth response can be offered. In all other experiments conducted in this laboratory, chicks fed HEDTA with only 5 ppm supplemental zinc in a soy protein diet grew as well or better than chicks fed instead an additional 40 or 80 ppm zinc.

An unexpected, but unconfirmed, result was that chicks fed 80 ppm supplemental zinc plus 0.5% glutamic acid had significantly less ($P < 0.05$) zinc in their tibias than did chicks fed 80 ppm supplemental zinc alone. Also, growth rate was significantly higher ($P < 0.05$) in the chicks fed supplemental glutamic acid with adequate zinc.

ACKNOWLEDGMENT

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Use of the Hysterectomy-obtained SPF Pig for Nutritional Studies of the Neonate [1]

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ABSTRACT Facilities and procedures were developed for use of the hysterectomy-obtained SPF (specific pathogen free) baby pig in routine nutritional studies from birth under essentially disease-free conditions. This provides an animal which can be used for nutritional studies in the neonatal period as well as in later stages of growth and development. SPF pigs fed a simulated sow's milk formula in these special facilities gained weight and grew as well as has been reported for normally born and suckled pigs.

A newborn laboratory animal that could be used routinely for nutritional studies from the day of birth would offer much toward gaining an understanding of the effects of early nutrition on growth and development in the human. However, the choice of a newborn animal which can be used for such exploratory research is limited. The rat is difficult to handle and feed as a newborn, and soon becomes intolerant to lactose, the principal carbohydrate in infant formulas. The monkey and dog are also difficult to feed from birth and require extensive breeding colonies and facilities which are usually prohibitive in cost. Until recently, the pig also was not available for study early in life because of problems of early feeding and incidence of infection.

With the increased use of SPF (specific pathogen free) swine for routine raising of pigs, numerous SPF laboratories have been established for delivery of pigs by hysterectomy under sterile conditions. Such baby pigs can be produced regularly in conjunction with a large-scale controlled breeding program, thereby providing a uniform strain for experimental studies. The size of the animal, the ease with which it can be handled, and the development of special facilities to prevent the many low-grade infections which often occur in animal colonies, support the use of the SPF pig as a newborn animal for nutritional research.

The present report describes facilities and procedures which have been developed to use the hysterectomy-obtained SPF baby pig as a laboratory animal for nutritional studies.

FACILITIES AND PROCEDURES

Facilities were designed to use up to 20 SPF baby pigs in each experiment. These [2] were obtained by hysterectomy on the 112th day of gestation (2 days prior to full term) from 2 or 3 sows of a Hampshire-Yorkshire cross, specially mated for delivery on the same date. The technique of Young et al. (1) was used, in which the total uterus, with the pigs *in utero*, was aseptically removed from the anesthetized sow and passed through a disinfectant solution into a sterile delivery hood. Within the hood, the pigs were removed from the uterus, the umbilical cords tied and the pigs placed in previously sterilized transfer cases. Each transfer case was covered with a sterile canvas bag when removed from the hood.

Transportation unit. Since a trip of 2 hours or more was necessary to transport the pigs by air or truck to the Research Center, an aluminum transportation unit [3] was designed to provide warm circulating air and maintain sterile conditions (fig. 1a). This self-contained unit consisted of the 3 transfer cases and an outside transport case which contained a thermostatically controlled heated-air supply system.

The transfer case (fig. 1c) was designed to fit the supplier's delivery hood. Each case contained a removable flexible canvas

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[1] A preliminary report of these studies was given at the 49th Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, New Jersey, 1965.

[2] Obtained from Pure-For-Sure, Flora, Indiana.

[3] A more detailed description of this unit will be made available upon request.

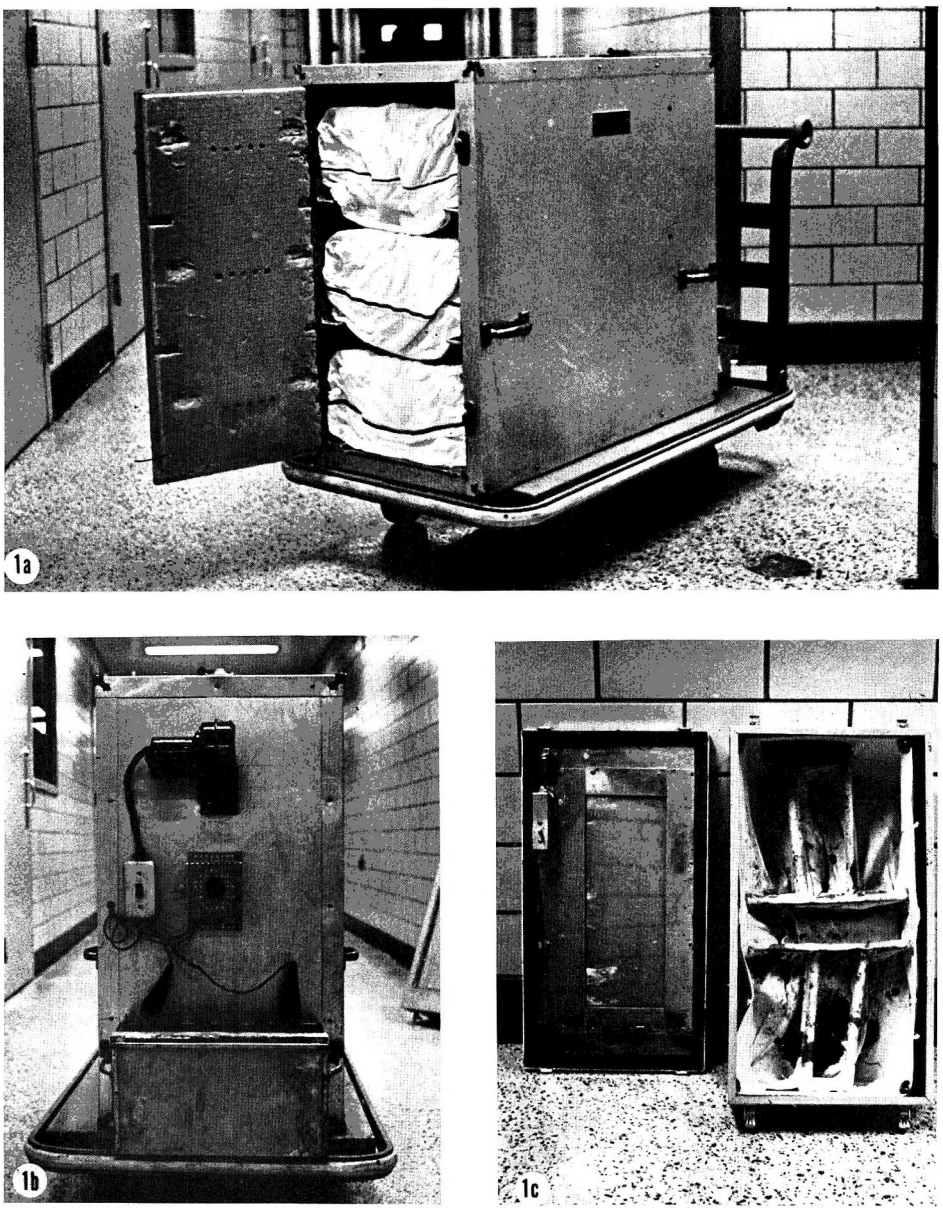

Fig. 1 Transportation unit: (1a) Complete unit with transfer cases in place; (1b) power supply, and (1c) transfer case and inner liner which holds 7 pigs. The blood seen on the inner liner is from umbilical bleeding which sometimes occurs during shipping because of the activity of the pigs crowded together.

inner liner divided into compartments for 7 pigs to protect the pigs during shipment, and a thermostat for controlling the temperature of the air which was blown through bacteriological filters[4] located on the ends of the case.

The transport case held the 3 transfer cases (see fig. 1a) permitting the shipment of 21 baby pigs. The transfer cases, covered with canvas bags, were attached by

[4] American Air Filter Media, 50 FG, Air Filter Supply Company, Louisville, Kentucky.

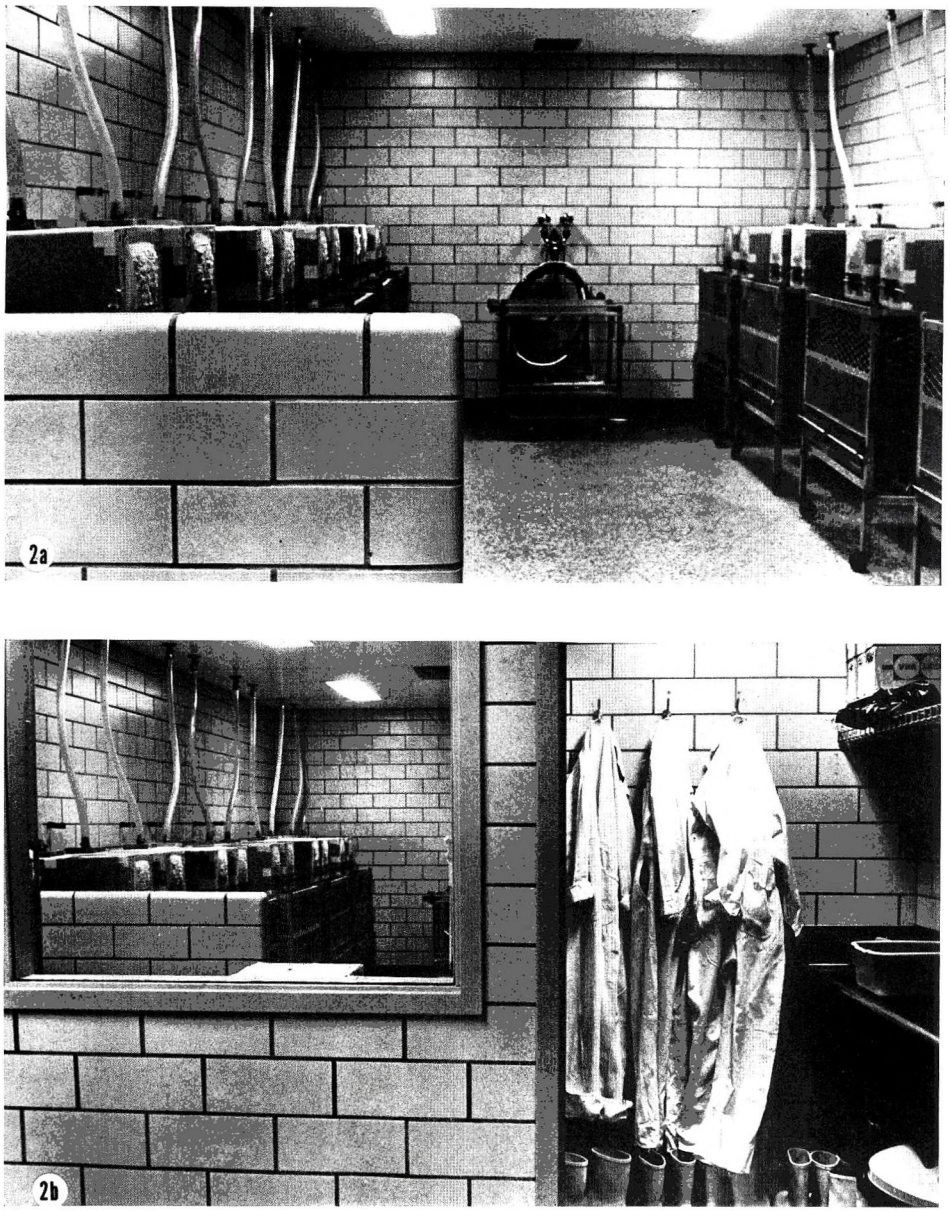

Fig. 2 Animal room: (2a) View from outside window; (2b) includes view of anteroom.

adaptors in the unit to an air manifold. Outside air was swept into the manifold which contained 3 sets of small lamps, each of which heated the air going into one of the cases. This unit provided 9 to 12 air changes/hour and maintained temperature at 34° to 35°. The unit was powered by a 12-volt storage battery (fig. 1) which supplied sufficient power for about 5 hours.

Animal rooms. Each animal room consisted of a main room (fig. 2a) and an anteroom (fig. 2b) for changing clothes.

The room was prepared for the pigs a few days before starting the experiment. The room and cages were aseptically cleaned with a germicidal detergent [5] and

[5] Vesphene, Vestal Laboratories, St. Louis.

all the equipment necessary to last throughout the experiment was put in place, i.e., incubators and canned formulas were placed in the animal room, and autoclaved clothing packs and boots in the anteroom. The rooms were sealed, dampers in the air conditioning system were closed, and the rooms fumigated with formaldehyde (dispersed by a fog generator[6]) using 35.3 ml of formaldehyde/m^3 of space. After 24 hours, the dampers were opened and the room aerated for at least 24 hours before use.

The air conditioning of each room was controlled individually, and conditions could be varied as desired. At the start of the experiment the temperature was set at 35° and 50% relative humidity. The temperature was lowered by 3.5° increments to 24° during the first 4 weeks of each experiment. To avoid contamination, the pressure in all of the rooms was maintained slightly higher than the environmental pressure so that air swept out of the rooms when doors were opened.

Twenty individual disposable cardboard incubators[7] in each room (seen in place in fig. 2a) were used to hold the pigs during the first 7 days of life. Each had an observation glass on top, an opening for a food cup in front, and was connected to an overhead exhaust system by plastic tubes. Air was pulled into the incubator through the air filter[8] on the front to provide 12 air changes/hour. The room also had 20 individual metal cages beneath the incubators (fig. 2a) which were used to house the pigs after the first week. These had open mesh floors and stainless steel food cups which could be locked in front of the cage.

The technicians who cared for the pigs changed clothes in the anteroom, putting on clean coveralls and rubber boots as well as disposable hats, masks, and gloves. They also stepped into a disinfectant[9] pan when entering and leaving the animal room.

Receiving the pigs. When the pigs were received, they were aseptically transferred into the animal room by the following procedure: The canvas bag was slipped off the transfer case as it was passed into the anteroom, a technician within the anteroom removed the lid, and another technician in the animal room reached through the doorway and removed the pigs from the transfer case. Once in the animal room, the pigs were sorted on the basis of weight, sex, and litter; initial blood samples were taken from the ear and the animals placed in the incubators.

The pigs were fed canned liquid formulas 3 times daily; at 7:00 and 11:30 AM and 5:00 PM. The volume of formula allotted to each pig was determined on an individual basis. This was estimated at each feeding by considering the condition of the animal, and how well his previous feeding was consumed. The pigs obtained their water requirements from the liquid formulas that were used. After the pigs were moved to the metal cages, 50 ml of distilled water were also allowed at each feeding. If a pig started to drink much of this, it usually indicated that the amount of formula should be increased. By these techniques, the feeding of the pigs appeared to be essentially on an ad libitum basis.

Other facilities. In the above facilities studies on baby pigs could be conducted for approximately 8 weeks of age. Adjacent animal rooms of similar size and design, but containing individual floor pens, were also available for study of further growth and development after 4 to 8 weeks.

RESULTS

The gross composition of the formulas used in this study is compared with published data on sow's milk (2) shown in table 1. Formula A was a commercially available formula[10] for feeding SPF pigs and formula E was prepared in our laboratory to more closely approximate sow's milk, especially in caloric density. Both contained skim milk, casein, vegetable fats, lactose, vitamins and minerals. The vitamin and mineral levels used in making formula E are shown in the footnote in table 1. These were based on the best estimate of the pigs' needs as determined from the current literature.

Table 2 shows the weight gains obtained in the present study with these formulas and weight gains of normally suckled pigs of the same strain, as well as literature

[6] Challenger Fog Generator, Z & W Manufacturing Corporation, Wickliffe, Ohio.
[7] Ft. Dodge Container Corporation, Ft. Dodge, Iowa.
[8] See footnote 4.
[9] Nolvasan, Ft. Dodge Laboratories, Ft. Dodge, Iowa.
[10] SPF-lac, The Borden Company, New York.

TABLE 1

Major constituents of formulas A, E, and sow's milk

	Formula A	Formula E [1]	Sow's milk [2]
Solids, g/liter	151	205	196
Protein, g/liter	52	66	61
Fat, g/liter	54	79	73
Carbohydrate, g/liter	37	53	53
Ash, g/liter	8.2	6.8	9.8
Kilocalories/liter	840	1185	1110
Caloric distribution			
Protein, %	25	22	22
Fat, %	58	60	59
Carbohydrate, %	17	18	19

[1] Formulated to contain the following vitamin and minerals/liter of formula: (in IU) vitamin A, 1000; vitamin D, 100; *d*-α-tocopherol, 2; (in mg) ascorbic acid, 50; thiamine, 0.8; riboflavin, 2; pyridoxine, 0.6; pantothenic acid, 5; choline, 300; niacin, 8.0; folic acid, 0.05; and vitamin B_{12}, 0.005; (in g) calcium, 2; phosphorus, 1.4; chlorine, 1.0; potassium, 1.5; sodium, 0.6; (in mg) magnesium, 150; iron, 25; copper, 3; manganese, 10; iodine, 0.2; and zinc, 15.

[2] Calculated from an average of Perrin's data (2) assuming a specific gravity of 1.0429.

TABLE 2

Comparison of weekly weight gains of pigs normally born and suckled, with hysterectomy-obtained formula-fed pigs

	Normally born, suckled			Hysterectomy-obtained, formula-fed	
				Formula A	Formula E
Breed of pigs and reference	Duroc (3)	Wessex Saddleback-Large White cross (4)	Hampshire-Yorkshire cross [1]	Hampshire-Yorkshire cross (present study)	
No. of animals	1296	(from 85 litters)	25	9	8
Birth wt, kg	1.3	1.6	1.6	1.0	1.0
Cumulative wt gain, kg					
Week 1	1.0	1.6	1.1	0.7	0.7
Week 2	2.3	3.2	2.4	1.9	2.4
Week 3	3.6	5.0	4.0	3.7	4.4
Week 4	4.8	6.6	6.0 ± 1.1 [2]	5.8 ± 0.9	7.4 ± 1.2
4-week formula intake, liters				34.8	33.2

[1] Data were supplied by Dr. and Mrs. S. F. Shipsides, Flora, Indiana.

[2] SD.

values for other normally born and suckled pigs. In 4 weeks the pigs gained 5.8 kg and 7.4 kg with formulas A and E, respectively. Pigs of the same Hampshire-Yorkshire strain born and raised on a clean SPF farm gained 6 kg in 4 weeks, when normally suckled and allowed creep feed (containing antibiotics) during the latter 2 weeks. Similarly, Duroc pigs (3) which were only nursed gained 4.8 kg and Wessex Saddleback-Large White cross (4) pigs gained 6.6 kg.

DISCUSSION

The SPF baby pig offers much promise as a laboratory neonate for nutritional studies. In addition to its physiologic and metabolic similarity to man, the litter size, ability to feed itself from birth, and ease of handling experimentally makes the baby pig quite useful. The present report shows that facilities and procedures can be developed which allow one to maintain animals which never received colostrum in an essentially disease-free state so that nutritional studies can be conducted routinely without the contamination and infection which often occur in pigs.

Antibiotics and γ-globulin were not given to these animals, since they might alter the results and interpretation of nutritional studies. The success of the techniques de-

scribed was shown by good growth of the pigs, and by the fact that there was a low-grade infection in only one of the 12 experiments conducted thus far. The cardboard incubators used during the first 7 days apparently gave the pigs extra protection against airborne infections.

Weight gains of pigs raised under these conditions appeared to be as good as those observed in pigs raised under natural conditions. The higher weight gain obtained with formula E as compared with that found with formula A was probably due mainly to the higher caloric density of formula E, since the volumes of formula consumed by the 2 groups were approximately the same. Comparisons with data on normally suckled pigs suggest that during the first week, slightly faster rates of weight gain may be obtained with nursed pigs than with formula-fed pigs. This slower weight gain in the formula-fed pigs may be due in part to their delivery 2 days earlier, the trauma of hysterectomy and transportation, and the feeding of formula only 3 times daily. However, in succeeding weeks, the formula-fed pigs gained as well as those which were suckled.

The techniques and procedures presented here show that the SPF pig can be utilized as a research animal for nutritional studies during the first few weeks of life. Its size and availability permit the determination of body composition and the measurement of many biological parameters. It should provide much useful information on the nutrition of the newborn, and the effect of early nutrition on growth and development.

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Effects of Cystine Deficiency and Trypsin Inhibitor on the Metabolism of Methionine [1,2]

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ABSTRACT While seeking methods capable of determining the cystine nutriture of animals, it was found that the addition of L-cystine to diets made with purified soybean protein resulted in an increased production of $^{14}CO_2$ from labeled methionine when compared with animals not receiving cystine. A similar enhancement of CO_2 production occurred if unheated soybean meal was fed, rather than heated meal, or if purified soybean trypsin inhibitor was given orally to rats maintained with either the heated or unheated meal. It is proposed that both cystine and trypsin inhibitor diminish the conversion of methionine to cysteine through the cystathionine pathway. Two mechanisms of soybean trypsin inhibitor action are proposed, one an interference with the incorporation of cystine into protein which increases the body pool of cystine which represses cystathionine synthase and the other, an interference of soybean trypsin inhibitor directly with cystathionine synthase.

While the mechanisms of the metabolism of amino acids are well known, it is difficult or impossible to detect and characterize a deficiency of any specific amino acid. The available methods based on measurement of nitrogen balance or growth are cumbersome and nonspecific. The multiple functions of the essential sulfur amino acids, cystine and methionine, suggest that it may be possible to develop a specific measure of deficiency for them. Studies of the sulfur amino acids are especially relevant because they are commonly encountered, limiting nutrients in human diets (1). The present experiments were conducted with animals made cystine-deficient either by feeding a diet low in the essential sulfur-containing amino acids or by combining such a diet with agents which increase the sulfur requirement by requiring cysteine for metabolic clearance.

Kwong and Barnes (2) have proposed that the magnitude of the production of CO_2 from methionine is related to cystine nutriture. They reported that growing rats receiving adequate cystine released less $^{14}CO_2$ from administered methionine-2-^{14}C than did animals deficient in cystine. We were not able to confirm this observation and found instead that the deficient rats converted less of the labeled methionine to CO_2. The studies were extended with animals maintained in conditions identical with those described by Kwong and Barnes. This involved the use of diets containing unheated soybean protein which added the complicating uncertainty of the influence of antitrypsin action. The history and status of soybean antitrypsin (SAT) have been summarized by Rackis (3). SAT consistently produces an impairment of absorption of nitrogen and pancreatic hypertrophy which is associated with an increase of pancreatic secretion. It is not established whether SAT also impairs some endogenous, anabolic phase of nitrogen metabolism and of sulfur amino acid metabolism in particular, although Kwong and Barnes (2) have proposed that SAT decreases the incorporation of sulfur amino acids into protein. We found that SAT, like cystine feeding, enhanced the production of CO_2 from methionine. These data suggest that SAT interferes with the utilization of sulfur amino acids.

EXPERIMENTAL

Three methods for producing cystine deficiency were used. A basal diet, made with purified soy protein [4] was used to pro-

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[3] Career Investigator, National Heart Institute, National Institutes of Health, K56-HE8288.
[4] C-1 Assay Protein, Skidmore Enterprises, Cincinnati, Ohio.

duce a marginal deficiency of cystine and methionine (table 1). L-Cystine was included at a level of 0.4% in the diets fed control animals. The addition of 0.75% cholic acid to the basal diet enhanced the cystine deficiency. The addition of 0.75% bromobenzene to the basal diet produced an even more severe deficiency. Animals fed according to one of these 3 regimens and appropriate control groups were studied.

TABLE 1

Composition of cystine-deficient diet

	%
Isolated soybean protein [1]	15
Sucrose	64
Non-nutritive fiber [2]	5
Hydrogenated vegetable oil [3]	10
Salt mixture [4]	4
Vitamin mixture [5]	2

[1] C-1 Assay Protein, Skidmore Enterprises Company, Cincinnati.
[2] Alphacel, Nutritional Biochemicals Corporation, Cleveland.
[3] Crisco, Procter and Gamble Company, Cincinnati.
[4] Formulation of Hubbell, R. B., L. B. Mendel and A. J. Wakeman 1937 A new salt mixture for use in experimental diets. J. Nutrition, 14: 273; obtained as HMW Salt Mixture from Nutritional Biochemicals Corporation.
[5] Prepared from individual vitamins to supply per 100 g diet: (in milligrams) thiamine·HCl, 2; riboflavin, 2; pyridoxine, 2; Ca pantothenate, 6; niacin, 6; p-aminobenzoate, 10; inositol, 10; biotin, 0.04; folate, 0.2; vitamin B_{12}, 0.003; menadione, 0.045; vitamin A, 5.3; vitamin D_2, 1; α-tocopheryl acetate, 12; and choline chloride, 150.

In the first series of experiments 36 male, weanling, rats of the Sprague-Dawley strain were fed the basal deficient diet for 4 weeks. Cholic acid was then added to the diet of 12 rats and six of these received L-cystine as well. Another 12 rats received bromobenzene and six of these also received L-cystine. Six of the remaining rats were continued with the basal diet and the other six received the basal diet with added L-cystine. Thus there were 3 deficient groups each with L-cystine-fed controls. After another month on these regimens the animals were fasted for 16 hours and injected intraperitoneally with 1 μc/kg body weight of either DL-methionine-1-^{14}C, specific activity 3.7 mc/mmole, or DL-methionine-2-^{14}C, specific activity 10.0 mc/mmole. The injected animals were placed at once in individual respiration chambers which allowed collection of the expired carbon dioxide. Air was drawn through the chamber at a rate of 1 liter/minute. Carbon dioxide was absorbed with the ethanolamine-ethylene glycol monomethyl ether system of Jeffay and Alvarez (4). Radioactivity of the CO_2 collected was determined by counting an aliquot of the absorbant in a liquid scintillation system. The collections were continued for 6 hours and the absorbers were sampled hourly.

In the second series of experiments 24 male, weanling rats of the Holtzman strain were fed diets similar to those used by Kwong and Barnes (table 2). Half of the animals fed each diet, heated and unheated, received a supplement of 0.4% L-cystine in the diet. The animals were fed the diets for 15 days before being given intraperitoneal radioactive methionine following a 16-hour fast. In certain experiments, as indicated, the rats received, by stomach tube, 50 mg of Kunitz-type crystalline soybean trypsin inhibitor[5] in one milliliter of water 2 hours prior to injection of the labeled methionine.

TABLE 2

Composition of diets made with soybean meal

	Diet	
	Unheated soybean	Heated soybean
	%	%
Raw soybean meal [1]	50	—
Heated soybean meal [2]	—	40
Dextrose [3]	29	39
Salt mixture [4]	4	4
Hydrogenated vegetable oil [5]	15	15
Vitamin mixture [6]	2	2

[1] Central Soya Company, Chicago.
[2] Autoclaved at 107° for 30 minutes.
[3] Cerelose, Corn Products Company, Argo, Illinois.
[4] Hubbell et al. J. Nutrition, 14: 273, 1937.
[5] Crisco, Procter and Gamble Company, Cincinnati.
[6] See table 1, footnote 5.

RESULTS

Series 1 — Animals fed diets made with assay protein. The growth responses of the animals are a crude reflection of cystine-methionine nutriture with these conditions (fig. 1). With each of the 3 treatment variations the control groups fed L-cystine showed significantly ($P < 0.05$) greater growth after one month of receiving the supplemented diets and the differences steadily increased.

The cumulative, hourly production of radiocarbon dioxide, expressed as percent-

[5] Kunitz Type from Mann Research Laboratories, New York.

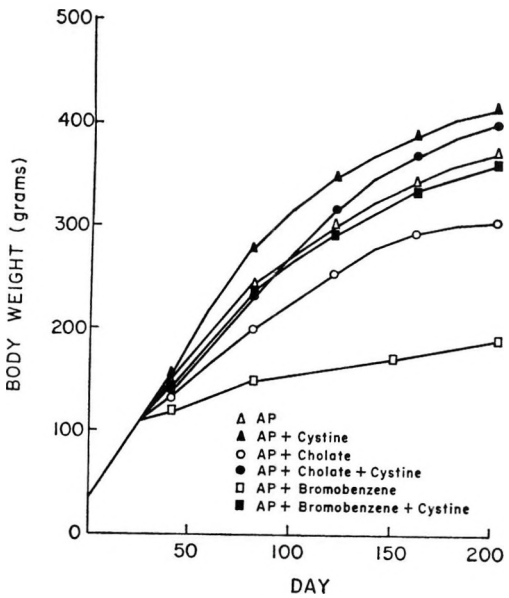

Fig. 1 Growth curves for rats fed assay protein (AP) diets. All the rats were maintained with assay protein diets with no supplement for the first month and then transferred to the diets indicated. The levels of supplement were: L-cystine, 0.4%; cholic acid, 0.75%; bromobenzene, 0.75%. The points are means for 6 animals.

age of administered dose, of animals fed the basal diet or basal plus L-cystine diet following injection with methionine-1-^{14}C is shown in table 3. The animals supplemented with L-cystine showed significantly greater $^{14}CO_2$ production at each sampling interval. The 6-hour recovery of labeled carbon dioxide for all treatment groups following injection of methionine-1-^{14}C is shown in table 4. For each of the dietary variations the $^{14}CO_2$ production was significantly ($P < 0.05$) higher in the animals receiving the L-cystine supplement. Both cholate and bromobenzene lowered the conversion of labeled methionine to CO_2 (table 4).

This enhancement of $^{14}CO_2$ production from methionine-1-^{14}C in L-cystine-supplemented animals was not found by Kwong and Barnes (2), who, however, used methionine-2-^{14}C. A supply of methionine labeled in the second carbon atom was obtained for trials with another group of animals prepared similarly to determine whether the position of the label was accounting for the differences. Results for the various diet groups following intraperitoneal injection with methionine-2-^{14}C are shown in table 5. As with the carboxyl-labeled methionine, the L-cystine-supplemented animals in each of the 3 dietary divisions showed a significantly greater production of labeled carbon dioxide. The position of the isotopic label did not influence the result.

Series 2 — Animals fed diets made with heated and unheated soybean meal. Since Kwong and Barnes (2) had used diets made with raw soybean meal which contains SAT, it might be supposed that the presence of SAT would lead to the different response obtained by these authors. For the examination of an antitrypsin effect the 6-hour recovery of labeled carbon dioxide from animals fed either heated or unheated soybean diets with and without L-cystine was determined (table 6). The L-cystine supplement did not significantly increase the production of labeled CO_2 in these ani-

TABLE 3

Recovery of methionine-1-^{14}C as $^{14}CO_2$ in rats fed assay protein diets

Hours	Cumulative recovery		P values [1]
	Assay protein	Assay protein + L-cystine	
	% administered dose		
1	5.11 ± 0.39 [2,3]	9.70 ± 0.43	< 0.01
2	11.46 ± 0.75	17.63 ± 0.75	< 0.01
3	14.66 ± 0.78	21.27 ± 0.81	< 0.01
4	16.64 ± 0.77	25.08 ± 0.97	< 0.01
5	18.09 ± 0.76	28.49 ± 1.24	< 0.01
6	20.11 ± 0.80	30.12 ± 1.22	< 0.01

[1] Probability that the observed difference between groups might occur by chance using Student's *t* test.
[2] Mean ± SE.
[3] Six animals were in each diet group.

TABLE 4

Recovery of methionine-1-^{14}C as $^{14}CO_2$ in rats fed cystine-deficient diets containing cysteine diverters

Diet	Six-hour cumulative recovery
	% administered dose
Assay protein	20.1 ± 0.8 [1,2]
Assay protein + L-cystine	30.1 ± 1.2
Assay protein + cholate	19.2 ± 0.8
Assay protein + cholate + L-cystine	22.9 ± 0.8
Assay protein + bromobenzene	12.2 ± 0.5
Assay protein + bromobenzene + L-cystine	18.5 ± 0.6

[1] Mean ± SE.
[2] Six animals in each diet group.

TABLE 5

Recovery of methionine-2-^{14}C as $^{14}CO_2$ in rats fed cystine-deficient diets

Diet	Six-hour cumulative recovery
	% administered dose
Assay protein	13.05 ± 0.98 [1,2]
Assay protein + L-cystine	23.03 ± 4.06
Assay protein + cholate	15.92 ± 3.19
Assay protein + cholate + L-cystine	21.18 ± 4.38
Assay protein + bromobenzene	9.33 ± 1.01
Assay protein + bromobenzene + L-cystine	15.54 ± 1.39

[1] Mean ± SE.
[2] Seven animals in each diet group.

TABLE 6

Recovery of $^{14}CO_2$ from methionine-2-^{14}C in rats fed diets made with soybean meal with and without L-cystine supplements

Diet	Six-hour cumulative recovery
	% administered dose
Unheated soybean meal	21.0 ± 1.7 [1,2]
Unheated soybean meal + L-cystine	22.9 ± 1.2
Heated soybean meal	16.6 ± 0.8
Heated soybean meal + L-cystine	18.7 ± 1.9

[1] Mean ± SE.
[2] Six animals in each diet group.

mals receiving large amounts of protein in the diet. The diets contained about 20 to 25% protein. However, the animals receiving the unheated soybean diet did show an increase in production of $^{14}CO_2$ when compared with those animals receiving the heated diet ($P < 0.05$).

Since the major difference between the 2 diets is the absence of SAT from the heated diet, the effect of purified trypsin inhibitor administered to animals fed the 2 diets was examined. Crystalline soybean trypsin inhibitor,[6] 50 mg/rat, was administered orally 2 hours before the labeled methionine. This treatment was found to significantly increase the production of radiocarbon dioxide in all diet groups (table 7).

These experiments consistently showed that the administration of SAT, or of

[6] See footnote 5.

TABLE 7

Recovery of $^{14}CO_2$ from methionine-2-^{14}C in rats fed diets made with soybean meal and given trypsin inhibitor

Diet	Oral trypsin inhibitor [1]	Six-hour cumulative recovery	P values [2]
		% administered dose	
Unheated soybean	−	21.0 ± 1.7 [3,4]	< 0.05
	+	26.8 ± 2.7	
Unheated soybean + L-cystine	−	22.9 ± 1.2	< 0.05
	+	28.5 ± 2.3	
Heated soybean	−	16.6 ± 0.8	< 0.05
	+	19.0 ± 1.0	
Heated soybean + L-cystine	−	18.7 ± 1.9	< 0.05
	+	24.6 ± 2.6	

[1] 50 mg/animal orally 2 hours before labeled methionine.
[2] Probability that the observed difference between groups might occur by chance using Student's *t* test.
[3] Mean ± SE.
[4] Six animals in each diet group.

L-cystine to deficient animals, produced an increased conversion of methionine to carbon dioxide. Cystine deficiency regularly impaired this conversion.

DISCUSSION

These results cannot be explained by the hypothesis of Kwong and Barnes (2) proposing that SAT causes cystine deficiency which then enhances the conversion of methionine to cystine. In view of the observation of Kato et al. (5) of an inhibitory action of dietary cystine on cystathionine synthase activity, it might be expected that animals receiving supplemental dietary cystine would show depressed conversion of methionine to cysteine. However, we find that cystine administration enhances the yield of either the first or second carbon of methionine as CO_2. The greater yield of $^{14}CO_2$ in animals fed either L-cystine or SAT suggests that methionine is catabolized more quickly under these circumstances by routes other than through cystathionine leading to cystine. It appears unlikely that these alternate pathways would provide an increased rate of catabolism of methionine unless cystine and SAT, in addition to inhibiting the cystathionine synthase pathway, also stimulate or potentiate some other methionine-catabolizing pathway such as the deamination of methionine to α-keto-γ-methiolbutyric acid. Such an action is not demonstrated.

It was consistently observed in animals maintained with diets made with soybean meal that either feeding an unheated diet, i.e., containing SAT, or the addition of SAT separately increased the production of $^{14}CO_2$ from labeled methionine. The animals receiving unheated diets or SAT behaved, with respect to the $^{14}CO_2$ production from methionine, as though they had received supplemental cystine. This observation might be explained in several ways.

The utilization of cystine for protein synthesis may be blocked by SAT leading to an accumulation of cystine. Kwong and Barnes (2) reported that the addition of L-cystine to diets made with soybean meal gave improvement of growth of rats only in the instance of the diets made with the unheated protein. Since the cystine content of both diets was the same, they suggested that feeding unheated soybeans must have increased the requirement of the animals for cystine and that animals fed an unheated soybean diet containing trypsin inhibitor would be effectively cystine-deficient. The authors hypothesized that this deficiency might be caused by a block in tissue utilization of cystine for protein synthesis in the presence of trypsin inhibitor. In such a "deficiency" the body pool of cystine could be paradoxically high. Thus an animal which by growth studies appeared to require cystine might behave as though it had an excess when examined for methionine conversion, as did the animals examined here.

Alternatively SAT may interfere directly with the conversion of methionine to cystine. A block in the methionine to cystine pathway would mimic the feedback inhibition reported by Kato et al. (5) to be present when cystine is fed to animals. Thus the animals fed SAT would respond as though they had received cystine, i.e., they would produce more $^{14}CO_2$ from labeled methionine, not being able to carry methionine through the cystathionine pathway. If methionine storage is limited, it would be catabolized by alternate channels, recently summarized (6), and the CO_2 production rate would reflect this. By either hypothesis, shown schematically in figure 2, a defect in tissue utilization of cystine or a block in the conversion of methionine to cystine, the feeding of unheated soybeans or trypsin inhibitor should enhance production of $^{14}CO_2$ from labeled methionine, a result which was observed here.

The first explanation would be supported by finding an increased body pool of cystine in the presence of SAT, the second by demonstration of an effect of SAT on the methi-

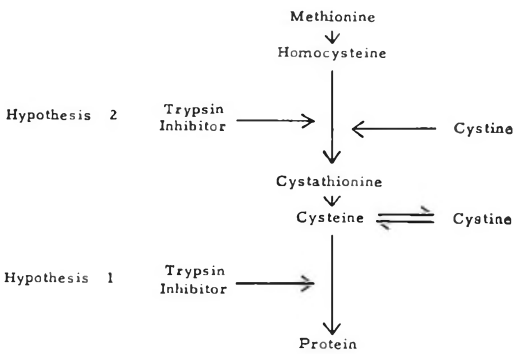

Fig. 2 Postulated actions of soybean trypsin inhibitor on methionine metabolism.

onine-catabolizing enzymes and perhaps an accumulation of intermediates in the cystathionine pathway after a methionine load.

The data of Kato et al. (5) have suggested that cystine, and to a lesser extent cysteine, combines in vitro with a protein to form an inhibitor of cystathionine synthase. It may be that trypsin inhibitor, or a peptide from the inhibitor is, preformed, a substance with an action similar to that of cystine on cystathionine synthase. If that were true, SAT should not lead to an increase of the body pool of cystine. If SAT blocks cystine utilization, the inhibition of cystathionine synthase may be secondary to cystine excess, as it is with cystine feeding. In either instance this mechanism of trypsin inhibitor action implies that an intermediate in the cystathionine pathway may accumulate after either trypsin inhibitor or cystine supplements in the presence of a methionine load. Thus homocystinuria may be expected to occur. These hypotheses are being investigated.

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Effect of Deficiencies of α-Tocopherol, Retinol and Zinc on the Lipid Composition of Rat Testes

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ABSTRACT To determine whether lipid changes noted previously in α-tocopherol-deficient rat testes were specific for vitamin E, a comparison was made with testes in deficiencies of retinol or zinc. Testicular degeneration was produced in rats fed purified diets deficient in either α-tocopherol, retinol or zinc for 12 to 19 weeks. Total lipid was reduced in testes in all 3 deficiencies with decreased amounts of phospholipid accounting for most of the change. α-Tocopherol-deficient testes had the lowest phospholipid content. The proportion of 20:4 ω6 was twice normal in α-tocopherol-deficient testes, moderately increased in zinc deficiency and unchanged in retinol deficiency; 22:4 ω6 increased only in α-tocopherol deficiency. The proportion of 22:5 ω6 was about two-thirds of normal in retinol and zinc-deficient testes but only one-third normal in α-tocopherol-deficient tissue. Dietary selenium had no effect on testes composition either in the presence or absence of α-tocopherol.

The relatively high content of polyunsaturated fatty acids in testis (1, 2) led us to an initial study of the lipid composition of this tissue from α-tocopherol (vitamin E)-deficient rats (3). Marked changes were produced by vitamin E deficiency — a reduction in phospholipid to about one-half normal, a sharp decrease in the docosapentaenoic acid and increases in eicosatetraenoic and docosatetraenoic acids. Since the rat testis damaged by a lack of vitamin E shows primarily a degeneration and loss of germinal epithelium, pathology which is not unique to this vitamin deficiency, it was of interest to determine whether testicular damage produced by other nutritional deficiencies would result in similar lipid changes. In this study, the effects of two other deficiencies in which testicular atrophy has been described, vitamin A (4) and zinc (5), are compared with those from vitamin E deficiency.

EXPERIMENTAL

Weanling rats were of the Holtzman [1] or Sprague-Dawley [2] strain. The animals were caged individually in stainless steel suspended cages at a room temperature of 23 ± 1°, and food and water were supplied ad libitum. Body weights were recorded weekly.

Two vitamin E-deficient diets for rats differed only in the protein source. One, diet R-8, contained 20% of isolated soy protein [3] and 0.3% of DL-methionine. The other, R-11, contained 22% of vitamin-free casein.[4] The remainder of the diets was the same and contained: (in per cent) salt mixture of Fox and Briggs (6), 6; stripped lard,[5] 5; vitamin mix in sucrose, 2; sucrose, to make 100. The vitamin mixture provided/kg of diet: (in milligrams) thiamine·HCl, 15; riboflavin, 15; pyridoxine·HCl, 15; Ca pantothenate, 45; niacin, 50; choline chloride, 1000; folic acid, 2; biotin, 1; 2-methyl-1,4-naphthoquinone, 1; vitamin B_{12}, 0.03; vitamin D_3, 0.075. In addition, 6.3 mg of stabilized retinyl acetate [6] were added /kg and 0.1 ppm of selenium as sodium selenate.

Testis damage from retinol (vitamin A alcohol) deficiency was produced with a diet containing retinoic acid (vitamin A acid). This compound permits normal body growth but will not maintain testicular function (7). By this means, the complicating effects of general inanition in acute vitamin A deficiency are avoided. The basal depletion diet was R-11 with

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[1] Holtzman Company, Madison, Wisconsin.
[2] Sprague-Dawley Company, Madison, Wisconsin.
[3] ADM Assay Protein C-1, Archer-Daniels-Midland, Minneapolis.
[4] General Biochemicals, Chagrin Falls, Ohio.
[5] Distillation Products Industries, Rochester, New York.
[6] Nopcay 20, Type V, Nopco Chemical Company, Newark, New Jersey.

retinyl acetate omitted and with additions of 50 mg/kg each of *dl*,α-tocopherol and *dl*,α-tocopheryl acetate and of 0.02% ethoxquin.[7] Supplements of retinoic acid and retinyl acetate were added to the diets of 2 groups as indicated below. After 7 weeks, the stripped lard in this diet was replaced by 4% cottonseed oil[8] since the lard contained sufficient vitamin A activity to permit slow growth of the unsupplemented rats. This diet was designated R-9.

To produce zinc deficiency, diet R-8 was prepared omitting zinc carbonate from the salt mixture and adding 200 mg/kg of *dl*,α-tocopheryl acetate.

For histological examination, a portion of each testis was fixed in Bouin's solution, dehydrated with 70% ethanol and sections stained with hematoxylin and eosin. Evaluation of the degree of damage was made according to the scale of Mason (8). The remainder of the testis was frozen at $-20°$ until analyzed. For chemical analysis, the tissue was extracted with chloroform-methanol (2:1) by the procedure of Folch et al. (9) using a high-speed, blade-type homogenizer. Aliquots of the extracts were analyzed for total lipid, total cholesterol and phosphorus as described previously (2). In the early series of experiments, total lipid was determined gravimetrically. This procedure gave slightly higher values than did the chromic acid oxidation (10) subsequently used. Fatty acids were analyzed by gas-liquid chromatography using a 200 cm $\times$ 4 mm column of 15% ethylene glycol succinate on Chromosorb W at 180°. Detailed conditions and the method of identification and quantification of peaks have been reported previously (2, 11). Polyunsaturated fatty acids derived from linoleic acid are indicated as ω6 according to the number of terminal carbon atoms after the last double bond.

RESULTS

α-Tocopherol-deficient rat testis. A decrease in the weight of the testes was first noted after 14 to 16 weeks of depletion. At this time, the total lipid content had not changed, although the phospholipid was slightly reduced (table 1). By 19 weeks, when the weight of the testes was about one-half normal, total lipid was down significantly with the loss accounted for entirely by the phospholipid fraction. After 28 weeks, when the testes were less than one-half normal weight, the phospholipid had decreased to about 60% of that for the control tissue. By 32 weeks, phospholipid was less than one-half that of the control testes. Cholesterol was not altered by the deficiency.

In several experiments diet R-8 was fed with 0.1 ppm of selenium as sodium

[7] 1,2-Dihydro-6-ethoxy-2,2,4-trimethyl-quinoline, Monsanto Chemical Company, St. Louis.
[8] Wesson Oil, Wesson Sales Company, Fullerton, California.

TABLE 1

Lipid composition of α-tocopherol-deficient rat testis

Diet	No. weeks	No. rats	Body wt	Testes wt	Total lipid [1]	Phospholipid	Total cholesterol
			g	*g*	*mg/g*	*mg/g*	*mg/g*
				Experiment 1 [2]			
With vitamin E [3]	14–16	3	409 ± 33	3.17 ± 0.16	22.9 ± 0.6	14.8 ± 0.2	2.4 ± 0.1
Without vitamin E	14–16	5	365 ± 16	1.81 ± 0.17	22.9 ± 1.5	11.4 ± 0.6	2.3 ± 0.1
Without vitamin E	19	4	418 ± 20	1.49 ± 0.30	17.7 ± 1.4	8.6 ± 1.1	2.4 ± 0.1
				Experiment 2 [4]			
With vitamin E	28	6	389 ± 21	3.25 ± 0.12	21.7 ± 0.8	12.0 ± 0.3	2.4 ± 0.2
Without vitamin E	28	6	371 ± 14	1.53 ± 0.13	15.4 ± 1.0	7.2 ± 0.6	2.2 ± 0.2
				Experiment 3 [4]			
With vitamin E	32	5	435 ± 19	3.10 ± 0.07	22.4 ± 1.2	11.8 ± 0.7	— [5]
Without vitamin E	32	6	362 ± 24	1.63 ± 0.11	14.4 ± 0.7	5.0 ± 0.3	—

[1] Gravimetric determination (see text).
[2] Fed diet R-8. Values are means ± SE.
[3] 200 mg dl,α-tocopheryl acetate/kg.
[4] Fed diet R-8 but with 15% protein. One-half of the rats in each group of these experiments received 0.1 ppm selenium in the diet. Since there were no observed effects of the selenium, the −Se and +Se groups were combined.
[5] Values not determined.

selenite. In the absence or presence of α-tocopherol, selenium had no effect on the condition of the testes nor on the lipid composition (table 1). Fatty acid content also was unaffected (data not presented).

An example of the lipid changes in the early stages of α-tocopherol deficiency was provided by rats in which one testis was small and had markedly damaged germinal epithelium, whereas the partner testis was normal in size and histology (table 2). (This is a rare phenomenon since paired testes are usually very similar in weight and histology (9)). There was no decrease in phospholipid in the damaged testis of rat no. 7, which was depleted for 16 weeks, but in rat no. 18, depleted for 20 weeks, the phospholipid of the small testis was about 65% of the normal value (14 to 15 mg/g). In rat no. 14, in which both testes were damaged, the phospholipid was only slightly depressed. From a variety of data such as that in tables 1 and 2, it was apparent that the phospholipid decrease did not correlate well with the extent of injury to the germinal epithelium during the early onset of testicular degeneration. When the degeneration was prolonged, e.g., beyond 22 weeks, the phospholipid was always about one-half normal.

Table 2 also illustrates the changes in the C-20 and C-22 polyunsaturated fatty acids reported previously (3). In both rats 7 and 18 the 22:5 ω6 was lower in the small testis but the difference was not marked in rat no. 7. α-Tocopherol deficiency also increased 20:4 ω6 and 22:4 ω6 although the right testis of no. 14 did not show these changes. As with the phospholipid, a direct correlation between the alterations in these 3 fatty acids and the degree of germinal epithelial damage was not always consistent during the first few weeks of testicular damage but when stage 5 (loss of most of the germinal epithelium) had persisted for several weeks, a consistent pattern developed.

Retinol-deficient rat testis. As noted above, by feeding retinoic acid a specific lesion of the testis is produced without complications from inanition as occurs in classical vitamin A deficiency. The rats listed in table 3 that received neither retinol nor retinoic acid were chronically deficient in vitamin A for 7 weeks, at which time they were changed to a rigorously vitamin A-free diet. Body weight loss ensued in 2 to 3 weeks, when they were killed. Weights of the testes were one-half those of the control (retinol) rats and all had moderate-to-severe degeneration of the germinal epithelium. Rats maintained with retinoic acid for 3 to 14 weeks had testes weights which were proportionately smaller with respect to body weight. There were slight decreases in total lipid and in phospholipid in the 8-week group, but after 14 weeks a marked increase in both lipid fractions occurred. The difference indicates an accumulation of triglyceride.

Fatty acid analyses of the testes (table 4) revealed some similarities between the non-supplemented rats and those receiving retinoic acid for 8 weeks. Linoleic acid was considerably lower in the former group, but 20:4 ω6, 22:4 ω6 and 22:5 ω6 were about the same in the 2 types of vitamin A-deficient testes. Compared with the control rats fed retinol, the 22:5 ω6 was decreased about one-third in the 8-week-deficient tis-

TABLE 2

Composition of individual testes from α-tocopherol-deficient rats

Rat no.[1]	Testis wt	Phospho-lipid	% of total fatty acids			Histological score [2]
			20:4 ω6	22:4 ω6	22:5 ω6	
	g	*mg/g*				
7 L	0.65	13.9	20.9	3.9	11.7	4–5
7 R	1.50	14.1	13.0	1.5	15.8	0–2
18 L	0.73	9.4	22.0	3.5	5.7	5
18 R	1.58	12.3	18.4	2.1	20.1	0–1
14 L	0.97	11.3	22.6	4.8	3.9	5
14 R	0.97	11.4	12.2	2.3	2.0	5

[1] Depletion times: no. 7, 16 weeks; nos. 14 and 18, 20 weeks. L = left testis, R = right testis. Rats fed diet R-8.

[2] According to Mason (8); 0 = normal, 5 = severe degeneration of germinal epithelium.

TABLE 3

Effect of retinol deficiency on the lipid composition of rat testes

Vitamin A in diet	No. rats	No. weeks [1]	Body wt	Testes wt	Histological score [2]	Total lipid	Phospholipid	Total cholesterol
mg/kg			*g*	*g*		*mg/g*	*mg/g*	*mg/g*
None	4	2–3	249 ± 22	1.71 ± 0.12	3–5	16.4 ± 1.4	12.1 ± 1.4	2.6 ± 0.1
Acid, 20	3	3	380 ± 7	1.52 ± 0.30	3–5	17.7 ± 2.1	12.8 ± 0.9	2.3 ± 0.1
Acid, 20	3	8	414 ± 30	1.41 ± 0.05	4–5	14.0 ± 0.5	10.1 ± 0.2	2.3 ± 0.1
Acid, 20	5	14	484 ± 17	1.59 ± 0.12	5	26.8 ± 1.2	14.4 ± 0.7	3.1 ± 0.2
Alcohol,[3] 6	3	3	401 ± 17	3.31 ± 0.15	0	15.1 ± 0.4 [4]	14.5 ± 0.8	1.7 ± 0.1
Alcohol, 6	3	8	406 ± 14	3.36 ± 0.03	0	18.2 ± 0.3	13.1 ± 0.5	2.1 ± 0.0
Alcohol, 6	2	14	497 ± 12	3.46 ± 0.06	0	17.8 ± 0.0	14.2 ± 0.6	2.0 ± 0.1

[1] Number of weeks fed the vitamin A-free diet (R9) following 7 weeks of feeding a vitamin A-low diet (R11). Supplements were fed from the start of the experiment. Values are means ± SE.
[2] See footnote to table 2.
[3] As stabilized retinyl acetate.
[4] Apparent absence of triglyceride due to variation in methods.

TABLE 4

Fatty acid composition of retinol deficient rat testes [1]

Fatty acid	Dietary supplement			
	None	Retinoic acid		Retinol [2]
	2–3 weeks	8 weeks	14 weeks	8 and 14 weeks
16 ald.	1.7 ± 0.3	0.7 ± 0.2	1.5 ± 0.2	1.7 ± 0.3
16:0	30.7 ± 0.4	26.7 ± 3.0	24.5 ± 2.6	32.1 ± 0.9
16:1	2.9 ± 0.4	2.0 ± 0.9	6.8 ± 1.0	3.3 ± 0.4
18:0	7.4 ± 0.4	9.5 ± 1.2	7.8 ± 0.9	5.3 ± 0.3
18:1	23.2 ± 1.5	27.0 ± 0.3	31.0 ± 2.0	20.2 ± 1.1
18:2 ω6	3.5 ± 0.3	6.5 ± 0.1	8.3 ± 0.7	5.1 ± 0.4
20:4 ω6	15.3 ± 0.7	14.6 ± 0.8	11.7 ± 1.1	13.3 ± 0.7
22:4 ω6	2.0 ± 0.1	1.8 ± 0.2	1.3 ± 0.1	1.4 ± 0.1
22:5 ω6	11.6 ± 0.3	10.1 ± 1.8	5.7 ± 1.1	15.5 ± 0.9

[1] Values are means ± SE expressed as percentage of total fatty acids. Acids present at less than 1% are not shown. Numbers of animals are the same as in table 3.
[2] Since the data from the 8- and 14-week groups were similar, they are combined.

sues. The accumulation of triglyceride in the testes of rats fed retinoic acid for 14 weeks must be considered in interpreting the changes in proportions of fatty acids at this time period. The apparent decrease of 22:5 ω6 from 10.1% at 8 weeks to 5.7% at 14 weeks does not represent an absolute decrease in the amount of this fatty acid, but rather a dilution due to the additional fatty acids, not polyunsaturated, from the increased triglyceride. A similar explanation can also be given for the decrease in 20:4 ω6.

Zinc-deficient rat testis. In 2 separate experiments, zinc deficiency was manifested by a slow growth rate after only 2 or 3 weeks of feeding the diet. Varying degrees of alopecia occurred in many of the rats and all had soft, fine-textured hair. In the first experiment, testes from 2 rats were analyzed after 3, 5 and 7 weeks. Although the testes were about one-half the weight of the control testes, and had marked degeneration of the germinal epithelium, no significant variations in lipid composition were noted. The second experiment was therefore continued for a longer period and the control rats were pair-fed to the deficient rats. Analyses of the testes at intervals of 6, 9, 12 and 15 weeks (table 5) showed that by 9 weeks the weights of the testes from the deficient rats were less than one-half of those of the controls, and this relationship continued until 15 weeks. There was a moderate increase in total lipid in the deficient testes at 6 weeks, primarily as triglyceride. At all subsequent periods, total lipid and phospholipid in deficient testes were slightly lower and cholesterol slightly higher than in normal testes. As noted by Millar et al. (5), pair-feeding of the control rats did not in

any way alter the testes from those of rats fed ad libitum.

Analyses of the testicular fatty acids revealed that after the sixth week the pattern for the deficient or control tissues did not change significantly. Thus, only the data from the combined 12 and 15 week periods are given in table 6. The most apparent difference in the zinc-deficient testis was a moderate decrease in 22:5 ω6.

DISCUSSION

To facilitate comparison of the testis lipid composition in the 3 deficiencies, representative data have been compiled in table 6. These data were selected to represent periods of chronic deficiency, i.e., the animals were permitted to live a prolonged time after the testes were known to be damaged. These periods were approximately: α-tocopherol, 5 weeks; retinol, 5 weeks;

TABLE 5

Effect of zinc deficiency on the lipid composition of rat testes [1]

Zinc addition	No. rats	No. weeks	Body wt	Testes wt	Total lipid	Phospholipid	Total cholesterol
mg/kg			*g*	*g*	*mg/g*	*mg/g*	*mg/g*
None	3	6	117 ± 8	1.39 ± 0.17	20.2 ± 0.6	12.3 ± 0.5	1.85 ± 0.11
75 [2]	3	6	136 ± 1	2.03 ± 0.40	15.7 ± 0.7	11.6 ± 0.5	1.60 ± 0.11
None	3	9	134 ± 23	1.35 ± 0.18	17.8 ± 0.9	11.7 ± 1.2	2.15 ± 0.08
75	3	9	155 ± 4	2.81 ± 0.14	18.0 ± 0.1	13.9 ± 0.2	1.77 ± 0.02
None	2	12	165 ± 22	1.32 ± 0.21	17.0 ± 1.6	12.6 ± 0.1	2.45 ± 0.50
75	2	12	183 ± 2	3.07 ± 0.09	18.6 ± 1.4	14.6 ± 0.3	1.87 ± 0.00
None	2	15	197 ± 13	1.44 ± 0.01	16.0 ± 2.9	11.2 ± 2.0	1.96 ± 0.27
75	2	15	187 ± 3	2.97 ± 0.19	17.5 ± 0.4	13.3 ± 0.2	1.85 ± 0.05
Avg of weeks 9, 12 and 15:							
None			—	1.37 ± 0.21	17.0 ± 2.3	11.8 ± 1.7	2.18 ± 0.38
75			—	2.93 ± 0.21	18.0 ± 1.0	13.9 ± 0.6	1.82 ± 0.07

[1] Values are means ± SE. Rats fed diet R-8.
[2] Added as zinc carbonate. Supplemented rats were pair-fed. Rats fed ad libitum weighed 226 ± 9 g at 6 weeks.

TABLE 6

Summary of lipid and fatty acid composition of testes from normal, α-tocopherol, retinol and zinc-deficient rat testes [1]

	Normal	α-tocopherol-deficient	Retinol-deficient [2]	Zinc-deficient
No. of rats	4	4	4	4
Age, weeks [3]	12–15	19	15	12–15
Wt of testes, g	3.02 ± 0.09	1.49 ± 0.30	1.41 ± 0.05	1.38 ± 0.10
Total lipid, mg/g	18.1 ± 0.7	17.7 ± 1.4 [4]	14.0 ± 0.5	16.5 ± 1.4
Phospholipid, mg/g	13.9 ± 0.4	8.6 ± 1.1	10.1 ± 0.2	11.9 ± 1.0
Cholesterol, mg/g	1.8 ± 0.0	2.4 ± 0.1	2.3 ± 0.1	2.2 ± 0.1
Fatty acids				
16 ald.	1.6 ± 0.4	0.8 ± 0.2	0.7 ± 0.2	0.9 ± 0.2
16:0	33.0 ± 0.9	25.2 ± 0.5	26.7 ± 3.0	30.6 ± 2.5
16:1	2.1 ± 0.4	3.2 ± 0.4	2.0 ± 0.9	1.8 ± 0.5
18:0	5.6 ± 0.2	9.8 ± 0.7	9.5 ± 1.2	8.7 ± 0.8
18:1	20.8 ± 2.0	22.1 ± 1.9	27.0 ± 0.3	19.9 ± 2.4
18:2 ω6	2.6 ± 0.1	3.4 ± 0.1	6.5 ± 0.1	2.9 ± 0.1
20:4 ω6	12.9 ± 0.5	24.3 ± 1.1	14.6 ± 0.8	17.8 ± 0.6
22:4 ω6	1.3 ± 0.1	3.8 ± 0.2	1.8 ± 0.2	2.1 ± 0.2
22:5 ω6	17.3 ± 1.0	5.3 ± 0.9	10.1 ± 1.8	12.4 ± 0.7

[1] Values are means ± SE.
[2] This diet contained retinoic acid, 20 mg/kg. The fat was 4% of cottonseed oil; the diets of the other groups contained 5% of stripped lard.
[3] Post-weaning.
[4] This value determined gravimetrically (see text).

zinc, 6 to 9 weeks. This was done because it was found that the lipid changes in the α-tocopherol-deficient testis became more pronounced as the deficiency progressed (table 1). Since deficiencies of retinol and zinc can be produced in a much shorter time than for α-tocopherol, the comparison of an acute versus a chronic deficiency would probably not be valid. Because of the accumulation of triglyceride in the testes of rats fed retinoic acid for 14 weeks, noted above, the testes from the 8-week period have been used in making comparisons. Typical histological pictures of the testes in the 3 deficiencies at the above time periods are shown in figures 2, 3 and 4.

The testes in all 3 deficiencies had reduced amounts of phospholipid, most pronounced in the case of α-tocopherol where the values were about 60% of normal. The retinol-deficient testes at 8 weeks had about 75% of the normal amount of phospholipid, whereas the zinc-deficient testes had only a slight reduction in phospholipid (table 6).

Changes in polyunsaturated fatty acids were characteristic for each of the deficiencies. The most pronounced alteration in the α-tocopherol-deficient testis was the decrease in 22:5 ω6 to about one-third the normal amount and an increase in 20:4 ω6 to twice normal. In retinol deficiency, although there was a decrease in 22:5 ω6, it was not as great as for α-tocopherol and the 20:4 ω6 did not change. In the zinc-deficient tissue there was a slight reduction in 22:5 ω6 and a moderate increase in 20:4 ω6. The apparent increase in 18:2 ω6 in the retinol-deficient testes was probably due to the higher linoleic acid content of the diet (2.0% vs. 0.5% in the lard-containing diets). In contrast with other tissues, the linoleic acid content of testis varies only slightly with changes in dietary linoleate except when the dietary level is increased several fold (2).

One unique change in the fatty acid pattern of the α-tocopherol-deficient testis is a two- to threefold increase in 22:4 ω6 (3). This fatty acid increased slightly in zinc deficiency but it was not affected by retinol deficiency.

The biochemical significance of these changes in phospholipid and in polyunsaturated fatty acids as related to vitamins A and E or zinc can only be speculated upon at this time. Although there is a distinct difference in the pathology of the lesions in the early stages of deficiencies of vitamins A and E, Mason (4) stated that "in advanced stages of A-deficiency the testes often become more or less indistinguishable in their histology from advanced E-deficiency." A histological comparison of testes in vitamin E and zinc deficiencies has not been made, but the evidence indicates that the pathology in the 2 conditions is similar[9] (figs. 2 and 4). In all 3 deficiencies there is a loss of germinal epithelium; thus, the changes in chemical composition could be due primarily to a loss of this portion of the tubules. Since the tubules make up about 90% of the fresh weight of the rat testis (12), it is not likely that the interstitial cells make a significant contribution to the analyses of the total testis.

The fact that the decrease in 22:5 ω6 was considerably greater in the α-tocopherol-deficient testis than in either the retinol- or zinc-deficient testes suggests a specific relationship between this fatty acid and α-tocopherol. If there was a general peroxidative loss of polyunsaturated fatty acids due to an antioxidant deficiency, 22:4 ω6 and 20:4 ω6 would also be expected to decrease. The observed increase in these 2 fatty acids may indicate a block in their further conversion to 22:5 ω6. An alternative consideration proposed by Bernhard et al. (13) is that vitamin E exerts a regulatory effect on the formation of 20:4 ω6 and recent work of Horwitt (14) supports this hypothesis. Since a moderate increase in 20:4 ω6 was observed in the zinc-deficient testis, as well as a decrease in 22:5 ω6, it appears that α-tocopherol and zinc deficiencies may to some extent mediate their effects through a similar mechanism.

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[9] Personal Communication from Dr. Karl E. Mason.

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PLATE 1

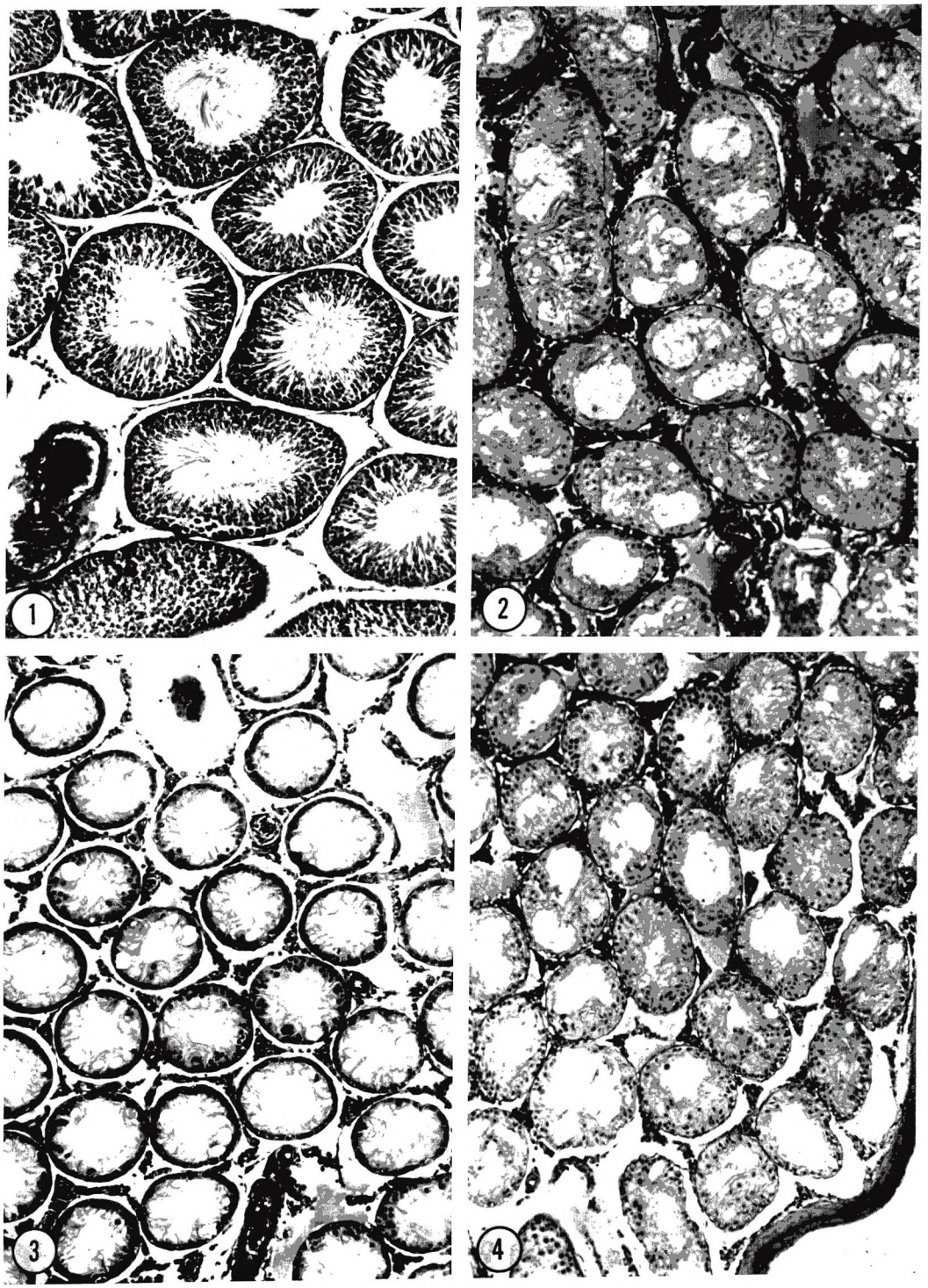
EXPLANATION OF FIGURES

1 Testis from a normal rat fed diet R-9 with adequate α-tocopherol and retinol for 8 weeks. H & E. × 79.

2 Testis from a rat deficient in α-tocopherol for 19 weeks. According to Mason (8), this represents ". . . stage 5, . . . with reduction in tubule size and almost complete removal of germ cells leaving only a fibrous Sertoli syncytium." H & E. × 79.

3 Testis from a retinol-deficient rat fed retinoic acid for 8 weeks. Similar to that described by Mason ((8), figs. 9 and 11) for vitamin A deficient testes "in an advanced stage of degeneration (55–90 days),— a greatly reduced size of the tubules with retention of one or two layers of residual germ cells." The vacuolation noted by Howell et al. (7) is also apparent. H & E. × 79.

4 Testis from rat deficient in zinc for 15 weeks. Note the similarity with the α-tocopherol-deficient testis (fig. 2). H & E. × 79.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Essential Fatty Acid Requirement of Young Swine [1]

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ABSTRACT Uncastrated male pigs, 3 weeks of age, were fed purified diets containing 6 levels of linoleic acid. One testicle and a sample of scrotal fat were removed by orchiectomy at 5 and 10 weeks to provide biopsy tissue for gas-liquid chromatographic analysis of fatty acid composition. Marked alteration in tissue fatty acids was evident after 5 weeks and these differences were accentuated at 10 weeks. A progressive depression of the dienoic and tetraenoic fatty acids occurred, with a corresponding elevation of the trienoic fatty acids, as the dietary level of linoleic acid was decreased. Extensive dermal lesions, typical of EFA deficiency, were observed on all pigs in the group receiving the basal diet. Dermal lesions were also observed in the groups receiving linoleic acid as 0.25 and 0.50% of the dietary calories. Feeding linoleic acid as 1.0% of the dietary calories either prevented or remitted the dermal symptoms, or both. Weight gain was not significantly affected by dietary linoleate level. A plot of the triene-to-tetraene ratio versus the dietary linoleate level indicates that the linoleic acid requirement of the young pig is not more than 2.0% of the dietary calories.

The linoleic acid requirement of swine has been estimated, using tissue fatty acid which showed changes in polyunsaturated fatty acid (PUFA) concentrations that were characteristic of essential fatty acid (EFA) deficiency (1, 2). Using the triene-to-tetraene ratio as an index, the linoleate requirement of swine was found to be equivalent to that of the rat when expressed as a percentage of the total caloric intake. Since the swine used in these experiments failed to develop dermal lesions, characteristic of EFA deficiency, no attempt was made to relate the linoleate requirement to gross skin lesions.

In recent studies, gross dermal lesions have been produced in young swine fed EFA-deficient diets in addition to the alteration of tissue PUFA typical of EFA deficiency (3). It was the purpose of the present study to determine the linoleic acid requirement of young swine, using observations on gross dermal lesions as well as variations in tissue PUFA as criteria of adequacy.

EXPERIMENTAL

Thirty-six crossbred uncastrated male pigs were removed from their dams at 3 weeks of age, weighing an average of 6.4 kg. The pigs were assigned to groups of 6 pigs each on the basis of litter and initial weight, with the groups being allotted at random to treatment. Each group was placed in a pen with an expanded metal floor and equipped with an automatic waterer and feeder.

TABLE 1
Composition of basal diet

	%
Casein	17.88
Glucose monohydrate	75.60
Cellulose [1]	3.00
Defluorinated phosphate	2.10
Mineral mixture [2]	1.14
Vitamin mixture [3]	0.28

[1] Solka Floc, Brown Company, Chicago.
[2] Supplied the following quantities/kg of diet: (in grams) NaCl, 2.99; K_2HPO_4, 2.22; KCl, 3.83; $MgCO_3$, 1.52; $FeSO_4 \cdot 7H_2O$, 0.55; (in milligrams) $MnSO_4 \cdot H_2O$, 135; $CoCl_2$, 26; $CuSO_4$, 5.50; $ZnCO_3$, 50; and KI, 3.0.
[3] Supplied the following quantities/kg of diet: (in IU) vitamin A, 6600; vitamin D_2, 1760; α-tocopheryl acetate, 22; (in milligrams) menadione, 4.40; ascorbic acid, 55; thiamine·HCl, 5.5; riboflavin, 11; Ca pantothenate, 22; niacin, 66; choline chloride, 1100; *p*-aminobenzoic acid, 18; inositol, 198; pyridoxine·HCl, 5.50; streptomycin sulfate, 22; chlortetracycline, 22; and (in micrograms) biotin, 66; folic acid, 440; and vitamin B_{12}, 44. An antioxidant (ethoxyquin) was added at a level of 0.0125% to all diets.

A casein-glucose monohydrate basal diet as shown in table 1 was used in the experiment. Total lipid content of the basal diet by analysis was 0.18%, with only a trace of linoleic acid being present. Corn oil was added to the basal diet in varying amounts to obtain the following levels of linoleic

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acid expressed as a percentage of the dietary calories: zero, 0.25, 0.50, 1.0, 2.0 and 4.0. These various levels of linoleic acid formed the 6 treatments studied.

When the pigs had received the various diets for a period of 5 weeks, an orchiectomy was performed on each pig in which the left testicle and a sample of scrotal fat were removed and immediately frozen until analysis for constituent fatty acids could be performed by gas-liquid chromatography. The remaining testicle and a scrotal fat sample were obtained from each pig for fatty acid analysis after they had received the experimental diets for a period of 10 weeks.

The lipid components were extracted from the tissue samples using the method of Folch et al. (4) and the methyl esters were prepared. Analyses of the fatty acid methyl esters were performed on a hydrogen flame ionization detector-type gas-liquid chromatographic apparatus using diethylene glycol succinate polymer as the participating agent. Identification of the fatty acid esters was by the use of standards obtained from Hormel Institute, Austin, Minnesota. Standards were chromatographed to determine retention time of the various fatty acids. Quantitative results agreed with the stated composition data with a relative error of less than 2% for major components (> 10% of total mixture) and less than 6% for minor components (< 10% of total mixture).

Individual weights were obtained on the pigs at 2-week intervals and group feed consumption was recorded.

Statements of significance are based on results of analysis of variance of data collected.

RESULTS AND DISCUSSION

No significant differences were noted in daily weight gains between any of the treatment groups over the 10-week experimental period. Data presented in table 2 show that weight gains of pigs receiving the higher levels of linoleic acid were slightly greater than those fed the lower levels. The absence of a marked effect on weight gains with pigs fed EFA-deficient diets corroborates earlier results (3). It is possible that a more pronounced variation in weight change might have occurred had the pigs been maintained with the EFA-deficient diets for a longer period of time.

The efficiency of feed utilization was increased as the linoleic acid content of the diet was increased. At least a part of this increase is due to a slightly greater caloric density of the diets containing the higher levels of linoleic acid. However, since an increase in metabolic rate has been reported to be an early symptom of EFA-deficiency in the rat (5), it may be that this effect also exerted some influence.

Dermal lesions, typical of EFA-deficiency, were observed in the pigs fed the low fat basal diet after 6 to 7 weeks on experiment and had increased in severity by the time the experiment was terminated at 10 weeks. Dermal lesions were also observed among the pigs receiving linoleic acid as 0.25 and 0.5% of the dietary calories. No skin lesions were present among the pigs receiving 1.0% or more of the dietary calories as linoleic acid. A scaly, dandruff-like desquamation of the skin over the dorsal surface was the first noticeable symptom. Later, a brownish, gummy exudate appeared around the ears, axillary spaces and under the flanks. Skin eruptions were also present about the ears, axillary spaces and flanks in the severest cases.

Highly significant differences were found in the fatty acid composition of the testis tissue obtained from pigs fed the various levels of linoleic acid. These differences were quite evident in the analyses of the testis tissue taken after the experimental diets had been fed for 5 weeks, but became

TABLE 2

Effect of linoleic acid level on weight gain and feed utilization [1]

Dietary linoleate, [2] % of calories	0.00	0.25	0.50	1.00	2.00	4.00
Daily gain, kg	0.44	0.46	0.43	0.48	0.50	0.51
Feed/gain	2.71	2.33	2.33	2.27	2.14	2.05

[1] Six pigs/group.
[2] Supplied as corn oil.

more pronounced by the end of the 10-week experimental period. Consequently, only the 10-week data are presented here. The fatty acid analysis data for the testis tissue obtained after 10 weeks on experiment are presented in graphical form in figures 1 and 2 so that the relationships of the response curves for the various fatty acids as influenced by dietary linoleate level can be seen clearly.

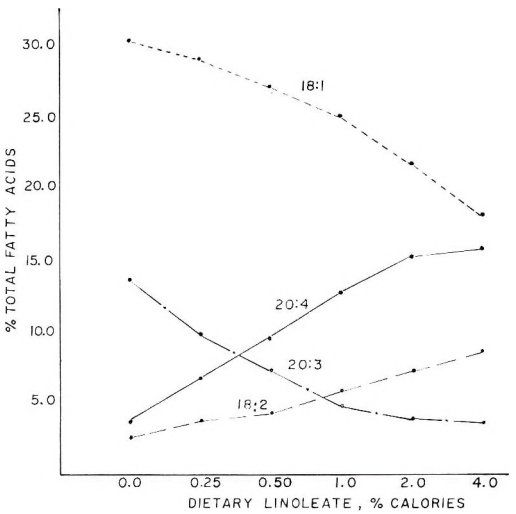

Fig. 1 Influence of varying levels of dietary linoleic acid provided as corn oil on the linoleic (18:2), arachidonic (20:4), eicosatrienoic (20:3) and oleic (18:1) acid content of testes tissue from young swine.

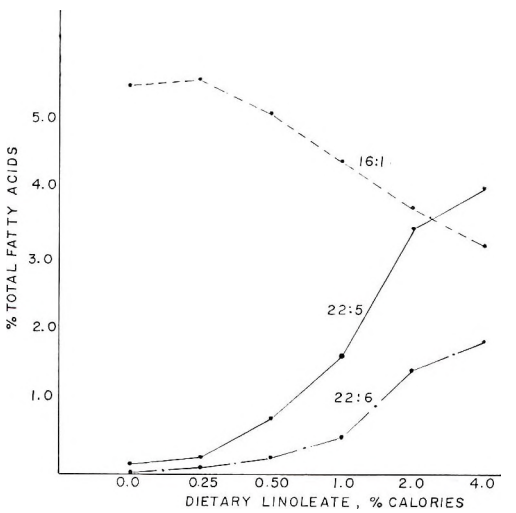

Fig. 2 Influence of varying levels of dietary linoleic acid provided as corn oil on the palmitoleic (16:1), docosapentaenoic (22:5) and docosahexaenoic (22:6) acid content of testes tissue from young swine.

Figure 1 shows that as the level of linoleic acid was increased in the diet there was a highly significant progressive increase in the dienoic and tetraenoic fatty acids with a corresponding decrease in the trienoic fatty acids. The oleic acid (18:1) content of the testis tissue decreased progressively as the dietary linoleate level was increased.

A highly significant decrease in the palmitoleic acid (16:1) content of the testis tissue as the dosage level of linoleic acid was increased may be observed in figure 2. There was also an increase in the content of docosapentaenoic (22:5) and docosahexaenoic (22:6) acids as the level of corn oil was increased in the diet. Linolenic acid, by analysis, constituted 2.14% of the fatty acids in the corn oil used, so that as the corn oil was increased in the diet to raise the linoleate level, the linolenate level was also increased proportionally. It is not known whether these 22-carbon acids are members of the linoleate or linolenate families, since their structure was not determined.

The diminished levels of dienoic and tetraenoic fatty acids and elevated trienoic fatty acids at the low levels of linoleate intake are characteristic of EFA deficiency reported for various species (6–9). The relationship between these 3 fatty acids was reversed rapidly as the level of linoleic acid was increased in the diet up to 2.0% of the dietary calories. Increasing the dietary linoleate level beyond this point resulted in only negligible changes in the arachidonic and eicosatrienoic acid content of the tissue lipids.

The fatty acid content of the adipose tissue obtained from the scrotum is shown in table 3. There was a highly significant increase in linoleic acid content of the scrotal fat as the level of dietary linoleate was increased. No significant variations were found in any of the other fatty acids studied.

A negative correlation between the triene and tetraene fatty acid fractions is clearly evident from these data (fig. 1) and this relationship is related to dietary linoleate. The triene-to-tetraene ratio has been used

TABLE 3

Fatty acid composition of scrotal fat at 10 weeks

Fatty acid	Dietary linoleate, % of calories					
	0	0.25	0.50	1.00	2.00	4.00
	% total fatty acids					
14:0	1.81	1.84	1.74	1.86	1.78	2.03
16:0	23.46	23.10	21.56	23.58	21.24	22.15
16:1	9.36	9.24	9.80	8.11	9.05	8.84
18:0	8.72	8.82	8.50	9.37	8.36	8.33
18:1	52.52	51.93	52.43	50.38	51.28	47.91
18:2 [1]	2.64	3.27	4.09	4.26	6.16	8.91
18:3	1.12	1.23	1.34	1.28	1.66	1.21

[1] Highly significant difference due to treatment ($P < 0.01$).

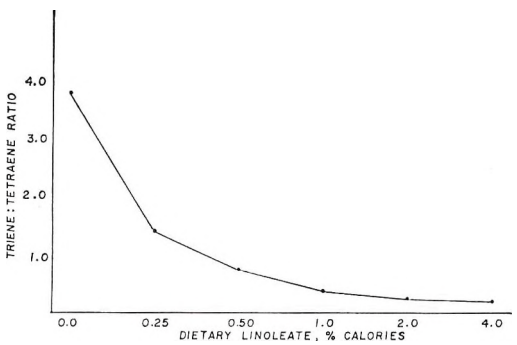

Fig. 3 Relationship of triene-to-tetraene ratio of testes tissue from young swine fed varying levels of dietary linoleic acid.

to estimate minimal EFA requirements (1, 2, 10). Consequently, a curve was constructed from a plot of the triene-to-tetraene ratio versus the dietary linoleate level as shown in figure 3. The ratio decreased markedly as the dietary level of linoleate was increased from zero to 1.0% of the dietary calories, with only a slight decrease occurring beyond this level of linoleate intake. It therefore appears from these data that the requirement for linoleic acid in the 3-week-old male pig is not greater than 2.0% of the dietary calories. Linoleic acid fed as 1.0% of the dietary calories appears to be approaching adequacy under these conditions, since this level clearly prevented the occurrence of dermal lesions. The triene-to-tetraene ratio at the 1.0% level of linoleate intake was 0.38, which is comparable to the figure of 0.4 that has been suggested by Holman (10) as being the point at which the minimal linoleate requirement of the rat has been met. These observations are in general agreement with previously reported estimates for the pig (1) and rat (10).

To determine whether linoleic acid alone would cure the dermal manifestations of EFA-deficiency produced in this study, the pigs that had received the low fat basal diet for 10 weeks and showed severe dermal lesions were divided into 2 groups. One group of 3 pigs was fed the basal diet plus methyl linoleate as 1.0% of the dietary calories. The other group was fed the basal diet plus methyl oleate as 1.0% of the dietary calories. The methyl linoleate used was, upon analysis, 98.7% pure with the contaminant being almost entirely methyl oleate. Analysis of the methyl oleate showed a purity of 94.3% with traces of myristic and palmitoleic acids present. The pigs were fed these diets for 31 days of the post-experimental period. At the end of this time the dermal lesions had been completely remitted among the pigs receiving the diet containing linoleic acid, whereas no observable changes had occurred in the dermal manifestations among the pigs fed the diet containing oleic acid. Data presented in table 4 show that pigs receiving the linoleic acid diet also gained at a slightly faster rate and had greater efficiency of feed utilization than

TABLE 4

Performance of EFA-deficient pigs fed either linoleic acid or oleic acid as 1% of dietary calories [1]

	Methyl linoleate	Methyl oleate
Daily gain, kg	0.69	0.55
Feed/gain	3.02	3.17

[1] Three pigs/group; pigs had received EFA-deficient diet for 10 weeks prior to being fed these diets, which were supplied for 31 days.

pigs receiving oleic acid. Thus, linoleic acid per se apparently is adequate in remitting the symptoms of EFA deficiency in the pig.

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Effect of Diets Marginal in Methionine and Choline with and without Vitamin B_{12} on Rat Liver and Kidney [1,2]

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ABSTRACT The effects of diets marginal in methionine and choline, without cystine, and with varying vitamin B_{12} were investigated in rats for a period of 6 months. Weight gains were slower for the first 12 weeks in rats fed the diets low in methionine and choline but at 6 months these values were equal to or higher than the control groups. A high proportion (87 to 90%) of the rats fed the low methionine and low choline diets showed striking microscopic lesions in the inner cortex of the kidney and non-calcified, mucoid structures in the urinary bladder. Addition of methionine, choline and vitamin B_{12} to the diet prevented all abnormalities. The liver was histologically normal and unaffected by the low methionine and low choline intake. Total plasma proteins and single fractions were unaffected except for a decrease in γ-globulin in rats fed the low methionine, low choline diet.

It is often assumed that diets which support reasonable increments in growth and permit attainment of a mature weight within the normal range for a given species are adequate for functional and morphologic normality. However, it is recognized that increased longevity has been associated with retardation of growth and sexual maturity (1, 2). Much of the research concerned with diet adequacy has been limited to relatively short periods of time with emphasis on gain in body weight and selected biochemical parameters. A majority of the material in this area of nutritional research has gone without adequate histologic assessment.

A few studies have dealt with the acute effects of methionine deficiency (3–5) but there appears to be a singular lack of studies relative to the feeding of marginal levels of sulfur-containing amino acids over prolonged periods of time. Since these amino acids are often limiting in diets consumed by population groups, particularly in the technologically underdeveloped countries, it seemed worthwhile to test the effect of diets marginal but not devoid in methionine and choline — without cystine — on the growth and development of the rat. Vitamin B_{12} content of the diets was varied as was methionine and choline; in one group, sulfur as ammonium sulfate was added on a molar basis equivalent to that contributed by the basal diet supplemented with 0.6% methionine. Results of 2 trials, each of 6 months duration, are presented in this report.

MATERIALS AND METHODS

Weanling male rats of the CD strain [3] were used in both experiments. Animals were caged individually in screen-bottom metal cages and kept at 21 to 27°. The diets and water were supplied ad libitum. Composition of the diets used in the studies is shown in table 1. Isolated soybean protein,[4] was used as the source of protein because of its low methionine content (approximately 1.0%) and its lack of cystine. Methionine and choline were added to the basal diets at levels previously shown (6) to support adequate weight gains. A group fed a 20% casein diet was also included in each trial to act as a further control. Diets were mixed weekly and stored at 4°. Body weights were recorded at weekly intervals. Blood was withdrawn by cardiac puncture under light ether-anesthesia at time of kill-

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[2] Contribution no. 769 from the Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts.

[3] Obtained from Charles River Breeding Farms, Brookline, Massachusetts.

[4] Obtained from Central Soya Company, Chicago.

TABLE 1

Dietary ingredients

	Diet number						
	1	2	3	4	5	6	7
	% of diet	*% of diet*	*% of diet*	*% of diet*	*% of diet*	*% of diet*	*% of diet*
Casein	—	—	—	—	—	—	20.0
Isolated soybean protein	20.0	20.0	20.0	20.0	20.0	20.0	—
Sucrose	67.8	67.8	67.6	67.6	66.8	66.8	67.7
Soybean oil	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Salt mix [1]	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Vitamin mix [2]	2.0	2.0	2.0	2.0	2.0	2.0	2.0
DL-Methionine	0.1	0.1	0.1	0.3	0.6	0.6	0.0
Choline [3]	0.1	0.1	0.3	0.1	0.6	0.6	0.3
Vitamin B_{12}, μg/100 g diet	0.0	5.0	5.0	5.0	0.0	5.0	5.0

[1] Wesson, L. G. 1932 A modification of the Osborne-Mendel salt mixture containing only inorganic constituents. Science, 75: 339. Purchased from Nutritional Biochemicals Corporation, Cleveland.

[2] The vitamin premix in sucrose carrier contained the following in mg/100 g diet: thiamine·HCl, 1.6; pyridoxine·HCl, 1.6; riboflavin, 1.6; Ca pantothenate, 4.0; nicotinamide, 1.2; folic acid, 0.5; inositol, 25.0; menadione, 1.0; vitamin E (*dl*-α-tocopheryl acetate), 11.0; vitamin A, 2300 USP units; vitamin D_2, 230 USP units.

[3] Added to the diet as a water solution in the concentrations indicated.

ing. Total plasma protein was determined with the aid of a Goldberg refractometer (7) and fractionated electrophoretically on cellulose acetate strips in Coleman chambers. Necropsy was performed on all animals at time of killing, with tissues preserved in appropriate fixatives for paraffin embedding and microscopic study. All sections were stained routinely with hematoxylin and eosin; selected sections were stained with Alcian blue-Periodic Acid Schiff (PAS) and with alkaline tetrazolium for protein-bound disulfide bonds (8). The von Kossa stain was used for identification of calcium.

RESULTS

Table 2 summarizes the body weights of the various dietary groups on both trials during the 6-month period of study. During the early period of study, when most rapid gain was expected, the low methionine-low choline groups gained weight more slowly than the other dietary groups. However, by 6 months the average body weight of the low methionine-low choline groups was not significantly different from that of the group fed a 20% casein diet. A decrease in body weight occurred in animals fed the high methionine-choline diet, with or without vitamin B_{12}, during the period between 3 and 6 months. The reason for this result is not clear.

Total plasma protein did not vary appreciably among the various groups after they had received the diet for 6 months (table 3). The only consistent difference among groups was in the reduced γ-globulin in rats fed diets low in methionine and choline, with or without vitamin B_{12}. Although albumin varied, no consistent alterations could be attributed to any of the dietary treatments.

At necropsy all animals appeared normal grossly with no evidence of liver or kidney damage. Firm, greyish-white mucoid structures, varying in size from 2 mm to about 1.0 cm in diameter, were observed in the urinary bladder of some of the animals. They were not calcified but were firm and rubbery in the fresh state; they cut without fragmenting when processed in paraffin blocks.

The liver was normal on gross and microscopic examination in all groups; sections stained with hematoxylin and eosin or frozen sections stained with oil red O did not show fatty infiltration in the low methionine-low choline groups. Although the kidneys appeared normal on gross observation, microscopically there was a striking lesion involving most of the inner cortex (fig. 1) in rats fed diets 1–5. Figure 2 is a photograph of a section taken from an animal fed the high methionine-choline diet (diet 6). Compare this with figures 3 and 4 which illustrate hypertrophic and degenerate epithelium observed in kidneys of rats fed diets 1–5; figures 2–4 are the same magnification. The lumen of many of the tubules contained material histochemically identical to the mucoid structures found in the urinary bladder of some

TABLE 2

Body weight of rats fed diets varying in methionine and choline, with and without vitamin B_{12}, for 6 months

Diet no.	Treatment	No. of animals	Body weights			
			Initial	4-week	12-week	6-month
			g	*g*	*g*	*g*
		Trial 1				
1	Low methionine-low choline	10	42 ± 1 [1]	118 ± 3	278 ± 7	490 ± 6
2	Low methionine-low choline + vitamin B_{12}	19	42 ± 2	139 ± 6	402 ± 14	534 ± 16
3	Low methionine-moderate choline + vitamin B_{12}	12	45 ± 3	143 ± 9	399 ± 16	502 ± 18
4	Moderate methionine-low choline + vitamin B_{12}	12	44 ± 3	158 ± 15	430 ± 19	504 ± 20
5	High methionine-high choline	10	39 ± 4	143 ± 7	432 ± 9	344 ± 8
6	High methionine-high choline + vitamin B_{12}	20	44 ± 3	194 ± 11	476 ± 13	392 ± 11
7	Casein control	16	40 ± 5	198 ± 8	390 ± 30	498 ± 15
		Trial 2				
1	Low methionine-low choline	20	42 ± 1 [1]	133 ± 5	407 ± 10	515 ± 10
1a	Low methionine-low choline + $(NH_4)_2SO_4$	20	44 ± 3	140 ± 7	420 ± 14	500 ± 16
2	Low methionine-low choline + vitamin B_{12}	20	42 ± 3	156 ± 7	440 ± 18	540 ± 20
5	High methionine-high choline	20	43 ± 2	189 ± 12	465 ± 33	326 ± 16
6	High methionine-high choline + vitamin B_{12}	20	40 ± 2	198 ± 13	450 ± 22	492 ± 14
7	Casein control	20	42 ± 2	194 ± 11	382 ± 11	488 ± 10

[1] Mean ± SD.

TABLE 3

Total plasma protein and electrophoretic analysis of plasma protein in rats fed diets varying in methionine and choline, with and without vitamin B_{12}, for 6 months

Diet no.	Treatment	No. of animals	Total protein	Albumin	Globulin		
					$\alpha_1 + \alpha_2$-	β-	γ-
			g/100 ml	%	%	%	%
	Trial 1						
1	Low methionine-low choline	10	6.7	54	18	19	8
2	Low methionine-low choline + vitamin B_{12}	18	6.7	60	12	19	8
5	High methionine-high choline	10	7.0	58	12	15	15
6	High methionine-high choline vitamin B_{12}	15	6.8	64	10	10	16
7	Casein control	5	7.0	56	12	17	14
	Trial 2						
1	Low methionine-low choline	18	7.1	62	10	17	10
1a	Low methionine-low-choline + $(NH_4)_2SO_4$	15	7.0	56	12	16	11
2	Low methionine-low choline + vitamin B_{12}	12	6.8	58	10	13	15
5	High methionine-high choline	20	6.2	61	10	15	14
6	High methionine-high choline + vitamin B_{12}	15	6.7	56	12	14	15
7	Casein control	10	7.0	52	13	15	14

animals. Occasional mineralized casts were observed in some of the renal tubules. The mucoid structures (fig. 5) seen in the urinary bladder were largely homogeneous with some focal granularity. They stained red with eosin, negative with the von Kossa stain for calcium and positive with PAS and disulfide stains. This placed them in the category of glycoproteins, but they were not identified further.

Table 4 lists the incidence of kidney lesions and occurrence of urinary bladder structures in the various dietary groups. Rats fed the low methionine-low choline diets (diets 1–3) showed the highest incidence of kidney lesions (87 to 90%). Supplementation of the low methionine diet with 0.3% choline (diet 3) reduced slightly the incidence of kidney lesions but had no effect on the presence of urinary bladder structures (diets 2 and 3). However, maintenance of the low choline intake together with a moderate methionine (0.3%) supplementation (diet 4) markedly reduced the incidence of kidney lesions, with approximately 16% of the animals showing lesions. A further significant decrease was noted when the diets were supplemented with 0.6% methionine and 0.6% choline (diet 5), but normal renal morphology and absence of urinary bladder structures was observed only after the addition of vitamin B_{12} to the diet (diet 6). Addition of ammonium sulfate (diet 1a) to supply sulfur equivalent to that in diets 5 and 6 did not reduce the incidence of kidney lesions or urinary bladder structures below that observed with diet 1. Urinary bladder structures appear to be related to kidney damage. They were reduced in number by feeding a moderate methionine diet and were completely absent with diets 5 and 6 (high methionine) and with the control casein diet.

DISCUSSION

Results of these experiments indicate that rats can survive and appear clinically normal when fed diets marginal in sulfur-containing amino acids, yet bear significant pathologic lesions which are not grossly visible. Normal liver morphology in animals fed the low methionine-low choline diet was unexpected. However, Sidransky and co-workers (5) have shown that rats

TABLE 4

Incidence of kidney lesions and urinary bladder structures in rats fed diets for 6 months varying in methionine, choline, vitamin B_{12} and $(NH_4)_2SO_4$

Diet no.	Treatment	Kidney lesions		Urinary bladder structures	
		No.	%	No.	%
1	Low methionine-low choline	27/30	90	19/30	63
1a	Low methionine-low choline + $(NH_4)_2SO_4$	18/20	90	12/20	60
2	Low methionine-low choline + vitamin B_{12}	34/39	87	8/39	20
3	Low methionine-moderate choline + vitamin B_{12}	7/9	78	3/9	33
4	Moderate methionine-low choline + vitamin B_{12}	2/12	16	1/12	8
5	High methionine-high choline	2/30	7	0/30	0
6	High methionine-high choline + vitamin B_{12}	0/40	0	0/40	0
7	Casein control	0/36	0	0/36	0

fed diets containing 0.12% methionine and 0.25% choline do not develop fatty livers in comparison with rats fed a similar diet but devoid of choline. Furthermore, it appears that in the studies of these investigators choline was more critical than methionine in the development of severe pathological lesions in the liver with diets low in methionine. It may reasonably be assumed therefore that the lipotropism of the low methionine and low choline diet under our conditions is the result of an adequate endogenous biosynthesis of choline. It is also noteworthy that the present study involved use of male rats and that the force-feeding of choline-containing diets devoid of methionine for 3 to 6 days induces fatty liver in adult females but not in males (9). That a relatively normal liver was maintained in the rats fed diets 1 to 3 is also supported in part by the ability of these animals to synthesize normal amounts of albumin. These observations may be misleading insofar as the long-term welfare of the animal is concerned; the reticuloendothelial system was unable to supply normal amounts of γ-globulin and the integrity of the renal tubules was compromised.

Urolithiasis is not uncommon in domestic farm animals (10) and it has been reported in rats by a number of investigators. Van Reen et al. (11) have shown that a strain of rats (NMRI-D) used in their laboratory is particularly susceptible to formation of kidney and bladder stones, composed predominantly of calcium citrate, when fed a diet of 15% casein and 4% of Hubbell, Mendel and Wakeman salt mix (12). They were able to prevent stone formation by increasing the protein level to 30% with either casein or soybean protein or by supplementation with both methionine and lysine. They postulate an effect of acidic groups as a primary factor in preventing stone formation.

We were unable in our studies to demonstrate calcium histochemically in the structures observed. Thus it appears that these structures were different in chemical composition from those usually reported as renal or urinary bladder calculi. Evidence suggests that both the structures in the bladder and the material in the lumen of some renal tubules resulted from secretion or excretion, or both, by damaged renal tubules.

The lesion in the kidney tubule requires further study. Bizarre cells such as those observed in this study are sometimes observed in a variety of conditions as isolated single cells or as small groups of cells associated with a damaged segment of the nephron. The extent of the lesion in this

study was much greater than any previously observed in rats in our laboratory. Descriptions of lesions of this nature and magnitude, associated with dietary treatment, were not found in the accessible literature.

The induction of these lesions appears to be related most critically to the low methionine content of the diet since methionine supplementation prevented their occurrence to the greatest degree. However, the choline content of the diet also appears to be important. The fact that the addition of methionine, choline and vitamin B_{12} prevented the development of a pathologic kidney suggests a relationship to methyl group or 1-carbon metabolism.

It seems unlikely that these lesions are a result of lack of acidic groups since addition of ammonium sulfate had no alleviating effect. Renal lesions commonly associated with choline deficiency are of a different nature (13) than those seen in this investigation.

The urinary bladder structures may have resulted from the renal excretion of an abnormal or incomplete protein, in quantities sufficient for it to accumulate, as a result of limiting dietary sulfur amino acids and of choline.

The possibility of a toxic factor has not been overlooked, and approaches to answer both the question of identity of the material and its pathogenesis are now underway in our laboratories.

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PLATE 1

EXPLANATION OF FIGURE

1 Section of kidney from rat fed low methionine-low choline diet without vitamin B_{12} for 6 months. Lesions in tubules are visible at this relatively low magnification. The upper border of photograph is at the junction of the outer and inner cortex; the lower margin joins the medulla. The lesions encompass the entire inner cortical zone. Some tubules contain precipitated material in the lumen (arrows). H & E. × 89.

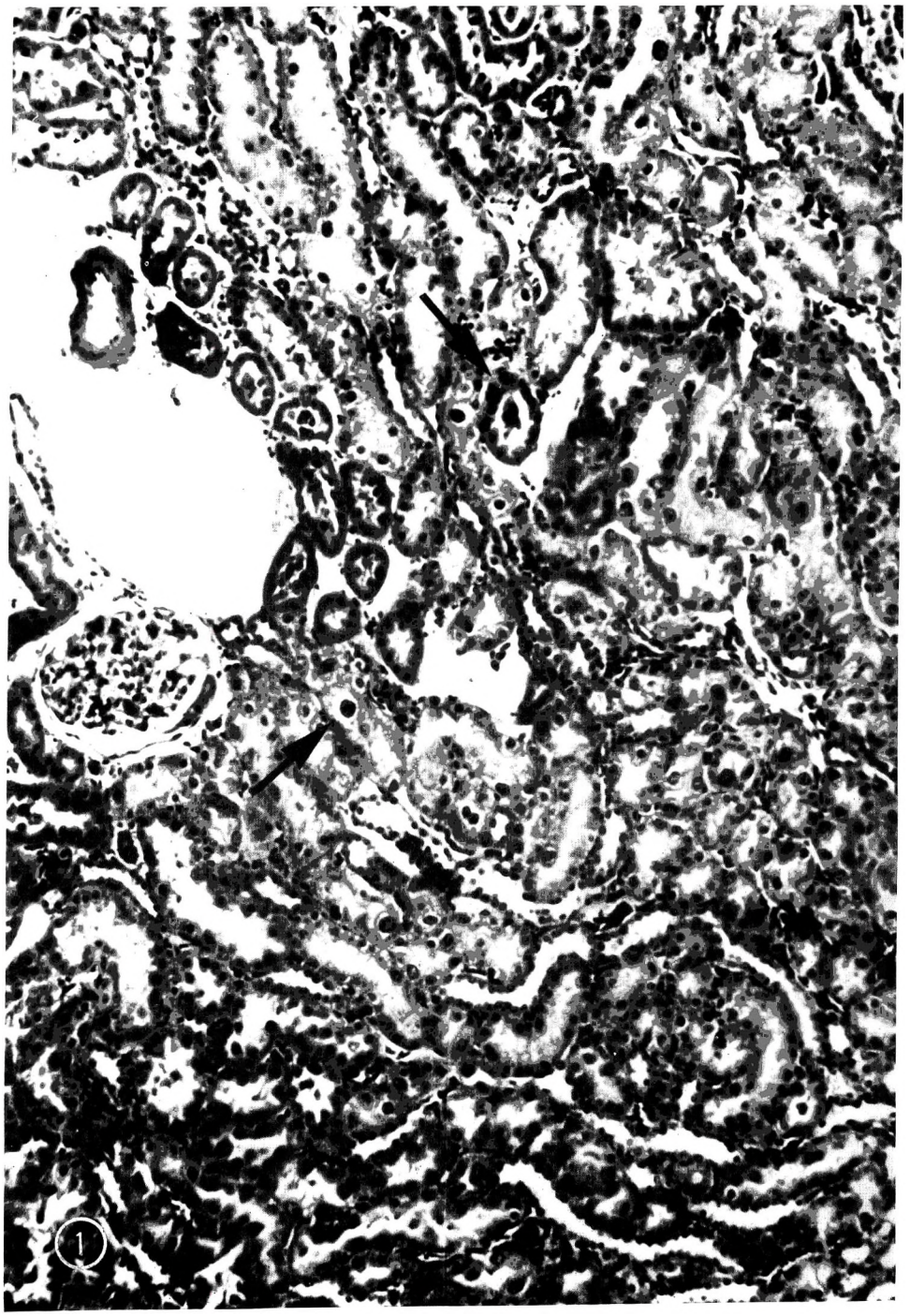
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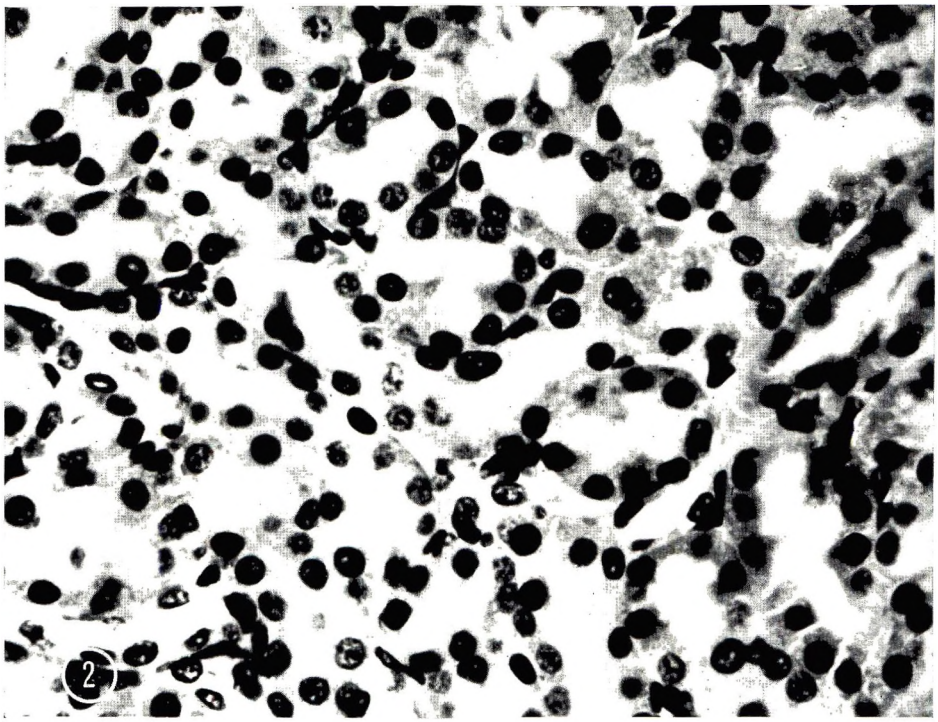
PLATE 2

EXPLANATION OF FIGURES

2 Section of kidney from rat fed casein control diet (no. 7) for 6 months. Tubular epithelium is normal. H & E. × 383.

3 Section of kidney from rat fed low methionine-low choline diet (no. 1) for 6 months. Note degenerative changes in tubular epithelium and bizarre shape of most of the nuclei. H & E. × 383.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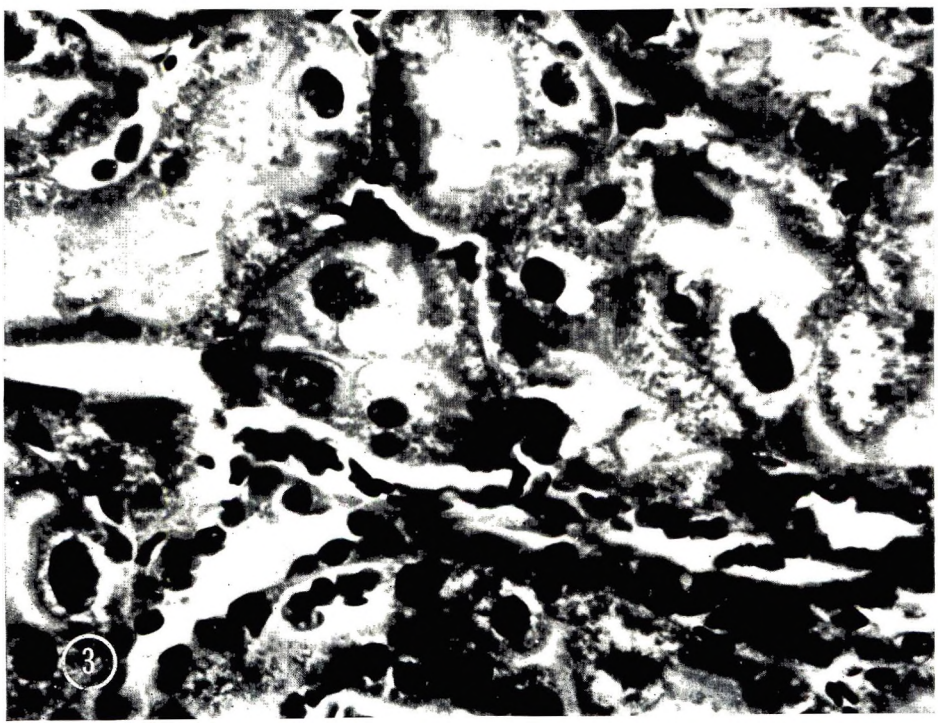
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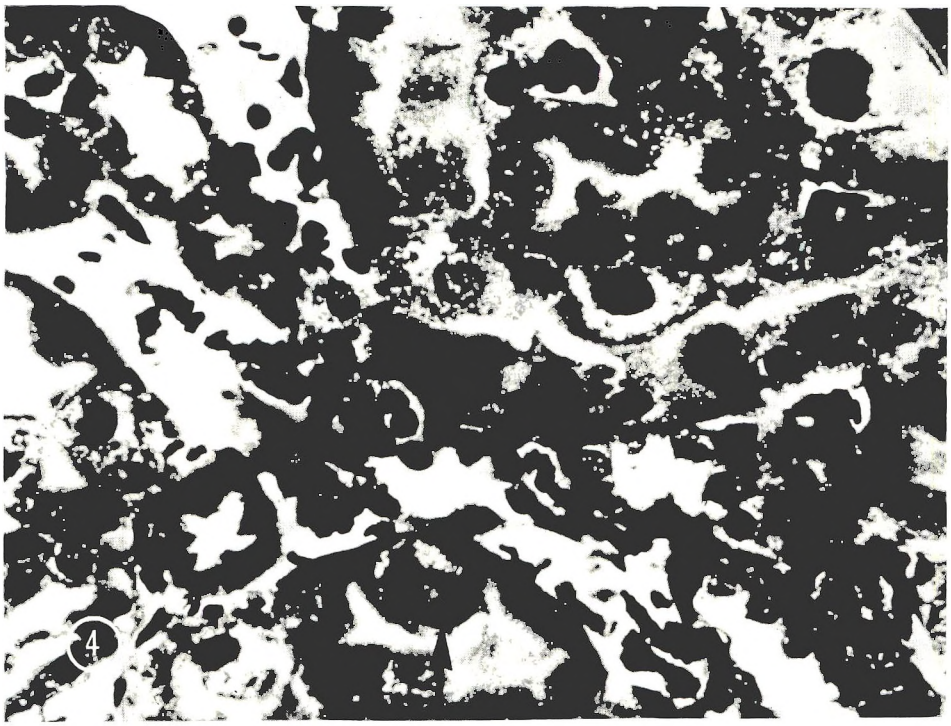
PLATE 3

EXPLANATION OF FIGURES

4 Section of kidney from rat fed low methionine-low choline diet plus vitamin B_{12} (no. 2) for 6 months. Lesions are identical to those seen in figure 3 except that one of the nuclei has an intranuclear inclusion-type structure (arrow). These structures were commonly seen in kidneys of all animals with the more generalized renal lesion. H & E. × 383.

5 Mucoid structure in bladder of rat fed low methionine-low choline diet (no. 1) for 6 months. Some areas are homogeneous while others are granular and vacuolated. Fragmentation, a finding common to mineralized calculi, did not occur as a sectioning artifact in structures seen in this study. H & E. × 71.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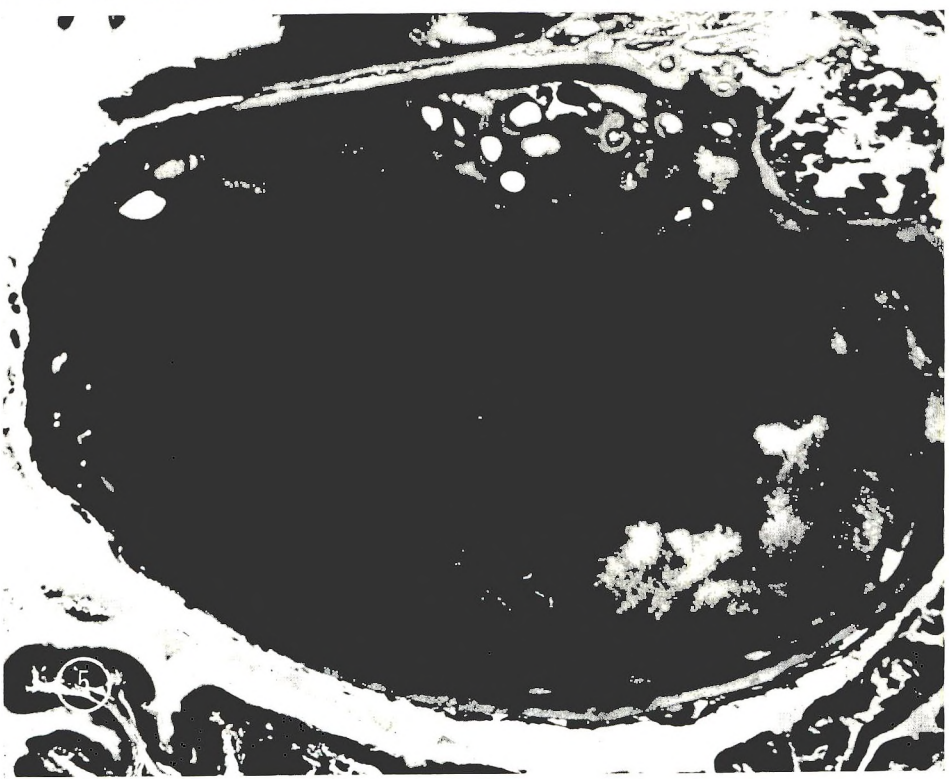
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Effect of Amino Acid Imbalance on the Fate of the Limiting Amino Acid [1]

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ABSTRACT The fate of the most limiting amino acid (i.e., tracer quantities of threonine-U-^{14}C or histidine-U-^{14}C) was studied in rats fed a single meal of a low protein diet in which an amino acid imbalance had been created by the addition of an amino acid mixture devoid of threonine or histidine. The amino acid imbalances did not increase the rate of oxidation of labeled amino acids as indicated by measurement of radioactivity in expired carbon dioxide; they did not result in enhanced excretion of radioactivity in urine or feces; nor did they result in enhanced incorporation of radioactivity into liver glycogen or fat. The accumulated evidence indicated that amino acid incorporation into liver proteins was enhanced by the amino acid imbalances. Incorporation into other tissue proteins was not depressed, and total retention of label in carcass was slightly greater as a result of the imbalance. On the basis of these and other observations, a hypothesis was formulated to explain the effects of amino acid imbalances. It is suggested that an imbalance leads to more efficient incorporation of the growth-limiting amino acid into tissues with the result that its concentration in blood plasma decreases within a few hours after ingestion of the imbalanced meal. This phenomenon, which resembles the response of an animal to a severely deficient diet — a protective response — results in a signal to an appetite-regulating center indicating that the diet is much more deficient than it actually is. Food intake is subsequently depressed and the food intake depression results in retarded growth.

Rats fed a low protein diet to which has been added a mixture of all but one of the indispensable amino acids gain less weight than control rats fed the low protein diet alone. The growth retardation is attributed to an amino acid imbalance and can be prevented by a small supplement of the amino acid missing from the mixture. Although considerable information about the nutritional effects of amino acid imbalances has accumulated (1), the metabolic events responsible for these effects are not known.

Ten years ago, Salmon (2, 3) postulated that the addition of an incomplete mixture of amino acids to a low protein diet might increase the catabolism of all amino acids and, hence, result in increased destruction of the amino acid in shortest supply in the diet (limiting amino acid). To test this hypothesis which would explain the growth-depressing effect of an amino acid imbalance, 2 groups of workers studied the metabolism of isotopically labeled tryptophan in rats fed a low protein diet lacking niacin and to which threonine had been added to create an amino acid imbalance. Florentino and Pearson (4) obtained results supporting the hypothesis, whereas Wilson et al. (5) found no indication of increased catabolism of tryptophan, the limiting amino acid in the diet used. The rats used in these experiments had been fed the imbalanced diet for a week or more and showed a depressed rate of growth. As the growth depression is mainly the result of low food intake (6–9), it is difficult in experiments conducted under such conditions to distinguish between metabolic changes due directly to the amino acid imbalance and those due to low food intake. The initial metabolic effects of an amino acid imbalance should be detectable before, or at least at the same time as, the food intake of the experimental animals is affected; hence experiments of relatively short duration appear to be needed to resolve the dilemma posed by these disparate results.

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A decrease in the concentration of the limiting amino acid in blood plasma is one of the earliest biochemical changes observed in rats fed an amino acid-imbalanced diet (10, 11), and the possibility of a close relationship between the regulation of food intake and plasma amino acid pattern has been suggested (9, 12, 13). Increased catabolism of the limiting amino acid could account for this type of change in the plasma amino acid pattern. In addition, the extra amino acids added to create an imbalance might interfere with absorption of the limiting amino acid from the gastrointestinal tract, with uptake of the limiting amino acid from body fluids into tissues, or with synthesis of protein in the tissues.

In an effort to determine the metabolic basis for the gross effects of an amino acid imbalance and for the reduction in the concentration of the most limiting amino acid in blood plasma, the metabolic fate of ^{14}C-labeled limiting amino acids was studied in rats immediately after they had ingested equal quantities of a balanced or an imbalanced diet.

EXPERIMENTAL PROCEDURE

Young adult male rats of the Sprague-Dawley strain were trained to consume about 10 g of a 15% casein agar-gel diet within 2 hours by feeding them for only 2 to 3 hours daily for about 3 weeks.

Two amino acid imbalances were studied: a threonine imbalance, and a histidine imbalance. These imbalances were produced by adding a mixture of indispensable amino acids lacking either threonine or histidine to a low protein basal diet. The basal diet used in the study of the threonine imbalance contained: (in per cent) casein, 6; L-methionine, 0.3; vitamin mixture in sucrose (14), 0.5; salt mixture (14), 5; corn oil, 5; choline·Cl, 0.2; sucrose, 28; and dextrin, 55. To prepare the threonine-imbalanced diet, 10% of the carbohydrate in the basal diet was replaced by an amino acid mixture lacking threonine. The amounts of individual amino acids added were as follows: (in per cent) L-leucine, 1.67; L-isoleucine, 1.26; L-valine, 1.26; L-histidine·HCl, 0.73; L-phenylalanine, 1.67; L-lysine·HCl, 2.60; L-tryptophan, 0.35; and L-methionine, 0.47. The basal diet used in the study of the histidine imbalance was similar except that the dietary level of casein was reduced to 5%, and 0.2% of L-threonine was added to ensure that this amino acid would not be limiting. To create a histidine imbalance, 5.4% of an amino acid mixture lacking histidine was added to the basal diet in place of carbohydrate. The amounts of individual amino acids added were as follows: (in per cent) L-methionine, 0.3; L-phenylalanine, 0.9; L-leucine, 0.9; L-isoleucine, 0.6; L-valine, 0.6; L-lysine·HCl, 0.9; L-arginine·HCl, 0.6; L-threonine, 0.45; and L-tryptophan, 0.15.

These amino acid mixtures were known from previous work (1, 9, 15,) to cause amino acid imbalances that resulted in growth depressions in rats fed ad libitum. The growth depression caused by the amino acid mixture lacking histidine is prevented by a supplement of histidine; that caused by the amino acid mixture lacking threonine is prevented by a supplement of threonine.

Each diet was dispersed in an equal amount of hot water containing 3% of agar and upon cooling the mixture set as a gel. Threonine-U-^{14}C (6.2 μg) was incorporated into the threonine-imbalanced diet and histidine-U-^{14}C (5.4 μg) was incorporated into the histidine-imbalanced diet such that each rat ingested about 8.3 μc. The quantities of the limiting amino acids added were insufficient to affect growth. Although the diets contained about 50% of water, food intakes are reported on a dry-weight basis. The control diets in these studies contained about half as much nitrogen as the experimental diets; hence, in essence, what is being studied is the effect of a surplus of all of the indispensable amino acids except one on the metabolic fate of the indispensable amino acid that is limiting for growth. The control and experimental diets contained the same amount of the limiting amino acid.

For the collection of carbon dioxide, animals were placed in glass metabolism cages through which dry, carbon dioxide-free air was passed. The expired carbon dioxide was collected in columns containing a mixture of ethanolamine and ethyleneglycol monomethylether (1:2, v/v), and the radioactivity was determined according to the method of Jeffay and Alvarez (16).

All of the samples were counted in a Packard liquid scintillation spectrometer. An internal standard of ^{14}C-toluene was used to determine the disintegrations per minute (dpm).

Spilled food was suspended in 1 N HCl, heated overnight, filtered and an aliquot was used for the determination of radioactivity with aqueous scintillation solution I (17) after neutralizing and decolorizing a 2-ml aliquot with 0.2 ml of 30% hydrogen peroxide for 4 to 6 hours at 100°. Recoveries of radioactivity from protein-^{14}C and threonine-^{14}C using this procedure were 97% and 85%, respectively. Urine was diluted to constant volume, filtered and a 2-ml aliquot was also decolorized and counted as described above.

Feces were homogenized in a mortar with water, made to volume and centrifuged, and 2 ml of the supernatant fluid was counted as described for urine.

In some experiments 0.1 ml of blood was collected hourly from the tail vein, mixed with 5 ml of cold 5% trichloroacetic acid and centrifuged, and 2 ml of the supernatant fluid was used for the determination of radioactivity.

Before the rats were killed, they were anesthetized with ether; blood was taken by heart puncture. Then the gastrointestinal tracts, livers, kidneys and left gastrocnemius muscles were taken out rapidly, frozen, and stored at −29° until analysis was performed. The remainder of each carcass was dissolved in about 600 ml of NaOH in methanol (160 g/liter) and heated at 95° for 2 to 4 hours. The solution was diluted to 1 liter with water and an aliquot taken, neutralized, and aqueous scintillation solution added and the sample counted.

Gastrointestinal contents were washed out with water, dissolved in dilute alkali, neutralized and centrifuged; an aliquot was decolorized and counted as described above. Portions of liver and kidney homogenates were diluted with 2 N NaOH, heated, then neutralized and the radioactivity of the whole tissue was determined as described above. Tissues were homogenized in water, and acid-soluble and protein fractions were obtained acccording to the method of Hutchinson and Munro (18). Protein fractions were dissolved in 2 N NaOH, and a 1-ml aliquot containing about 10 mg of protein was transferred to a counting vial and partially hydrolyzed by heating at 95° for 24 hours. The solution was then neutralized with HCl and mixed with the aqueous scintillation solution and counted. The addition of 1 to 2 ml of absolute ethanol to this counting mixture was necessary to prevent the separation of the aqueous phase from the organic solvent phase. The ethanol also minimized the formation of precipitates.

Another aliquot of the protein solution in 2 N NaOH was used for the determination of nitrogen by the Kjeldahl method. From these data the specific activity of the protein was calculated. For some samples, isolated protein was suspended in methanol, and a portion of it was transferred to a tared counting vial, and the weight determined directly. The nitrogen content of tissue homogenates was also determined by the Kjeldahl method. In some of the samples radioactivity of liver glycogen and liver lipids was measured. To prepare the glycogen a portion of fresh liver was dissolved in 30% KOH, and glycogen was precipitated with ethanol; the precipitate was dissolved in 5% trichloroacetic acid, and the contaminating protein was removed by centrifugation. Glycogen was again precipitated with ethanol, hydrolyzed with dilute HCl, neutralized and counted in aqueous scintillation solution. Glycogen content was determined by a colorimetric method using phenol and sulfuric acid (19). Liver lipids were extracted with diethylether from the dried, powdered, liver homogenates. The lipids were dissolved in petroleum ether, washed with distilled water and counted in toluene containing 0.4% 2,5-diphenyloxazole (PPO).

Uniformly labeled L-threonine and L-histidine were purchased from the New England Nuclear Corporation which had tested them for purity by chromatography in 4 solvent systems and guaranteed impurities of less than 2%. They were used as received. Later, both amino acids were tested for purity in our laboratory by placing a sample in a Technicon Amino Acid Analyzer and passing the effluent from the column through the flow cell of a Nuclear Chicago scintillation spectrometer. For labeled threonine 96 to 98% of the radioactivity

coincided with the standard threonine. For the labeled histidine 75% of the radioactivity coincided with the standard histidine. The rest of the radioactivity was scattered along the chromatogram in small peaks which were apparently breakdown products of histidine. Histidine in very small quantities is known to be light-sensitive. Not more than 4% of the contamination corresponded with peaks for known standard amino acids. All results are based on the amounts of radioactivity absorbed by individual rats.

RESULTS

Shown in figure 1 is the percentage of radioactivity expired as carbon dioxide by animals fed 7 g of either the control diet (4 rats) or the threonine-imbalanced diet (6 rats) containing threonine-U-^{14}C. Carbon dioxide was collected hourly for the first 8 hours; then at 4-hour intervals to the end of 24 hours, and finally at 6-hour intervals until 48 hours. At 24 hours the animals were again fed 7 g of their respective diets, but without threonine-U-^{14}C. Animals fed the control diet expired somewhat more radioactivity in CO_2 than did those of the imbalanced group over the 48 hours of the experimental period, but the difference between the 2 groups was not statistically significant. During the second 24 hours the curves for cumulative radioactivity in CO_2 expired by the 2 groups were almost parallel.

The amounts of radioactivity excreted in the urine and feces of the imbalanced group did not exceed those for the control group (table 1) so that, overall, no more ^{14}C was eliminated by the threonine-imbalanced group than by the control group. The livers of rats fed the threonine-imbalanced diet contained more radioactivity from threonine-U-^{14}C than did those of the control group, and more ^{14}C was also retained in the carcasses of rats fed the imbalanced diet.

To test the effect of dietary amino acid imbalance on the absorption of the first-limiting amino acid, gastrointestinal contents were collected and their radioactivity determined at 3.5 and 8 hours after feeding control and imbalanced diets containing ^{14}C-labeled threonine or histidine. Animals were fed 3 g of diet in the experiment on the histidine imbalance. Absorption was calculated as the difference between the amount of radioactivity ingested and that recovered from the gastrointestinal tract (table 2). By 3.5 hours after the feeding period 65% and 59% of the ingested radioactivity had been absorbed from the intestinal tracts of the control and threonine-imbalanced groups, respectively. The difference between the 2 groups was not statistically significant. By 8 hours 97% of the ingested radioactivity had been absorbed by the animals of the control group and 95% by those of the imbalanced group.

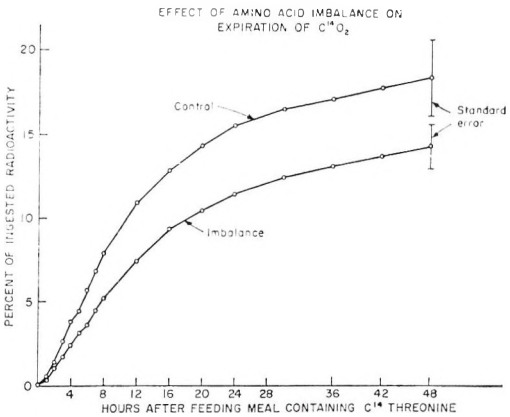

Fig. 1 Expiration of $^{14}CO_2$ after feeding diets containing threonine-U-^{14}C. Rats were fed 7 g of either the control diet or threonine-imbalanced diet containing threonine-U-^{14}C. After 24 hours, each rat was fed 7 g of the same diet without threonine-U-^{14}C. Each point represents the average of 4 rats for the control group and 6 rats for the imbalanced group. Standard errors of the mean are shown at 48 hours.

TABLE 1

Distribution of radioactivity at 48 hours after feeding diets containing threonine-U-^{14}C [1]

	% of ingested ^{14}C	
	Control	Imbalanced
	%	%
Expired CO_2	18.4 ±2.3 [2]	14.3 ±1.3
Urine	2.08±0.25	2.20±0.17
Feces [3]	1.65±0.17	1.25±0.20
Liver	5.87±0.29	7.24±0.26
Carcass	70.1 ±1.87	74.6 ±2.37
Recovery	98.1 ±1.27	99.5 ±2.16

[1] Rats were fed 7 g of either the control or threonine-imbalanced diet containing 8.3 μc of threonine-U-^{14}C. After 24 hours, animals were fed 7 g of the same diet without threonine-U-^{14}C.
[2] Mean ± SE of mean.
[3] Water-soluble portion of feces.

TABLE 2

Percentage absorption [1] of threonine-U-^{14}C or histidine-U-^{14}C after feeding the threonine- or histidine-imbalanced diet

Hours after feeding diet	Threonine-U-^{14}C		Histidine-U-^{14}C	
	Control	Imbalanced	Control	Imbalanced
3.5	64.6 ± 5.79 [2]	58.9 ± 2.84	—	—
8	97.4 ± 0.72	95.7 ± 0.83	92.1 ± 1.89	89.3 ± 1.55

[1] % absorption = $\frac{\text{radioactivity ingested} - \text{radioactivity in g.i. contents}}{\text{radioactivity ingested}} \times 100$.

[2] Mean ± SE of mean.

In the experiment on histidine imbalance 92% of the ingested radioactivity was absorbed within 8 hours by animals fed the control diet and 89% by those fed the imbalanced diet. Thus, amino acid mixtures lacking either threonine or histidine did not appear to affect the extent of absorption, respectively, of threonine and histidine, the limiting amino acids.

A statistically significant difference ($P < 0.05$) was observed between the amounts of radioactivity remaining in the TCA-soluble fraction of blood from control and imbalanced groups 8 hours after the feeding period (fig. 2). A time study was carried out only for the threonine imbalance. In one experiment blood was collected from each group of rats at 0.5, 1.5, 2.5 and 3.5 hours; in another experiment at 8 hours. In the experiment on histidine imbalance, blood was collected only at 8 hours. After feeding the diets containing threonine-^{14}C, the TCA-soluble fraction of blood from animals fed the threonine-imbalanced diet contained less radioactivity at 3.5 hours, and by 8 hours the difference was highly significant. A significant difference was also observed at 8 hours between the experimental and control groups in the study of histidine imbalance. Thus, absorbed threonine-U-^{14}C and histidine-U-^{14}C disappeared more rapidly from the peripheral circulation when animals were fed the threonine- or histidine-imbalanced diets.

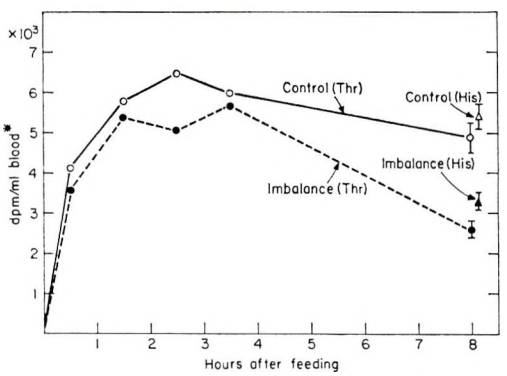

Fig. 2 Effect of amino acid imbalance on disappearance of radioactivity from the acid-soluble fraction of blood. Plasma was used in the histidine study. Differences at 8 hours were significant in both experiments ($P < 0.05$).

The values for total protein content of the livers of animals fed the threonine- and histidine-imbalanced diets were slightly higher than those for their respective controls, but the differences were not significant (table 3.). Values for the percentage of absorbed radioactivity incorporated into liver were significantly higher for both threonine- and histidine-imbalanced groups than for the respective control groups, except for the experiment terminated at 3.5 hours (table 4). The greatest difference was observed in the experiment on histidine imbalance in which the livers of the control group contained 11.2% of absorbed radioactivity, whereas those of the imbalanced group contained 16.1%. Values for the percentage of ingested radioactivity in the TCA-soluble fraction of liver were similar for the control and the threonine-imbalanced groups; but in the experiment on histidine imbalance, the value of 1.56% for the control group was distinctly higher than that of 0.87% for the imbalanced group.

The percentage of ingested radioactivity associated with the total liver protein was significantly higher for the histidine-imbalanced group as also was the specific activity of the liver protein. Similar results were obtained in the experiment on threonine imbalance, but the differences were smaller, and no difference was observed at

TABLE 3

Composition of liver

Hours after feeding ^{14}C-containing diets	Type of diet	Body wt	Liver wt	Total protein	
		g	*g*	*mg/liver*	*%*
		Threonine imbalance			
3.5	Control	196 ± 10.0 [1]	6.1 ± 0.2	1250 ± 78	20.6 ± 0.8
	Imbalanced	201 ± 7.6	6.7 ± 0.4	1396 ± 60	21.0 ± 0.5
8	Control	129 ± 2.4	4.5 ± 0.1	814 ± 19	18.2 ± 0.5
	Imbalanced	132 ± 1.5	4.6 ± 0.2	892 ± 40	19.4 ± 0.7
48	Control	235 ± 2.8	5.6 ± 0.3	1172 ± 89	20.0 ± 0.8
	Imbalanced	230 ± 4.8	6.6 ± 0.3	1256 ± 59	18.8 ± 0.5
		Histidine imbalance			
8	Control	137.8 ± 5.9	6.3 ± 0.16	909 ± 28	14.4 ± 0.33
	Imbalanced	142.2 ± 6.6	6.3 ± 0.35	949 ± 48	15.1 ± 0.17

[1] Mean ± SE of mean.

TABLE 4

Distribution of radioactivity in liver

Hours after feeding ^{14}C-containing diet	Type of diet	% of absorbed ^{14}C			Specific activity of protein [1]
		Liver	Acid-soluble fraction	Protein	
		%	%	%	
		Threonine imbalance			
3.5	Control	5.73 ± 0.34 [2]	1.02 ± 0.08	3.78 ± 0.34	323 ± 27
	Imbalanced	5.84 ± 0.48	1.23 ± 0.46	3.83 ± 0.42	291 ± 25
8	Control	5.64 ± 0.26 [a]	0.85 ± 0.11	4.39 ± 0.17	926 ± 38
	Imbalanced	6.29 ± 0.10	0.77 ± 0.14	5.14 ± 0.37	989 ± 41
48	Control	5.87 ± 0.29 [a]	0.30 ± 0.03	5.04 ± 0.31	667 ± 41
	Imbalanced	7.24 ± 0.26	0.36 ± 0.03	6.18 ± 0.48	788 ± 49
		Histidine imbalance			
8	Control	11.2 ± 0.64 [b]	1.56 ± 0.18 [b]	10.3 ± 0.45 [b]	1638 ± 69 [b]
	Imbalanced	16.1 ± 0.62	0.87 ± 0.076	15.9 ± 0.44	2402 ± 115

[1] Specific activity = dpm/mg liver protein $\times \frac{\text{mean dpm absorbed of control and imbalanced groups}}{\text{dpm absorbed for each rat}}$.

[2] Mean ± SE of mean.

[a] Difference between control and imbalance is statistically significant ($P < 0.05$).

[b] Difference between control and imbalance is statistically significant ($P < 0.01$).

the 3.5 hour interval. The radioactivity of liver glycogen and liver lipids from rats used in the study of histidine imbalance was measured. Liver glycogen and liver lipids from the control group contained 0.15% and 0.073%, respectively, of absorbed radioactivity and values for the histidine-imbalanced group were, respectively, 0.12% and 0.055%. Differences between the control and experimental groups were not statistically significant.

Radioactivity per unit weight of muscle tended to be low for the imbalanced groups up to 8 hours (table 5). In the histidine imbalance experiment the value of 59.2 dpm/mg muscle for the control group was significantly higher than that of 42.6 dpm /mg muscle for the imbalance group. The decrease appeared to be due to a decrease in the amount of radioactivity in the TCA-soluble fraction of the muscle since the specific activity of muscle protein at 8 hours

TABLE 5

Incorporation of threonine-^{14}C or histidine-^{14}C into muscle

Hours after feeding diets	Type of diet	Radioactivity		
		Whole muscle	TCA-soluble fraction	Muscle protein
		dpm/mg muscle	*dpm/mg muscle*	*dpm/mg protein*
		Threonine imbalance		
3.5	Control	28.3 ± 5.1 [1]	—	25.8 ± 2.9
	Imbalanced	20.7 ± 1.1	—	36.7 ± 1.3
8	Control	49.2 ± 2.6	17.9 ± 0.09 [a]	221 ± 5.5
	Imbalanced	44.8 ± 1.5	7.5 ± 0.07	230 ± 5.9
48	Control	38.8 ± 3.3	—	—
	Imbalanced	45.8 ± 3.2	—	—
		Histidine imbalance		
8	Control	59.2 ± 3.7 [a]	36.9 ± 1.4 [a]	177 ± 8.9
	Imbalanced	42.6 ± 2.2	12.6 ± 0.53	175 ± 9.0

[1] Mean ± SE of mean.
[a] Difference between 2 groups is significant ($P < 0.01$).

TABLE 6

Incorporation of histidine-U-^{14}C or threonine-U-^{14}C into kidney and intestine

Hours after feeding diet	Type of diet	Kidney			Intestine
		Wet wt	Acid-soluble fraction	Protein	Protein
		g	*dpm/mg tissue*	*dpm/mg protein*	*dpm/mg protein*
		Threonine imbalance			
8	Control	0.84 ± 0.03 [1]	49.0 ± 1.6 [a]	846 ± 29	2226 ± 156
	Imbalanced	0.85 ± 0.02	40.4 ± 1.1	787 ± 23	2376 ± 247
		Histidine imbalance			
8	Control	1.04 ± 0.04	55.9 ± 1.8	1192 ± 33 [a]	2412 ± 399
	Imbalanced	1.07 ± 0.05	59.0 ± 2.5	1588 ± 86	2724 ± 120

[1] Mean ± SE of mean.
[a] Difference between 2 groups is statistically significant ($P < 0.01$).

was the same for the control and imbalance groups in both the threonine imbalance and histidine imbalance studies. At 3.5 hours in the threonine study, however, the value for the imbalance group was higher.

Analyses of kidney and intestine were carried out 8 hours after feeding diets containing either threonine-U-^{14}C or histidine-U-^{14}C (table 6). In the threonine-imbalance study the value for radioactivity in the TCA-soluble fraction and in the protein of kidney was lower for the imbalanced group than for the control; but in the histidine-imbalance study the values for the imbalance group were higher than the control values. The specific activity of kidney protein from the histidine-imbalanced group was significantly higher than that for the control ($P < 0.01$). The specific activity of intestinal protein tended to be higher in the imbalanced groups in both studies, but in neither study were the differences statistically significant (table 6).

DISCUSSION

From these and previous results we have developed a hypothesis concerning the sequence of events which leads to depressed growth and food intake in rats ingesting a diet in which an amino acid imbalance has been created. The imbalanced meal is evidently digested and absorbed normally, and this results in the flow of a surplus of all but one of the indispensable amino acids to the liver where protein synthesis is stimulated. More of the amino acid in short

supply is incorporated into liver proteins and hence the supply for peripheral tissues is reduced. It is not, however, reduced to the point where protein synthesis is suppressed by low substrate concentration because incorporation into muscle proteins apparently continues at the same rate as in the control animals (table 5). This eventually results in the muscle and plasma free amino acid patterns becoming severely unbalanced, and the patterns then resemble those resulting from ingestion of a severely deficient diet; a homeostatic mechanism is triggered and food intake is depressed. As a consequence of this depression of food intake, the supply of amino acids for protein synthesis is reduced, and the growth of the animal is retarded.

Incontrovertible evidence for the validity of some aspects of this hypothesis has been difficult to obtain. However, some additional indirect evidence from other sources tends to support it, and on the basis of evidence obtained so far, some alternative hypotheses can be eliminated.

The observations on the metabolism of threonine-U-^{14}C (fig. 1 and table 1) indicate that an amino acid imbalance created by adding a surplus of all but one of the indispensable amino acids to a low protein diet does not increase the catabolism of the limiting amino acid as suggested earlier (2, 3,). Actually, the amounts of ^{14}C in carbon dioxide, glycogen, lipids, urine and feces were somewhat lower for the group fed the imbalanced diet. The results of carcass and liver analyses confirmed that most of the ^{14}C had been retained in the body. If, as proposed above, a greater amount of the most limiting amino acid is removed from the circulation because of increased incorporation into proteins, a reduction in the amounts of radioactivity in carbon dioxide, urine, feces, glycogen and fat would be expected. In a study of the effect of an amino acid imbalance involving histidine on the oxidation of histidine-U-^{14}C, less radioactivity was found in CO_2 expired by rats fed the imbalanced diet.[3]

Competition for transport sites has been suggested as a possible explanation of the effects of amino acid imbalance (20). The rates of absorption of threonine-U-^{14}C and histidine-U-^{14}C from the intestine were not lower for the imbalanced groups than for the control groups (table 2). An amino acid imbalance, therefore, does not appear to affect significantly gastrointestinal absorption of the limiting amino acid. This may well be due to the large capacity of the intestine to absorb amino acids (21). Evidence that competition among amino acids during transport into cells does not occur as a result of feeding an imbalanced diet has been presented recently (22).

The observations on the incorporation of ^{14}C-labeled amino acids indicated that the amount of radioactivity incorporated into liver proteins of rats fed the imbalanced diets was greater than for controls, but the differences were not consistently significant. It takes only a small increase in body proteins to account on a quantitative basis for the decrease in the concentration of the growth-limiting amino acid in both muscle and plasma. In the first place the plasma pool represents only a small portion of the total amount of amino acids in the body (22). A small increase in incorporation into the proteins or pools of one or more large organs or tissues or a small decrease in the rate of protein breakdown could cause a substantial change in the amount of an amino acid circulating in blood plasma. An estimate of the total decrease in plasma and muscle free threonine made in another experiment amounted to 37 μmoles (22). A 12% increase (about 100 mg) in liver proteins or a 0.5% increase in total body proteins would account for a decrease of this size. Such changes could well be within the limits of accuracy of measurements made on intact animals owing to the variability among individuals. The development of techniques for the continuous monitoring of amino acid pools and amino acid incorporation into various tissues and organs in the living animal pose almost insuperable problems. The present experiments provide values only at specified time intervals and it must be assumed that the results indicate the true course of events.

In relation to the present results, Sidransky et al. (23–26) observed that liver protein content of rats force-fed a purified diet devoid of threonine was greater than that

[3] Dakshinamurti, K., and A. E. Harper, unpublished results.

of a control group, although muscle protein content was lower. Sanahuja (27) has recently reported higher protein content of the livers of rats fed an imbalanced diet. Sidransky has also reported that liver RNA increased in rats force-fed a diet devoid of threonine and that incroporation of ^{14}C-labeled amino acids into liver protein was enhanced in this condition (25, 26, 28).

At present it is not clear whether protein synthesis may be slightly enhanced in organs other than the liver after ingestion of an amino acid-imbalanced diet. The specific activity of muscle protein was almost the same for the control and imbalanced groups in both experiments despite the lower activity of the muscle TCA-soluble fraction for the imbalanced group. This would indicate that muscle protein synthesis is continuing steadily. The specific activity of kidney protein 8 hours after feeding the diet containing ^{14}C-threonine was slightly less for the imbalanced group, although the specific activity of kidney was clearly higher 8 hours after animals had ingested the histidine-imbalanced diet containing ^{14}C-histidine. The incorporation of the limiting amino acid into intestinal protein was also slightly higher. No evidence was obtained of a substantial depression of incorporation of labeled amino acid, and even if incorporation is unaffected in most organs and accelerated in only a few, the plasma concentration of the most limiting amino acid would decrease.

Quite apart from enhanced incorporation, a lowering of the plama concentration of the limiting amino acid could occur after ingestion of an imbalanced diet, if the imbalanced amino acid mixture decreased the rate of breakdown of body proteins. This mechanism is important in relation to metabolic adaptations of mammalian enzymes (29, 30). In the present study, such an effect could not be distinguished from increased protein synthesis.

It must be kept in mind that despite the apparent increase in protein synthesis after feeding a meal of an imbalanced diet, within a short time (a few hours) an amino acid imbalance results in depressed food intake, depressed growth and, therefore, in reduced overall protein synthesis. Henderson et al. (31) suggested that the increased niacin or tryptophan requirement of rats fed a low protein diet lacking niacin and supplemented with tryptophan might be due to accelerated protein synthesis. This would deplete the supply of tryptophan available for niacin synthesis and lead to the development of niacin deficiency. The above situation is a special case of amino acid imbalance in which an interrelationship with a vitamin is involved, but there is considerable similarity to the imbalances discussed in this paper. Henderson's idea was not generally accepted because the postulation of enhanced protein synthesis appeared to be incompatible with the observed reduction in the growth of rats fed the imbalanced diet.

If food intake can be maintained as by insulin injection (32), exposure to a cold environment (33), cortisol injection,[4] or forced-feeding,[5] no adverse effects of an amino acid imbalance are observed. When rats were fed an imbalanced diet after being protein-depleted, they gained weight a little more rapidly for a few days than rats similarly treated but fed the control diet (12). Also when food intake was stimulated by cold exposure in our laboratory, rats fed imbalanced diets gained somewhat more than those fed the control diet (15). Amino acid imbalances are frequently observed when relatively small amounts (less than 1%) of the second most limiting amino acid or acids are added to a diet. The effect is out of proportion to what would be anticipated. Such quantities are far below those tolerated readily if the amino acid is not second most limiting. If enhanced incorporation of the limiting amino acid is the basis for the changes in blood amino acid pattern as a result of feeding an imbalanced diet, then simply by mass action, provision of an excess of the second most limiting amino acid should lead to more efficient utilization of the most limiting one.

All of these observations indicate that, provided food intake can be maintained, the limiting amino acid in an imbalanced diet is used as efficiently, if not more efficiently, than the same amino acid in an appropriate control diet. These observations,

[4] Leung, P. M-B., Q. R. Rogers and A. E. Harper 1964 Effect of amino acid imbalance on food intake and preference. Federation Proc., 23: 185 (abstract).
[5] See footnote 4.

thus, lend support to the idea that the alteration in blood amino acid pattern as a result of feeding an imbalanced diet is the result of more, rather than less, efficient utilization of the limiting amino acid. The link between the alteration in blood amino acid pattern and depressed food intake is not clear, but it is known that the blood change resembles that observed in animals fed a much more deficient diet, a diet that causes a severe depression in food intake and that, when force-fed, causes the development of pathologic lesions (23–26). Thus, the response to an imbalanced diet appears to be a protective response to a signal that normally arises only when a much more severely deficient diet is fed. Rats given a choice between an imbalanced diet and a protein-free diet will select the protein-free diet (12, 34) which will not support life but will restore the blood amino acid pattern to normal and reject the imbalanced diet which will support growth. These observations suggest that the changed blood amino acid pattern serves as a signal that activates an appetite-regulating mechanism or that the pattern is an indirect reflection of some more subtle change, possibly at sites of protein synthesis, that serves as a signal.

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Effect of Threonine on the Toxicity of Excess Tyrosine and Cataract Formation in the Rat [1,2]

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ABSTRACT Typical symptoms, i.e., eye and paw lesions, of tyrosine toxicity were found when 3% L-tyrosine was included in a 6% casein diet fed ad libitum or when 5% L-tyrosine was included in a 6% casein diet fed only 2 hours each day. The addition of 0.8 to 1.25% of L-threonine to the high tyrosine diet prevented the appearance of the eye and paw lesions and improved the growth. However, when the diet containing high tyrosine and high threonine was fed for over 2 months, cataracts were observed in 80% of the rats. Growth was improved and the cataracts were completely prevented by a supplement of 0.2% tryptophan; although the eye and paw lesions caused by the high tyrosine were still observed when 0.2% tryptophan was added to the 3% tyrosine diet. It appears that the addition of 1.25% L-threonine to the 3% tyrosine diet increases the tryptophan requirement.

Growth rates and food intake are depressed when rats are fed a low protein diet containing excess tyrosine. After about one week young rats fed such a diet develop eye and paw lesions. These effects of tyrosine are well documented (1–3).

Benton et al. (4) observed that the addition of 0.3% DL-threonine to a 9% casein diet containing 3% tyrosine, partially prevented the growth depression without having much effect on the severity of eye and paw lesions. Since threonine was the most limiting amino acid in the casein diet supplemented with methionine and tryptophan used by Benton et al., Becker [3] investigated the effect of including threonine with 3% of tyrosine in a diet that contained fibrin as the protein source. Threonine is the least limiting amino acid in fibrin. Tyrosine retarded the growth of rats fed the 6% fibrin diet and caused eye and paw lesions. Addition of 0.4% DL-threonine improved growth slightly and caused a partial improvement of eye and paw lesions.

The interrelationship between threonine and tyrosine was therefore investigated in greater detail. Experiments were conducted to establish more clearly the extent to which threonine alleviates tyrosine toxicity. During the course of the feeding studies a more complex interrelationship involving tryptophan as well as threonine and tyrosine was discovered.

EXPERIMENTAL

Rats. Young male rats were used in all experiments. The weight ranges and strains are recorded with the results of each experiment. The rats were housed in individual suspended cages. Food and water were supplied ad libitum except in the studies on rats trained to eat during a single 2-hour period.

Interval feeding. Rats were allowed to adjust to the regimen of eating for only 2 hours daily by presenting the control diet to the rats for only 2 hours daily for a period of 2 to 3 weeks. Following this period of adjustment the rats were fed the experimental diets 2 hours each day. Water was supplied ad libitum.

Diets. In the first experiment, the results of which are shown in table 1, the diets were fed in dry form. In the remainder of the studies reported in this paper agar-gel diets, containing about 50% moisture, were used. The diets for the experiment in which fibrin was used differed from those used in the subsequent

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[2] Contribution no. 721 from the Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts.

[3] Becker, R. V., M.S. Thesis, University of Wisconsin, 1961.

TABLE 1

Effect of threonine on tyrosine toxicity at 2 levels of protein [1]

Diet			Wt gain, 14 days	Lesions, 14 days		
				Incidence	Severity score [2]	
Fibrin	L-Tyr	DL-Thr			Eye	Paw
%	%	%	g			
6	—	—	36 ± 1.7 [3]	0/5	0	0
6	3	—	10 ± 1.2	5/5	8.0	6.2
6	3	0.4	13 ± 1.0	5/5	3.6	3.2
6	3	0.8	13 ± 2.7	3/5	0.6	2.0
6	3	1.6	14 ± 1.3	3/5	0.6	0.6
9	—	—	60 ± 2.5	0/5	0	0
9	4.5	—	10 ± 1.7	5/5	7.2	8.4
9	4.5	0.4	22 ± 2.1	5/5	8.4	8.0
9	4.5	0.8	23 ± 1.4	5/5	8.0	3.6
9	4.5	1.6	24 ± 1.3	5/5	3.4	0.4

[1] In young male rats of the Holtzman strain weighing 55 to 60 g.
[2] Judged on a scale of zero to 10; see text.
[3] SE of mean.

experiments in that autoclaved starch [4] was used as the carbohydrate source and the vitamins [5] and mineral [6] mixtures (7) were slightly different. The composition of the casein basal diet was: (in per cent) casein, 6.0; DL-methionine, 0.2; salts (5),[7] 4.0; vitamin mixture (6),[8] 0.5; choline chloride, 0.2; corn oil,[9] 5.0; agar, 2.5; and dextrin,[10] 81.6. Other amino acids were added at the expense of carbohydrate. The diets were stored in a refrigerator at 4° throughout the experimental period.

To prepare the agar-gel diets the agar was dissolved separately in an amount of boiling distilled water equivalent to the weight of dry diet. All other ingredients less vitamin mixture and choline were well mixed in a Hobart mixer. Then the agar solution was added and mixing was continued until the material in the mixer had cooled to 60 to 70°. Next the vitamins and choline were added and the contents mixed again. The diet was then poured into polyethylene or aluminum trays, allowed to gel and stored in a refrigerator at 4° until it was used.

The diet remained in a firm gel at room temperature and was fed by placing cubes of the diet in the cage daily.

Eye and paw lesions. Observations were made on the severity of eye and paw lesions that developed in rats fed excess tyrosine. Generally these appeared within 5 to 8 days and were at the peak of their severity about 2 weeks after initiation of the high tyrosine feeding. After this time the eye lesions started to heal. Therefore the severity of the lesions was recorded after 2 weeks. A score of zero to 10 was used to describe the severity. The 2 sets of observations on lesions were made by different individuals; hence, although the comparisons within experiments are uniform, those among experiments are not necessarily directly comparable because of the subjective nature of the scoring procedure.

RESULTS

In the initial experiment the effects on tyrosine toxicity of higher dietary levels of DL-threonine (0.4, 0.8 and 1.6%) than were tested by Benton et al. (4) were determined using rats fed 2 levels of fibrin. The tyrosine was fed at levels proportional to the protein level. The growth of rats

[4] Moist starch heated in an autoclave at 121° for 2 to 3 hours, then dried and ground.
[5] The vitamin mix provided in mg/100 g diet: thiamine·HCl, 0.5; riboflavin, 0.5; niacin, 2.5; Ca pantothenate, 2.0; pyridoxine·HCl, 0.25; folic acid, 0.02; menadione, 0.05; biotin, 0.01; vitamin B_{12}, 0.002; inositol, 10.0; and ascorbic acid, 5.0; α-tocopherol, 10; vitamin A, 400 units; and vitamin D_3, 200 units.
[6] The salt mix provided in % of diet: Ca, 0.592; P, 0.394; K, 0.493; Na, 0.493; Cl, 0.760; Mg, 0.049; Fe, 0.0049; Cu, 0.00195; Mn, 0.00195; Zn, 0.0004; I, 0.000019; and Mo, 0.0000095.
[7] Wesson salt mixture contains in %: $CaCO_3$, 21.000; $CuSO_4 \cdot 5H_2O$, 0.039; $FePO_4$, 1.470; $MnSO_4$, 0.020; $MgSO_4$, 9.000; $KAl(SO_4)_2$, 0.009; KCl, 12.000; KH_2PO_4, 31.000; KI, 0.005; NaCl, 10.500; NaF, 0.057; and $Ca_3(PO_4)_2$, 14.900.
[8] The vitamin mix provided in mg/100 g diet: thiamine·HCl, 0.5; riboflavin, 0.5; niacinamide, 2.5; Ca D-pantothenate, 2.0; pyridoxine·HCl, 0.5; folic acid, 0.05; menadione, 0.05; D-biotin, 0.02; vitamin B_{12}, 0.003; ascorbic acid, 5; vitamin E acetate, 10; vitamin A acetate, 380 units; and vitamin D_2, 38 units.
[9] Mazola, Corn Products Company, New York.
[10] Corn Products Company, New York.

fed 3% of tyrosine with 6% of fibrin or 4.5% of tyrosine with 9% of fibrin was greatly depressed. Both groups developed severe eye and paw lesions (table 1). Although the higher levels of threonine were no more effective than the 0.4% level in alleviating the growth depression, they were much more effective in alleviating the eye and paw lesions.

In another experiment of the same type (results not shown) when 2.5% of DL-threonine was included in a diet containing 6% of casein and 3% of tyrosine, only 3 of 15 rats developed mild eye and paw lesions, and growth was substantially improved. However, 5 out of 9 rats fed this diet for a prolonged period of time developed cataracts. This prompted another study of the interrelationship between threonine and tyrosine and the occurrence of cataracts in rats fed excess threonine and tyrosine using L- instead of DL-threonine.

Several amino acid deficiencies including that of tryptophan are known to cause cataracts in rats. Since tryptophan is just slightly less limiting than threonine in a 6% casein diet supplemented with additional methionine, the effect of a supplement of tryptophan on the development of cataracts was determined.

To study the effects of excess tyrosine alone and the combination of tyrosine and threonine, 10 rats per group were fed the diets listed in table 2, for 3 weeks. All of the animals in groups 2, 4, 5 and 6, and five each from the rest of the groups were then killed, and the remaining rats were maintained with the experimental diets for seven more weeks. At the end of 3 weeks, the group fed 3% tyrosine (group 3) showed a 77% growth depression which was partially alleviated by threonine (group 4, 6 and 8). Levels of 0.8% and 1.25% of L-threonine were equally effective in alleviating the growth depression caused by 3% of tyrosine even though these levels of threonine fed with the basal diet depressed growth. The addition of 0.2% L-tryptophan stimulated the growth of rats fed 1.25% L-threonine, with or without 3% tyrosine (groups 9 and 10). The tryptophan response was greater when the diet contained both tyrosine and threonine (group 10). In fact, rats fed this diet grew somewhat better than those fed the control diet without tyrosine (group 9). The results obtained with tryptophan are representative of two separate experiments.

The observations on the severity of the lesions are reported in table 3. They indicate that, although 0.2% of L-threonine was almost without effect on the eye and paw lesions, higher levels of L-threonine (0.8% and 1.25%) prevented their development. With prolonged feeding, however, 4 out of 5 rats in the group fed 1.25% threonine with 3% tyrosine developed cataracts (table 4). Also, 1 out of 5 rats developed cataracts in the group fed 1.25% L-threonine without additional tyrosine. None of the rats in the groups fed diets supplemented with 0.2% L-tryptophan developed cataracts during the 10-week period.

TABLE 2

Effect of L-*threonine and* L-*tryptophan on growth of rats fed 3%* L-*tyrosine* [1]

Group	Additions to 6% casein diet			Weight gain	
	L-Tyr	L-Thr	L-Try	3 weeks [2]	10 weeks [3]
	%	%	%	g	g
1	–	—	—	45.3 ± 2.7 [4]	160.2 ± 6.1
2	–	0.2	—	49.9 ± 2.8	—
3	3	—	—	10.4 ± 1.4	77.8 ± 20.0
4	3	0.2	—	20.3 ± 1.5	—
5	–	0.8	—	41.9 ± 2.8	—
6	3	0.8	—	27.5 ± 2.9	—
7	–	1.25	—	31.8 ± 2.1	153.0 ± 9.8
8	3	1.25	—	25.7 ± 2.0	138.0 ± 5.8
9	–	1.25	0.2	50.2 ± 3.2	199.2 ± 3.7
10	3	1.25	0.2	59.5 ± 2.3	219.6 ± 5.3

[1] Young male rats weighing 55 to 80 g of the Holtzman strain.
[2] Ten rats/group.
[3] Four or five rats/group.
[4] SE of mean.

TABLE 3

Effect of different levels of L-*threonine on eye and paw lesions of rats fed 3%* L-*tyrosine*

Group	Additions to 6% casein diet			Lesions		
	L-Tyr	L-Thr	L-Try	Incidence	Severity score [1]	
					Eye	Paw
	%	%	%			
1	–	—	—	0/10	0	0
3	3	—	—	10/10	1.9	5.3
4	3	0.2	—	8/10	3.4	4.1
6	3	0.8	—	1/10	0	1.0
8	3	1.25	—	1/10	1.0	0
10	3	1.25	0.2	0/10	0	0

[1] Judged on a scale of zero to 10; see text for details.

TABLE 4

Effect of L-*threonine and* L-*tryptophan on cataract formation in rats fed high tyrosine diet*

Group	Addition to 6% casein diet			Cataract development	
	L-Tyr	L-Thr	L-Try	No. of rats showing cataracts	Time of appearance
	%	%	%		
1	–	—	—	0/4	—
3	3	—	—	0/4	—
7	–	1.25	—	1/5	8
8	3	1.25	—	4/5	6.5 to 8.5
9	–	1.25	0.2	0/5	—
10	3	1.25	0.2	0/5	—

TABLE 5

Effect of 2% L-*threonine on the toxicity of 5%* L-*tyrosine in interval-fed rats* [1]

Group	Additions to 6% casein diet		Wt gain, 15 days	Severity of lesions		
	L-Tyr	L-Thr		No. of rats [2]	Eye	Paw
	%	%	g			
1	–	–	18.5	0	0	0
2	5	–	1.0	4	1.3	1.3
3	5	2	10.2	0	0	0

[1] Young male rats, 60–70 g, of the Sprague-Dawley strain.
[2] Six rats/group; numbers in column indicate number of rats that developed lesions.

Effect of threonine on tyrosine toxicity in interval-fed rats. In a preliminary experiment rats trained to eat for only a single 2-hour period daily did not develop eye and paw lesions when they were fed a diet containing 6% of casein and 3% L-tyrosine. The excess tyrosine did, however, retard growth which was prevented by the concomitant feeding of 1.25% L-threonine. The level of tyrosine in the diet was raised to 5% in a subsequent experiment. The results obtained after feeding rats such a diet for only 2 hours daily are shown in table 5. This high level of tyrosine produced a severe growth depression and also led to the appearance of eye and paw lesions. The addition of 2% L-threonine slightly improved growth and completely prevented eye and paw lesions.

DISCUSSION

Ingestion of excessive quantities of most amino acids by the rat leads to retardation of growth, as was observed in these experiments on rats fed a low protein diet containing 3% of tyrosine. Threonine is the limiting amino acid in casein diets supplemented with methionine (8), and hence it

is not unexpected that the addition of threonine improved the growth of rats fed the high tyrosine diet. But, the fact that large amounts of L-threonine, i.e., 0.8% and 1.25%, three to four times the requirement, were more effective than 0.2% in improving both growth and the eye and paw lesions caused by ingestion of excess tyrosine, suggested that threonine was acting in some way unrelated to its role as the limiting amino acid. The observation that threonine also reduced the growth depression, and in high amounts (1.6% DL-) alleviated the eye and paw lesions in rats fed a fibrin diet containing 3 or 4.5% of tyrosine, in which threonine is the least limiting amino acid, further supports this idea.

The appearance of cataracts in the group of rats fed the high tyrosine, high threonine diet is clearly due to a tryptophan deficiency. This is shown by the lack of appearance of cataracts in any group of rats supplemented with 0.2% tryptophan. The occasional development of cataracts in rats fed only the control diet supplemented with high threonine also suggests that their appearance was caused by a deficiency of tryptophan and not by the toxicity of tyrosine. The increased need for tryptophan as a result of including 1.25% L-threonine with or without 3% tyrosine in the basal diet is reminiscent of imbalances involving tryptophan and produced by the addition of a small amount of threonine to a low protein diet lacking niacin (9). In these early studies growth inhibition produced by 0.078% of threonine was reversed by the addition of a small amount of niacin or tryptophan. In the present experiments the much larger amount of L-threonine caused a growth depression that was prevented by tryptophan in rats fed a diet containing an adequate amount of niacin. The growth-stimulating effect of tryptophan was greater in the presence of tyrosine than in its absence. One possible explanation may be that in the presence of 1.25% L-threonine and 0.2% L-tryptophan, phenylalanine or tyrosine become somewhat limiting for growth.

Cataracts may be produced in rats under a wide variety of experimental conditions, and many of them have been well-studied. Cataracts may be caused by excess galactose (10, 11) or xylose (12) in the diet or by deficiencies of almost any of the indispensable amino acids (13). The cataractogenic effect of a diet deficient in tryptophan was first observed by Totter and Day (14). Their observations were confirmed by Albanese and Buschke (15). In the present experiments the cataracts observed in the groups fed tyrosine and threonine appear to be due to such a deficiency produced by an interference in tryptophan utilization by excesses of these amino acids. The observation that 0.2% L-tryptophan not only stimulated the growth of rats fed the cataractogenic diet, but also prevented the appearance of cataracts, points to the conclusion that the tryptophan requirement is increased by adding 1.25% L-threonine to the 3% tyrosine diet.

In rats trained to eat for only 2 hours daily, 3% of tyrosine did not cause eye and paw lesions, but 5% of tyrosine did. When food is offered for only 2 hours a day, the rat is in the post-absorptive state for a considerable time. There may be therefore, under these conditions, a greater demand for energy from non-carbohydrate sources, which could lead to more efficient metabolism of the excess of tyrosine. The adrenals are known to increase in size in interval-fed rats. An increase in adrenal size may be indicative of increased production of corticosterone by the gland which may, in turn, increase the activity of tyrosine-α-ketoglutarate transaminase in the liver and result in a faster catabolism of tyrosine.

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Effect of Tyrosine and Threonine on Free Amino Acids in Plasma, Liver, Muscle, and Eye in the Rat [1,2]

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ABSTRACT Young rats fed a diet containing 3% tyrosine for 3 to 4 weeks had altered patterns of plasma amino acid concentrations. The greatest change occurred in the concentration of tyrosine, which increased 25- to 80-fold. When 2.5% DL- or 1.25% L-threonine was included in the 3% tyrosine diet, the plasma concentration of tyrosine was much lower than that of rats fed the 3% tyrosine diet without additional threonine; however, the threonine concentration in plasma increased 50- to 100-fold. The tyrosine concentration was increased in liver, muscle, and eye tissue as a result of feeding 3% tyrosine; and threonine supplementation also lowered the tyrosine concentration in these tissues.

Young rats fed a low protein diet containing 3% tyrosine develop eye and paw lesions and their growth is retarded. These adverse effects are alleviated when 1.25% L- or 2.5% DL-threonine is added to the high tyrosine diet (1).

Ingestion of a diet containing a disproportionately large quantity of an amino acid can alter the amino acid pattern in blood plasma and in various tissues of the body (2–4). The objectives of the studies described in the present paper were to determine the extent to which excessive tyrosine (the amount used in the growth studies (1)) affected the free amino acid pattern of plasma and tissue in the rat and to determine whether there was a correlation between severity of symptoms and tyrosine content of body fluids in rats ingesting a 3% tyrosine diet.

EXPERIMENTAL

Rats. Young, 50- to 80-g male rats were used, except in the experiment on rats trained to eat for only 1 to 2 hours daily. The rats in the latter study had been trained for 6 weeks, and weighed 100 to 120 g on the day of the experiment. All rats were housed in separate, suspended cages.

Diets. Agar-gel diets were used in all experiments. The basal diet had the following composition: (in % dry weight) casein, 6.0; DL-methionine, 0.2; salt mixture (5), 4.0; vitamin mixture (6), 0.5; choline chloride, 0.2; corn oil, 5.0; agar, 2.5; dextrin, 40.8; and sucrose, 40.8. The gel diet contained about 50% water. In the experiments reported in tables 1, 2, and 3, the carbohydrate was supplied entirely as dextrin, and in those reported in tables 2 and 3 the amount of agar was 3.6%, added at the expense of carbohydrate. The vitamin mixture for experiments 1–3 also differed slightly (7). Diets were fed ad libitum except in the experiment in which plasma amino acid concentrations of rats that had eaten a single meal were determined. These rats which had been trained to eat a single large meal daily, were offered 15 g of agar-gel diet (7.5 g dry diet). Water was provided ad libitum for all groups.

Plasma. Blood was drawn by heart puncture, sometime during the daylight hours, from rats anesthetized with ether. Because of the large numbers of rats involved, individual rats were taken from groups in rotation. In most of the experiments the blood from animals of the same group (about 1 ml of plasma from each of 4 to 10 rats) was pooled before being analyzed for plasma amino acids. In one experiment blood from individual rats was analyzed. After centrifugation of the blood, an aliquot of plasma was removed

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and mixed with 5 volumes of 1% picric acid to precipitate the proteins. The resulting precipitate was removed by centrifugation, and the protein-free picric acid extract was analyzed for free amino acids, according to the method of Moore and Stein (8), using a Technicon amino acid analyzer. Each value in the results represents samples from separate groups of rats, killed at the time indicated. The precision of the method depends upon the quantity of the amino acids on the analyzer. Generally, reproducibility is ± 3 to 7%; however, when the quantity is very large or very small the variation may exceed 10%.

Muscle. About 1 g of muscle was excised from the thigh and immediately frozen in dry ice. Samples were kept frozen at −20° until the time of analysis when each sample was cut into small pieces with scissors; samples from 4 to 10 rats were pooled and then homogenized thoroughly in 1% picric acid in a Virtis homogenizer. Proteins and cellular debris were removed by centrifugation, and the protein-free picric acid extracts were analyzed for free amino acids as described above. Since muscle has substantial quantities of asparagine and glutamine, these samples were hydrolyzed by heating with 3 N HCl for one-half hour at approximately 100°. The hydrolyzed extracts were used for chromatography after removal of excess HCl under vacuum in a rotary evaporator. The hydrolysis was necessary for accurate determination of threonine, since glutamine and asparagine overlap the threonine peak. Most peptides require considerably longer than one-half hour for hydrolysis.

Liver. One- to two-gram samples of liver from individual rats were removed, homogenized, and treated in the same manner as the muscle tissue. Glutamine concentration was estimated by measuring the difference in glutamic acid concentration before and after the mild hydrolysis of the protein-free extract.

Eye tissues. The eyes of the rats were removed, weighed, pooled, and homogenized in 1% picric acid. The precipitate and cellular debris were removed by centrifugation, and the supernatant picric acid solution was used as described for plasma.

RESULTS

Effect of L-threonine with and without 3% tyrosine on plasma threonine and tyrosine concentrations. The concentrations of tyrosine and threonine in plasma from rats fed various levels of threonine for 3 weeks are shown in table 1. Tryosine concentration increased almost 25-fold in the plasma of rats fed the basal diet with 3% tyrosine added. Supplementation of this diet with 0.8 or 1.25% L-threonine led to a substantial decrease in plasma tyrosine concentration; however, supplementation with 0.2% L-threonine did not result in a decrease of plasma tyrosine concentration below that of the group fed the diet containing tyrosine alone. The lowest dietary level of threonine did not cause as great an increase in plasma threonine concentration as did the higher levels, nor did it alleviate the signs of tyrosine toxicity (1).

Threonine concentration in plasma increased with increasing levels of threonine in the diet (compare groups 2, 5, and 8).

TABLE 1

Effect of L-threonine on plasma tyrosine and threonine concentrations of rats fed 3% L-tyrosine for 3 weeks [1]

Group no.	Diet	Threonine	Tyrosine
		μmoles/100 ml plasma	
1	Basal (6% casein + 0.2% Met)	15	4
2	Basal + 0.2% L-Thr	157	7
3	Basal + 3% L-Tyr	18	101
4	(3) + 0.2% L-Thr	107	163
5	Basal + 0.8% L-Thr	965	6
6	(3) + 0.8% L-Thr	408	77
7	Basal + 1.25% L-Thr	1525	nd [2]
8	(3) + 1.25% L-Thr	1348	27
9	Basal + 1.25% L-Thr + 0.2% L-Try	1500	nd [2]
10	(3) + 1.25% L-Thr + 0.2% L-Try	616	104

[1] Male, Holtzman rats (55–80 g) were used. Samples were pooled from 5 rats/group.
[2] None detectable, with the amount of plasma used.

When tyrosine was present in the diet, the plasma concentration of threonine tended to be lower (compare groups 2 vs. 4, 5 vs. 6, 7 vs. 8, 9 vs. 10).

Effect of threonine on tyrosine and threonine concentrations in plasma and eye tissue of rats fed excess tyrosine. Results of 2 experiments in which tyrosine and threonine content of plasma and eye tissues were measured after feeding rats the experimental diets ad libitum are shown in tables 2 and 3. Results were similar with 1.25% L-threonine and 2.5% DL-threonine. Tyrosine concentration in plasma increased 30- to 80-fold after feeding the 3% L-tyrosine diet. Threonine at the levels used, reduced plasma tyrosine concentrations to about one-half by the end of 3 weeks and to about one-fourth to one-sixth by the end of 10 weeks. The threonine concentration was greatly increased.

The patterns of change in tyrosine and threonine concentrations in eye tissues were similar to those in plasma.

Effect of excess tyrosine with and without L-threonine on the amino acid pattern in plasma, liver, and muscle. The changes in free amino acid concentrations in plasma, muscle, and liver of rats fed diets containing 3% tyrosine or 3% tyrosine with 1.25% L-threonine for 26 days are shown in tables 4 to 7.

Plasma. Many of the amino acids were elevated in plasma from rats fed excess tyrosine (table 4). The most significant increase was in tyrosine concentration, which was elevated about 70-fold. When threonine was included with the high tyrosine, plasma tyrosine concentration was greatly reduced and threonine concentration increased more than a hundred-fold. Serine and glycine concentrations also increased when threonine was included.

Table 5 summarizes the results of 3 different experiments on the effects of feeding a 3% tyrosine diet for 3 to 4 weeks on the plasma amino acid pattern. The amino acids significantly elevated by excess tyrosine feeding were serine, glycine, valine, isoleucine, leucine, and tyrosine. Threonine concentration also increased but the increase was not statistically significant

TABLE 2

Effect of 1.25% L-threonine on tyrosine and threonine concentrations in plasma and eye tissues of rats fed 3% L-tyrosine [1]

Group no.	Diet	Threonine and tyrosine concentrations [2]							
		3 Weeks				10 Weeks			
		Threonine		Tyrosine		Threonine		Tyrosine	
		Plasma	Eyes	Plasma	Eyes	Plasma	Eyes	Plasma	Eyes
1	Basal (6% casein + 0.2% DL-Met)	0.25–0.35	trace	0.03	0.14	0.3	—	0.07	—
2	Basal + 3% L-Tyr	0.5–0.6	trace	2.4	6.0	0.17	trace	2.2	4.7
3	(2) + 1.25% L-Thr	11.8	4.6	1.3	3.3	8.0	6.4	0.35	1.1

[1] Male, Holtzman rats (60–70 g) were used. Samples were pooled from 4–5 rats/group.
[2] Tyr and Thr concentrations are expressed as μmoles/ml plasma and μmoles/g of wet eye tissue.

TABLE 3

Effect of 2.5% DL-threonine on tyrosine and threonine concentrations in plasma and eye tissues of rats fed 3% L-tyrosine [1]

Group no.	Diet	Threonine and tyrosine concentrations [2]				
		23 Days		10 Weeks		
		Threonine	Tyrosine	Threonine		Tyrosine	
		Plasma	Plasma	Plasma	Eyes	Plasma	Eyes
1	Basal (6% casein + 0.2% DL-Met)	0.08	0.03	0.1	0.4	0.06	0.25
2	Basal + 3% L-Tyr	0.17	2.7	0.2	0.3	2.0	4.3
3	(2) + 2.5% DL-Thr	9.8	1.1	9.6	6.0	0.55	1.1

[1] Male, Holtzman rats (50–70 g) were used.
[2] Concentrations of threonine and tyrosine are expressed as μmoles/ml plasma and as μmoles/g wet eye tissue of pooled samples from 4–6 rats/group.

TABLE 4

Effect of 1.25% L-threonine on plasma amino acid pattern of rats fed 3% L-tyrosine [1]

Amino acid	Control	3% L-Tyr	3% L-Tyr + 1.25% L-Thr
	μmoles/100 ml plasma		
Thr	9	33	1093
Ser	24	53	104
Glu	—	16	31
Gly	24	30	43
Ala	43	65	50
Val	13	14	10
Cys	trace	trace	trace
Met	trace	trace	trace
Ileu	5	7	4
Leu	8	11	7
Tyr	4	254	10
Phe	—	6	4
Lys	61	54	29
His	11	7	6
Arg	10	12	13

[1] Male, Sprague-Dawley rats (60–80 g) were used. Pooled samples for the control, 3% L-Tyr, and 3% L-Tyr and 1.25% L-Thr, respectively, of 6, 2, and 4 rats/group.

TABLE 5

Effect of 3% L-tyrosine on plasma amino acids [1]

Amino acid	Control	3% L-Tyr
	μmoles/100 ml plasma	
Thr	9 ± 2.1 [2]	23 ± 5.2
Ser	30 ± 3.8	52 ± 2.8 [3]
Pro	25	27
Gly	17 ± 3.2	29 ± 2.3 [3]
Ala	53 ± 8.2	81 ± 10.0
Val	12 ± 1.0	15 ± 0.8 [3]
Cys	trace	trace
Met	trace	trace
Ileu	5 ± 0.5	7 ± 0.2 [3]
Leu	8 ± 0.8	11 ± 0.2 [3]
Tyr	4 ± 0.4	257 ± 24.9 [3]
Phe	4	5
Lys	53 ± 7.8	57 ± 2.7
His	8	8
Arg	8	12

[1] Male, Sprague-Dawley rats (60–80 g) were used. The rats were fed ad libitum for 3–4 weeks.
[2] SE of the mean of 3 experiments. When there is no standard error indicated, the results were available from only 2 experiments.
[3] Significantly ($P \leq 0.05$) different from control value.

owing to the variability among experiments. The greatest increase, as had been observed consistently in previous experiments, was in tyrosine concentration.

Liver. Concentrations of free amino acids in liver of rats fed excess tyrosine, supplemented and unsupplemented with threonine, are shown in table 6. Tyrosine, alanine, and glutamine concentrations were elevated in livers of rats fed the 3% tyrosine diet. Only in tyrosine and alanine were the increases statistically significant; the other free amino acids did not show much alteration. In contrast with the observations for plasma, the concentration of free tyrosine in liver of rats fed the high tyrosine diet was not greatly increased; its elevation was approximately 2.5-fold compared with 70-fold in plasma.

Free tyrosine concentration in liver, as in plasma, was greatly reduced when threonine was included in the high tyrosine diet. Threonine concentration, however, was about 11 times higher than that of the control.

Muscle. As shown in table 7, there were few significant changes in the free amino acid concentrations in muscle of rats fed the 3% tyrosine diet. The exceptions were aspartic acid plus asparagine, tyrosine, and histidine; tyrosine concentration increased about 15-fold. Histidine and asparagine concentrations were significantly reduced, the reduction being much greater for histidine. Inclusion of threonine in the tyrosine diet led to a 34-fold increase in the concentration of free threonine in muscle and a marked decrease in tyrosine concentration.

Plasma amino acid concentrations of rats fed a high tyrosine diet ad libitum for one day. Table 8 shows the effects of the high tyrosine diet with or without extra threonine fed ad libitum on plasma amino acid concentrations. In most respects the amino acid patterns were similar to those observed after prolonged feeding of the experimental diets. Threonine, serine, glycine, tyrosine, and arginine concentrations were higher in the group fed high tyrosine. The greatest increase was in tyrosine. Amino acids which were somewhat reduced in concentration were alanine, isoleucine, valine, leucine, phenylalanine, lysine, and histidine. When threonine was included in the diet, the concentrations of these amino acids were further reduced. Threonine, though it did not separate well from serine on the column, showed a marked increase. The results of the one-day ad libitum feeding differed from those of the 3-week study in one important respect: the tyrosine concentration in plasma was markedly increased, rather than lowered, as a result of adding 1.25% threonine to the high tyrosine diet.

TABLE 6

Effect of 3% L-tyrosine with and without 1.25% L-threonine on free amino acids in liver [1]

Amino acid	Control	3% L-Tyr	3% L-Tyr + 1.25% L-Thr
	μmoles/g wet tissue	*μmoles/g wet tissue*	*μmoles/g wet tissue*
Thr	1.2 ± 0.3 [2]	1.3 ± 0.1	13.9 ± 1.4 [3]
Ser	6.0 ± 1.3	4.6 ± 0.5	5.3 ± 0.3
Glu	6.6 ± 0.4	6.3 ± 0.5	8.0 ± 1.1
Gly	4.3 ± 0.5	5.0 ± 0.3	5.1 ± 0.4
Ala	5.4 ± 0.3	9.5 ± 1.0 [3]	6.0 ± 0.6
Val	1.6 ± 0.4	1.3 ± 0.04	1.5 ± 0.06
Met	0.7 ± 0.2	0.7 ± 0.01	0.7 ± 0.05
Ileu	0.9 ± 0.2	0.8 ± 0.05	0.9 ± 0.06
Leu	2.8 ± 0.5	2.2 ± 0.1	2.3 ± 0.09
Tyr	1.0 ± 0.2	2.6 ± 0.6 [3]	0.9 ± 0.01
Phe	1.2 ± 0.2	1.1 ± 0.04	1.2 ± 0.03
Lys	2.8 ± 0.3	2.1 ± 0.03	2.0 ± 0.15
His	1.4 ± 0.2	1.1 ± 0.03	0.9 ± 0.07
Arg	trace	trace	trace
Cys	trace	trace	trace
Glu-NH_2	3.1 ± 1.4	5.1 ± 0.5	5.6 ± 0.7

[1] Male, Sprague-Dawley rats (60–80 g) were used.
[2] SE of the mean of 4 rats/group except for control which had three.
[3] Significantly ($P \leq 0.05$) different from control value.

TABLE 7

Effect of 3% L-tyrosine with and without 1.25% L-threonine on free amino acids in muscle [1]

Amino acid	Control	3% L-Tyr	3% L-Tyr + 1.25% L-Thr
	μmoles/g wet tissue	*μmoles/g wet tissue*	*μmoles/g wet tissue*
Asp + Asp-NH_2	0.7 ± 0.05 [2]	0.5 ± 0.05 [3]	0.7
Thr	0.4 ± 0.03	0.4 ± 0.04	13.4 ± 0.87 [3]
Ser	3.3 ± 0.55	1.9 ± 0.33	3.7 ± 0.32
Gly	4.0 ± 0.25	5.1 ± 0.70	4.0 ± 0.52
Ala	5.1 ± 0.95	4.0 ± 0.45	2.8 ± 0.09
Val	0.3 ± 0.04	0.2 ± 0.01	0.3 ± 0.06
Ileu	0.2 ± 0.04	0.1 ± 0.01	0.2 ± 0.03
Leu	0.3 ± 0.04	0.3 ± 0.01	0.4 ± 0.05
Tyr	0.2	3.0 ± 0.65 [3]	0.5 ± 0.05
Phe	0.2	0.2 ± 0.02	—
Lys	—	2.7	1.6
His	4.8 ± 0.57	1.8 ± 0.05 [3]	1.7 ± 0.02
Arg	0.4	0.4 ± 0.03	0.4 ± 0.03

[1] Male, Sprague-Dawley rats (60–80 g) were used.
[2] SE of the mean for 3–4 rats for each group. When no standard error is given, figures were available for only 2 rats.
[3] Significantly ($P \leq 0.05$) different from control value.

Plasma amino acid patterns of rats fed a single meal containing excess tyrosine with and without additional threonine. Figure 1 shows the results of feeding a single meal containing excess tyrosine on plasma tyrosine and threonine concentrations in rats. Except for glycine in all 3 groups, and glutamic in the tyrosine and tyrosine + threonine groups, the concentrations of all the amino acids increased in blood plasma as a result of ingestion of the meal. Increases in most of the amino acid concentrations had occurred by 6 hours. By 24 hours, when animals were in the post-absorptive state, most amino acid concentrations had decreased and approached the fasting values.

The greatest change in the plasma amino acid pattern as a result of feeding the 3% tyrosine diet was an increase in the tyrosine concentration by about 100-fold after 6 hours; after 24 hours, it was still 13 times as high as that of the control. Threonine supplementation, as in the one-day ad libitum feeding study, did not reduce plasma tyrosine concentration; in fact, after

TABLE 8

Effect of feeding for one-day (ad libitum) diets containing 3% L-tyrosine with and without 1.25% L-threonine on plasma amino acids [1]

Amino acid	Control	3% L-Tyr	3% L-Tyr + 1.25% L-Thr
	μmoles/100 ml plasma		
Thr	8	10	313 [2]
Ser	33	41	
Glu	21	29	26
Gly	24	35	47
Ala	86	58	33
Cys	4	6	—
Val	16	13	9
Met	trace	trace	trace
Ileu	6	5	3
Leu	12	8	5
Tyr	7	194	310
Phe	7	5	2
Lys	64	56	37
His	16	11	7
Arg	9	23	12

[1] Samples were pooled from 4 rats/group.
[2] Because of the large quantity of threonine, serine did not separate clearly; therefore, the total is given of which about 90% is threonine.

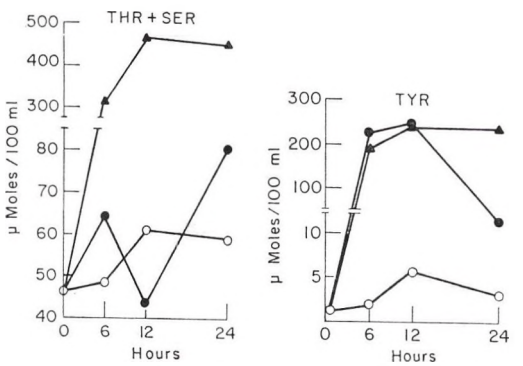

Fig. 1 Effect of a single meal on blood plasma tyrosine and threonine concentrations of interval-fed rats. Plasma was pooled from 3 rats/group before feeding and at 6, 12 and 24 hours after feeding the 3 diets. ○——○ rats fed the control diet; ●——● rats fed 3% tyrosine; ▲——▲ rats fed 3% tyrosine + 1.25% L-threonine.

24 hours the value was much higher than for the group that received no additional threonine.

Most of the plasma amino acid concentrations at 24 hours were higher in the group fed the 3% tyrosine diet than in the other 2 groups.

Although there were significant changes, especially in tyrosine and threonine + serine concentrations as a result of including 1.25% L-threonine in the high tyrosine diet, the values for most of the other amino acids resembled those for the control group.

DISCUSSION

The amino acid pattern in the plasma of rats fed a low protein diet was altered when 3% tyrosine was included in the diet. The most significant change was the 25- to 80-fold increase of tyrosine concentration. When animals are fed an excess of individual amino acids the plasma concentration of the amino acid fed usually increases substantially. The extent of the increase depends upon the nature of the amino acid and upon the amount fed (9, 4). Sauberlich (4) observed about a 60-fold increase in plasma tyrosine concentration in young rats fed for 4 weeks a diet containing 5% tyrosine. An increase of the same order of magnitude in plasma tyrosine has also been observed in monkeys (10) fed excess tyrosine.

The observation that the tyrosine concentration in plasma increased manyfold after the ingestion of only a single meal containing excess tyrosine, is also in accord with the results of Hier (1947) in dogs (9). The increase in the dog was not as large as that observed in the present study with the rat; however, the amount of tyrosine ingested by the dogs was also proportionately less.

When graded levels of threonine were fed with and without 3% tyrosine, with each dietary level of threonine tested, the threonine concentration in the plasma was lower when tyrosine was present in the diet. It is not known whether this lowering is due to a specific interrelationship between the 2 amino acids or simply to the lower food intake of rats fed excess tyrosine.

The addition of threonine to the 3% tyrosine diet brought about within 3 weeks a considerable reduction in plasma tyrosine concentration. The effect was more pronounced as the threonine content of the diet was increased. Lowering of plasma tyrosine concentration occurred only when plasma threonine concentration was very high.

A number of explanations may be advanced for the mode of action of threonine. Tyrosine and threonine are both neutral amino acids and share a common transport system. It is possible that the large amount of threonine that accumulates in the plasma of animals fed for some time a diet

containing 1.25% L-threonine could interfere with the reabsorption of tyrosine in the renal tubules. However, urinary excretion[3] of radioactivity was about the same for rats that had ingested a single meal of the various diets used in this study together with a tracer dose of L-tyrosine-U-^{14}C.

During intestinal absorption competition between certain amino acids had been demonstrated in vitro (11) and in vivo (12). Inhibition of intestinal absorption of certain amino acids in vivo has been shown to occur in the presence of an excess of other amino acids (13). The absorption of tyrosine from the intestine may be affected by the presence of excess threonine. Again, this hypothesis has apparently been ruled out experimentally by the use of ^{14}C-L-tyrosine. Twenty-four hours after rats ingested a single meal, a threonine-supplemented group had almost the same amount of unabsorbed radioactivity in their intestinal contents and their feces as did a group of rats fed tyrosine alone.[4] Thus, it seems highly improbable that inhibition of tyrosine uptake by threonine is important in the intestine. This may be due to the large total absorptive capacity of the intestine.

Another possible explanation for the reduced tyrosine concentration in plasma is that threonine may stimulate the oxidation of tyrosine in the liver, thus leading to lower plasma values in spite of an increased intake of tyrosine from the diet.

One-day feeding of threonine with tyrosine did not result in a lowering of plasma tyrosine concentration. Under such conditions tyrosine concentration in the plasma reflected the dietary intake of tyrosine. Since the addition of threonine to the high tyrosine diet increased the food intake of rats by at least 1.0 g, those receiving threonine and tyrosine together actually consumed more tyrosine. Also, plasma tyrosine concentration in rats fed a single meal containing extra threonine and tyrosine was not reduced. It appears that the mechanism whereby addition of threonine to the tyrosine diet brings about a lowering of the plasma tyrosine concentration comes into play only after feeding threonine for a prolonged period of time.

Feeding 3% tyrosine for 3 to 4 weeks significantly increased most of the other amino acid concentrations in the plasma in addition to the high tyrosine concentration. For some amino acids, i.e., threonine and alanine, because of the high variability the changes were not statistically significant. High variability in the free amino acids in plasma and in tissues has also been reported by other workers (14, 15). This is especially a problem when the diets are fed ad libitum and the level of the amino acid fed in the diet is high.

Table 9 shows that although the magnitude of increase in the tyrosine or threonine concentration in muscle and in liver was lower than in plasma after feeding a 3% tyrosine diet, the absolute concentrations in these tissues and the total pool size of tyrosine and threonine was much higher than in plasma. Tyrosine concentrations in muscle and in liver increased, respectively, 13- and 2.5-fold as a result of feeding 3% tyrosine for 26 days (table 9). Although the magnitude of increase was much less

[3] Alam, S. Q., Ph.D. Thesis, Massachusetts Institute of Technology, 1965.
[4] See footnote 3.

TABLE 9

Concentrations and pool size of threonine and tyrosine in plasma, liver and muscle of rats fed 3% L-tyrosine with or without 1.25% L-threonine [1]

Diets	Control	Tyr	Tyr + Thr	Control	Tyr	Tyr + Thr
	Tyrosine, μmoles/g water			*Tyrosine, μmoles/total tissue*		
Plasma	0.04	2.72	0.1	0.14	6.4	0.4
Liver	1.43	3.76	1.3	5.4	10.2	5.2
Muscle	0.32	4.05	0.6	12.3	100.0	23.6
	Threonine, μmoles/g water			*Threonine, μmoles/total tissue*		
Plasma	0.09	0.35	11.8	0.33	0.8	43.6
Liver	1.71	1.86	19.9	6.4	5.0	77.4
Muscle	0.49	0.51	17.9	17.5	12.5	704.0

[1] For the calculations of total pool size in liver, muscle, and plasma, liver weight was used as such: muscle was taken to be 40% of body weight and plasma as 3% of body weight. The moisture content was assumed to be: plasma, 93%; muscle, 75%; and liver, 70%.

in muscle and liver, as compared with plasma, the absolute concentrations of tyrosine (μmoles/g water) were higher. This occurs because the tyrosine concentration in the muscle and the liver of rats fed the control diet are initially high. It appears, therefore, that by feeding 3% tyrosine, the transport mechanisms of the liver and the muscle cells become saturated. Another possible explanation for the relatively small increase in tyrosine concentration, especially in the liver, is the faster rate of tyrosine catabolism when rats are fed high concentrations of this amino acid.

Apart from the increase in tyrosine concentration, tyrosine feeding apparently did not change appreciably the pattern of free amino acids in muscle and liver. In liver, alanine and glutamine (measured as glutamic acid) increased. Tyrosine-α-keto glutarate transaminase activity in the liver of intact rats increases as a result of tyrosine administration (16, 17). This increase could result in an elevation in glutamic acid concentration. Part of the glutamic acid may be converted into glutamine in the liver, thus accounting for increased glutamine concentration in the liver of rats fed excess tyrosine. Part of the glutamic acid may also be transaminated with pyruvic acid to yield alanine. Glutamic pyruvic transaminase is also induced by cortisol (18). Some evidence was obtained for an increase in corticosterone concentration in plasma of rats fed 3% tyrosine for 3 weeks.[5] This could result in increased activities of hepatic tyrosine-α-ketoglutarate transaminase and glutamic-pyruvic transaminase.

In liver, the concentrations of most of the free amino acids for the control group were higher than those reported in earlier literature (14, 19, 20). However, the difference might be attributed to the basal diet used in the present studies being a low protein diet. Hoshino (3) has shown that most of the free amino acids in liver are elevated by feeding a low protein diet.

A single feeding of a high tyrosine diet increases plasma tyrosine concentration. The eye and paw lesions take from a week to 10 days to develop. A high tyrosine concentration in the plasma apparently must be sustained for some time before rats develop pathological signs of tyrosine toxicity. The results obtained from threonine supplementation experiments show that low plasma and tissue tyrosine concentrations are always associated with the alleviation of toxicity symptoms. It was a consistent observation that 1.25% L-threonine fed with 3% tyrosine improved growth and prevented the appearance of eye and paw lesions.

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Influence of Various Phosphates and Other Complexing Agents on the Availability of Zinc for Turkey Poults [1]

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ABSTRACT The availability of zinc from its compounds with various inorganic phosphates, phytic acid and some organic chelates was studied for turkey poults fed purified diets containing isolated soybean protein. With 15 ppm zinc in the diet, the gain in weight of turkey poults was less with $CaHPO_4 \cdot 2H_2O$, sodium phytate or calcium phytate than with sodium hexametaphosphate, sodium tripolyphosphate, sodium acid pyrophosphate or sodium orthophosphate supplying one-half of the dietary phosphorus (0.6%). When mixed with enough zinc oxide to provide 15 ppm zinc before mixing with the purified diets at levels of 0.684 mmole, sodium tripolyphosphate, sodium hexametaphosphate and sodium phytate reduced its availability. Various phosphates did not improve the availability of zinc already present in the diets. Even though 1,2-diaminocyclohexanetetraacetic acid and diethylenetriaminepentaacetic acid were far less effective than ethylenediaminetetraacetic acid in making dietary-bound zinc available for turkey poults, zinc was equally available from its complexes with these compounds and citric acid but not as available from complexes with orthophosphoric acid, hexametaphosphoric acid, pyrophosphoric acid, tripolyphosphoric acid or phytic acid.

Various phosphates have been studied as sources of phosphorus for chickens and certain calcium salts such as metaphosphate, pyrophosphate or polyphosphate were found to be poor sources (1–4). Calcium acid pyrophosphate was better utilized than vitreous calcium metaphosphate or vitreous sodium metaphosphate. However, phosphorus from α-, β- and γ-forms of calcium pyrophosphate, or β- and γ-forms of calcium metaphosphate, or calcium phytate was virtually unavailable (3). Sodium pyrophosphate caused renal damage in rats (5). The reports concerning the availability of phosphorus from inositol hexaphosphate (or phytic acid) salts are highly conflicting, but it appears that organic phosphorus was more available for gain in body weight than for bone calcification (6). The earlier literature on this subject is well covered in another report (7).

The role of phytic acid in interfering with zinc availability for chicks (8–12) has been well established. The amount of dietary phytic acid in these studies varied between 0.18% to 1.8%. Phytic acid decreased the availability of zinc even in the absence of protein (11), or in the presence of its complexes with glucose or casein (9). A partial removal of phytic acid from isolated soybean protein reduced its zinc binding capacity in in vitro studies (13). The effects of sodium acid pyrophosphate, hexametaphosphate, tripolyphosphate and orthophosphate on the availability of zinc need investigation.

If zinc is made unavailable because of low solubility, it is worthwhile comparing the availability of zinc from its combinations with phosphate, pyrophosphate (Zn-PP), hexametaphosphate (Zn-HMP), tripolyphosphate (Zn-TPP), phytate (Zn-Phy) and oxide.

Earlier investigations have indicated that bound zinc is made available from purified diets for turkey poults (14, 15) and chicks (9, 11, 16, 17) when ethylenediaminetetraacetic acid (EDTA) is added to these diets. It has been suggested that 1,2-diaminocyclohexanetetraacetic acid (CDTA) and diethylenetriaminepentaacetic acid (DTPA) were ineffective in making dietary-bound zinc available to turkey poults because they

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TABLE 1
Zinc-deficient basal diet

	g/kg
Cornstarch	486.7
Isolated soybean protein [1]	330.0
Cellulose, powdered [2]	50.0
$CaHPO_4 \cdot 2H_2O$	30.0
$CaCO_3$	25.0
Mineral mixture [3]	23.1
Vitamin mixture [4]	10.0
Soybean oil	35.0
DL-Methionine	4.5
Choline chloride, 44% [5]	5.7

[1] Assay Protein C-1, Skidmore Enterprises, Cincinnati; contained 0.95% P.
[2] Solka Floc, Brown Company, New Hampshire.
[3] Supplied the following minerals: (in grams) NaCl (uniodized), 9.9; $MnSO_4 \cdot H_2O$, 0.297; $FeSO_4 \cdot 7H_2O$, 0.644; $CuSO_4 \cdot 5H_2O$, 0.079; cobalt acetate tetrahydrate, 0.02; KI, 0.009; $Al_2(SO_4)_3 \cdot 18H_2O$, 0.25; $MgSO_4 \cdot 7H_2O$, 3.97; KCl, 2.97; K_2HPO_4, 4.95; and $Na_2MoO_4 \cdot 2H_2O$, 0.009.
[4] Supplied the following: (in mg) riboflavin, 10; thiamine·HCl, 10; pyridoxine·HCl, 10; Ca pantothenate, 30; niacin, 120; folic acid, 5; menadione, 10; biotin, 0.4; and (in grams) BHT, 1; inositol, 1; vitamin A, 5000 IU; vitamin D_3, 4500 ICU; vitamin E, 88 IU; and vitamin B_{12}, 10 μg.
[5] 44% in wheat middling carrier.

complexed the zinc so strongly themselves that it was not released to the tissues (9). If this was correct, zinc should be less available from zinc complexes of CDTA or DTPA than from EDTA or ZnO.

The experiments conducted to answer these questions are described here.

EXPERIMENTAL

Broad Breasted Bronze turkey poults which were fed a practical poult starter for 2 days and purified diets for 3 days were weighed and divided into groups of approximately equal weight. Each group contained the same number of poults (from 8 to 10). The birds were housed in electrically heated battery cages either coated with an epoxy resin or made of stainless steel and had free access to feed and tap water over the experimental period of 21 days. The composition of the low zinc basal diet is shown in table 1 and it contained 17 mg/kg Zn. The diet contained about 0.3% organic P from the isolated soybean protein and 0.54% inorganic P supplied by $CaHPO_4 \cdot 2H_2O$ (30 g/kg). A slight modification in this diet was made for experiment 1 when sodium chloride was omitted from the salt mixture and levels of $CaHPO_4 \cdot 2H_2O$ and $CaCO_3$ were altered to 16.25 g and 33.9 g/kg, respectively. Sodium was supplied as sodium carbonate besides the sodium present in various phosphates and the chloride ion equivalent to 6.06 g/kg was supplied in the form of KCl. The modified diet contained about 0.3% organic P and about 0.29% inorganic P. Various phosphates such as dicalcium phosphate (CaP), sodium hexametaphosphate (NaHMP), sodium tripolyphosphate (NaTPP), sodium dihydrogen phosphate (NaP), sodium phytate (NaPhy) and calcium phytate (CaPhy) supplied 0.3% phosphorus to the diets. The necessary adjustments were made in starch content of the diets used in experiment 1.

In experiments 2 to 5, the various phosphates and EDTA were added at levels of 0.684 mmoles/kg diet without making any adjustments in the composition of the diets. Zinc oxide was used to supplement the diets with 15 ppm Zn. In experiments 2 and 3, necessary amounts of zinc oxide and the various phosphates were premixed together before mixing with the rest of the diet. In experiments 4 and 5, zinc was mixed with the feed first and the phosphate sources were added afterward.

In testing the availability of zinc from its various compounds, the compounds were analyzed for their zinc content by the atomic absorption method and the results of the analyses are shown in table 2. Necessary amounts of those compounds, some of which were prepared in our laboratory, were added to the diets to supply 15 ppm Zn.

The poults were weighed twice each week as a group and individually at the end of the experiment. The hocks of the birds were scored for perosis on a numerical scale of zero to 4, indicating variations from a normal condition to an extreme condition of slipped tendon. When needed for a bone ash determination, the left leg of the dead poults was severed and the analysis was carried out according to the procedure outlined in AOAC (18).

For the determination of zinc and phosphorus in the feed, samples were ashed in a muffle furnace and zinc was determined by atomic absorption spectra and phosphorus by the method outlined in AOAC (18) using molybdovandate as the reagent.

Various zinc phosphates were prepared by dissolving the sodium salt of the respective phosphate in water and adding to it a solution containing the necessary amount

TABLE 2

Zinc content of various zinc compounds

Name	Formula	Zn	
		Theoretical	Determined
		%	%
Zinc orthophosphate	$Zn_3(PO_4)_2 \cdot 4H_2O$	42.6	43.4
Zinc-EDTA	$ZnC_{10}H_{14}O_8N_2$	18.3	19.1
Zinc-HMP	$Zn_3P_6O_{18}$	29.2	29.8
Zinc-PP	$Zn_2P_2O_7 \cdot 4H_2O$	34.6	34.9
Zinc-TPP	$Zn_2HP_3O_{10}$	27.5	29.7
Zinc-Phy	$Zn_6C_6H_6P_6O_{24} \cdot 3H_2O$	35.6 [1]	19.1
Zinc-DTPA	$ZnC_{14}O_{10}H_{23}N_3$	13.9	10.2
Zinc-CDTA	$ZnC_{14}H_{20}O_8N_2$	15.9	14.3
Zinc citrate	$Zn_3(C_6H_5O_7)_2 \cdot 2H_2O$	31.9	27.7

[1] $Zn_3C_6H_{12}P_6O_{24} \cdot 3H_2O$ corresponds to 21.6% Zn.

TABLE 3

Effect of various sources of phosphorus [1] on the growth, tibia bone ash, and the perotic leg condition of turkey poults [2] over 21 days in experiment 1

Source of P		Added [3]			Exp. 1		
Code name	Formula	Zn	Ca	Na	Gain [4]	Bone ash	Perosis score
		ppm	%	%	*g*	%	
CaP	$CaHPO_4 \cdot 2H_2O$	0	2.1	0.4	57 [a]	41.6	0.1
CaP	$CaHPO_4 \cdot 2H_2O$	15	2.1	0.4	179 [b]	36.5	0.6
NaHMP	$(NaPO_3)_6$	15	1.8	0.4	235 [c]	31.9	0.9
NaTPP	$Na_5P_3O_{10}$	15	1.8	0.4	228 [c]	40.8	1.3
NaAPP	$Na_3HP_2O_7$	15	1.8	0.3	237 [c]	41.1	1.4
NaP	$NaH_2PO_4 \cdot 2H_2O$	15	1.8	0.7	229 [c]	42.7	2.7
NaPhy	$Na_{12}H_6P_6O_{24} \cdot 3H_2O$	15	1.8	0.7	160 [b]	34.9	1.0
CaPhy	$Ca_5H_8P_6O_{24} \cdot 3H_2O$	15	2.2	0.4	138 [b]	32.4	0.6

[1] The diets contained about 0.3% organic P, about 0.29% P supplied by $CaHPO_4 \cdot 2H_2O$; and an additional 0.3% P was supplied by the various sources. The overall P content of these diets was about 0.9%.
[2] Each group contained 8 poults.
[3] Calculated amounts.
[4] The numbers followed by different superscript letters in a column indicate statistical significance at the 1% level by Duncan's method (19).

of zinc chloride. The required amounts were calculated from the theoretical formula of the desired compound. In most cases precipitates appeared on mixing the solutions but the pH was adjusted to 3.5 in all cases with HCl and the mixtures were stirred thoroughly for 4 to 5 minutes and filtered over suction, washed with water and alcohol and dried at 100° for 2 hours.

For other complexes of zinc, equimolar quantities of EDTA (free acid), or DTPA, or CDTA and zinc chloride were mixed in solutions, and the solutions were filtered and evaporated to dryness in a current of air at room temperature. Zinc citrate was obtained commercially.

When each treatment was duplicated in an experiment the gain in weight of poults at 21 days was examined for statistical significance by Duncan's method (19). Otherwise, these were checked by Fisher's *t* test (19).

Sodium and calcium phytate were commercial preparations [2] and were assigned the following formulae:

Sodium phytate	$Na_{12}C_6P_6H_6O_{24} \cdot 3H_2O$
Found:	Na, 27.8%; P, 19.3 ± 0.3%
Calculated:	Na, 29.95%; P, 20.1%
Calcium phytate	$Ca_5C_6P_6H_8O_{24} \cdot 3H_2O$
Found:	Ca, 22.4 ± 0.4%; P, 19.4 ± 0.4%
Calculated:	Ca, 22.2%; P, 20.0%.

RESULTS AND DISCUSSION

There is reasonable agreement between the calculated and the observed zinc content of the various unpurified compounds (table 2) except zinc phytate. It appears that our preparation did not correspond to

[2] Purchased from Sigma Chemical Company, St. Louis.

the formula $Zn_6C_6H_6P_6O_{24}\cdot 3H_2O$ but had the formula $Zn_3C_6H_{12}P_6O_{24}\cdot 3H_2O$.

Supplementation of the basal diet with zinc gave marked improvement in the growth of turkey poults when CaP was the source of phosphorus (table 3). With 15 ppm Zn in the diets, CaP gave slightly better growth than NaPhy which was better than CaPhy although these differences were not statistically significant at the 1% level. These 3 sources of phosphorus were inferior to NaHMP, NaTPP or NaAPP or NaP in the presence of zinc. The growth rates of turkey poults with the latter sources were of the same order. The sodium content of the diets with NaP and NaPhy could not be reduced below 0.7% but these levels appeared to cause no deleterious effects.

There appears to be no correlation between the perosis score and the bone ash. With no supplemental zinc in diet, turkey poults grew very poorly and no perosis was observed. As the growth of poults improved, hock deformity appeared. Bone ash was high in the absence of zinc for poults fed CaP and in the presence of zinc for those fed NaTPP, NaAPP and NaP. It is difficult to be specific about the cause of reduction in bone ash but it may be related to the availability of zinc, phosphorus, and vitamin D_3 for normally growing poults. It is possible that a reduction in the availability of zinc as well as phosphorus might be responsible for lowered gains in weight as well as lower bone ash for birds fed CaP, NaPhy and CaPhy in the presence of 4500 ICU/kg dietary vitamin D_3. The observations concerning phytates are in agreement with the similar studies with chicks (8–12) but differ from the study with chicks (6) in which zinc was not deficient. Bone ash of 40.8 to 42.7% for turkey poults (table 3) appeared to be in the normal range (20). The cause of the reduction in bone ash but not the growth of turkey poults by NaHMP needs further investigation.

The levels of phosphates added in experiment 1 (table 3) were quite high. An earlier study indicated (15) that a level of even 0.684 mmole EDTA and other complexing agents markedly influenced the availability of zinc present in the purified diet containing isolated soybean protein.[3] The results on the effect of adding the same levels of various sodium phosphates on the gain in weight and severity of perosis of turkey poults are presented in table 4. None of the phosphates released zinc from the basal diet (exps. 2 and 3). NaTPP, NaHMP, NaPhy appeared to reduce the availability of supplementary zinc mixed with it prior to addition to the basal diet. However, if zinc was added to the feed first and these phosphates later, no deleterious effect of these phosphates was observed on zinc availability (exps. 4 and 5). Pero-

[3] Assay Protein C-1, Skidmore Enterprises, Cincinnati.

TABLE 4

Effect of various phosphates and EDTA when added at levels of 0.684 mmoles/kg diet on the availability of zinc for gain in weight and prevention of leg perosis of turkey poults over 21 days

Treatment	Exp. 2		Exp. 3		Exp. 4		Exp. 5	
	Gain	Perosis score	Gain	Perosis score	Gain	Perosis score	Gain	Perosis score
	g		*g*		*g*		*g*	
Basal diet	108 $^{b\,1}$	0.5	126 $^{a\,1}$	0.3	72 $^{a\,1,2}$	0	88 $^{a\,1,2}$	0
+ 15 ppm Zn	332 ^{d}	1.9	323 ^{c}	1.2	223 ^{b}	0.6	237 ^{b}	1.6
+ EDTA	298 ^{cd}	2.2	260 ^{b}	0.7				
+ NaHMP	100 ^{b}	0.2	124 ^{a}	0.1				
+ NaTPP	119 ^{b}	0.9	119 ^{a}	0.1				
+ NaAPP	82 ^{a}	0.6	116 ^{a}	0.1				
+ NaPhy	106 ^{b}	0.6	127 ^{a}	0.6				
+ 15 ppm Zn + NaHMP	310 ^{cd}	2.3	254 ^{b}	0.9			285 ^{cde}	2.1
+ 15 ppm Zn + NaTPP	259 ^{c}	2.3	217 ^{b}	0.8	220 ^{b}	0.5	252 ^{bc}	1.4
+ 15 ppm Zn + NaPhy	277 ^{cd}	2.5	241 ^{b}	0.8	203 ^{b}	0.3	245 ^{bcd}	2.0
+ 15 ppm Zn + EDTA							358 ^{e}	0
+ 15 ppm Zn + NaAPP					237 ^{b}	1.1	269 ^{bcd}	2.0

[1] Different superscript letters in a column indicate statistical significance at the 1% level by Fisher's *t* test.
[2] In contrast with experiments 2 and 3, the zinc was mixed with the bulk of diet and various phosphates were added later. In experiments 2 and 3, zinc and phosphates were mixed together and added to the diets.

TABLE 5

Effect of various zinc phosphates, zinc oxide, and other zinc chelates on gain in weight and leg perosis of turkey poults over 21 days

Treatment [1]	Exp. 6		Exp. 7		Exp. 8		Exp. 9		Exp. 10	
	Gain	Perosis score	Gain	Perosis score	Gain	Perosis score	Gain	Perosis score	Gain	Perosis score
	g		*g*		*g*		*g*		*g*	
Basal diet	79 [a 2]	0	79 [a 2]	0	76 [a 2]	0	78 [a 2]	0	49 [a 2]	0
+ZnO	236 [cd]	0.4	267 [cde]	0.8	247 [b]	0.6	257 [c]	0.4	237 [c]	0.9
+Zn-Phy	190 [bc]	0	228 [cd]	1.1			197 [b]	0		
+Zn-PP	179 [bc]	0.5	187 [bc]	0.6					180 [b]	0.4
+Zn-HMP	206 [bcd]	0.2	190 [bcd]	0						
+Zn-TPP	228 [cd]	0.6	166 [b]	0.5						
$+Zn_3(PO_4)_2$	146 [ab]	0.2	176 [b]	0.1			222 [bc]	0.4	164 [b]	0.6
+Zn-EDTA	264 [d]	0.3	318 [e]	1.6	263 [b]	0.5				
+Zn-CDTA	230 [cd]	0.7	317 [e]	1.2	243 [b]	0.3				
+Zn-DTPA			302 [e]	1.0	277 [b]	0.5				
+Zn-Citrate					295 [b]	0.5				
+ZnO (30 ppm Zn)	273 [d]	0.2								

[1] The various zinc salts supplied 15 ppm zinc, except in last diet which had 30 ppm Zn.
[2] Statistical significance at the 1% level by Fisher's *t* test is indicated by different superscript letters in a column.

TABLE 6

Effect of various zinc phosphates, zinc oxide, and other zinc chelates on gain in weight, bone ash and leg perosis of turkey poults over 21 days

Treatment	Dietary		Exp. 11		
	Zn [1]	P [1]	Wt gain [2]	Perosis score	Bone [1] ash
	ppm	%	*g*		%
Basal diet	17	0.92	36 [a]	0	46.2
+ZnO	34.7	0.92	242 [de]	0.4	43.8
+Zn-Phy	40.2	0.81	219 [de]	0.1	44.3
+Zn-PP	30.8	0.88	205 [d]	0.4	40.1
+Zn-HMP	32.2	0.85	154 [c]	0.5	46.0
+Zn-TPP	31.6	0.91	108 [b]	0	46.6
$+Zn_3(PO_4)_2$	31.4	0.85	161 [c]	0.1	46.3
+Zn-EDTA	26.2	0.93	257 [ef]	0.7	44.3
+Zn-CDTA	31.4	0.85	258 [ef]	0.3	44.2
+Zn-DTPA	35.5	0.86	316 [ef]	0.9	44.4

[1] Determined values.
[2] Statistical significance at the 1% level by Fisher's *t* test is indicated by different letters in a column.

sis is more related to the overall weight of the poults. Zinc-deficient poults, because of their poor growth, had low perosis scores.

To confirm the observations that phosphates might be interfering with the availability of zinc, the data of experiments 6 to 10 are presented (table 5). The data for Zn-EDTA, Zn-CDTA, Zn-DTPA and Zn citrate which have stability constants of 16.5, 18.7, 18.2 and 5.5, respectively, are also presented in this table. Zinc was equally available from its organic complexes even though in the present studies CDTA and DTPA did not make Zn available from the basal diet to the same extent as EDTA. In experiment 12 (not in tables) the average gain in weight of turkey poults fed the basal diet, basal supplemented with EDTA, CDTA, DTPA, ZnO, Zn-EDTA, Zn-CDTA and Zn-DTPA over 21 days were (in grams) 75, 231, 77, 143, 215, 301, 248, 294, respectively. This means that the original hypothesis that zinc was so strongly bound to CDTA and DTPA that it was not released to the tissues needs to be modified. Actually, the values given for stability constants are the maximal values for these complexes and are only attainable at a pH of 10 or above. Such pH values are hard to visualize in living systems. The relative binding constants for Zn-EDTA, Zn-CDTA, and Zn-citrate 1:1 complexes

were found to be 6.73, 6.69 and 4.53 at a pH of 7.4. Zn-DTPA did not form a 1:1 complex and the binding constant for 1:2 complex was 12.27 (21). This may imply that the binding constant of CDTA, DTPA and citrate are too low in comparison with that of EDTA to release zinc from its bound form in isolated soybean protein. Zinc was freely available from its complexes with these ligands (diets 8–11, exps. 6, 7, 8, table 5).

Zinc is less available from its phosphate compounds than from oxide or non-phosphate organic complexes, although in experiment 6, the availability of zinc from its phosphate compounds was higher in comparison with the rest of the experiments. In experiment 11 (table 6) the zinc from zinc phytate was slightly more available than from the phosphates but some of the improved growth was due to an error in mixing by which more than the intended 15 ppm of zinc was added by zinc phytate. In the absence of this 50% extra zinc, the growth of poults might have been less than observed. A poorer weight gain with Zn-EDTA than with Zn-CDTA was the result of a shortage of 5 ppm Zn less than calculated. The perosis scores were related only to the gain in weight and the amount of zinc needed for optimal growth, and prevention of perosis was not the same (12). The availability of zinc from CDTA and DTPA complexes was again confirmed in this experiment. The lowest bone ash was obtained for Zn-PP but was still in the same range as in experiment 1 for normal poults.

We feel that these studies indicate that the availability of zinc from various phosphates for animals depends upon its solubility in the intestinal tract. The importance of solubility considerations on availability of phosphorus from phytate (22, 23) and phosphate is already known (3, 20).

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Skin Lipids of Puppies as Affected by Kind and Amount of Dietary Fat [1]

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ABSTRACT Clinical evidence of a dietary requirement for linoleic acid in maintenance of a healthy skin prompted a study of fatty acid distributions in skin of 84 young dogs fed diets with and without linoleic acid. When weanling puppies were fed diets deficient in linoleic acid for 2 months, monoene fatty acids in whole skin greatly exceeded levels for saturated fatty acids. Linoleic and arachidonic acid levels were lower than for newborn puppies. Levels of these fatty acids decreased further in skin and serum after 4 months when definite deficiency signs were evident, but levels remained approximately the same during longer feeding periods. Small amounts of linoleic acid were always present in skin and serum. Step-by-step increases in dietary linoleate were reflected in increased levels of this fatty acid in triglycerides, cholesterol esters, and phospholipids in skin and serum. During linoleic acid-deficient states, 5,8,11-eicosatrienoic acid was always present in serum, but it was observed infrequently in skin and only in the phospholipid fraction. In deficient states arachidonic acid was present in most serum silicic acid fractions but appeared infrequently in small amounts only in skin phospholipids. When ethyl arachidonate was fed for 2 months after weaning, it was observed in skin and serum, but during recovery from the linoleic deficient state, it was not noted in skin after a 2-month feeding period. Phospholipids make up a small fraction of skin lipids, but changes in their fatty acid patterns appear to reflect alterations which occur in epidermal cells during a dietary deficiency of linoleic acid.

Dietary linoleate has been shown to be required for the maintenance of a healthy skin in young infants (1, 2) and many young animals (3–11). When the diet is deficient in linoleic acid, the most universal and striking clinical sign is dry, thickened and scaly skin. Among the histologic alterations which develop are increased density of the keratin layer with parakeratosis and thickening of the epidermal layer. Mitosis is evident in early stages of the deficiency, but in advanced deficiency there is increased thickening of the epidermis without mitotic activity (12).[2] These changes appear to indicate alterations in the normal maturation of the epidermal cells. Many feeding studies have demonstrated the reversibility of the gross and histologic changes in skin by incorporation of linoleic acid in the diet. Numerous investigators also have reported the chemical components of skin, particularly surface lipids, ductal or sebaceous gland lipids, and components of epidermis of various species. This literature has been reviewed by Nicolaides and co-workers (13–16), who have contributed additional comprehensive data. However, there has been little attempt to correlate the fatty acid composition of whole skin with dietary regimen. In view of evidence for the essentiality of linoleic acid in the maintenance of healthy skin, a 4-year study was undertaken to determine major fatty acid components in skin of young puppies (a) at birth, (b) when the diet fed from the time of weaning is deficient in linoleic acid, (c) when the diet fed from the time of weaning contains 1% or more of the calories as linoleic acid, and (d) when the diet fed during recovery from the deficient state contains 2% or more of the calories as linoleic or arachidonic acid. No attempt was made to characterize the many components present in epidermis or

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[1] This work was supported in part by Public Health Service Research Grant no. AM-04309 from the National Institute of Arthritis and Metabolic Diseases and was inaugurated under the direction of Dr. Arild E. Hansen (deceased).

[2] Sinclair, J. G., E. L. Pratt, V. Hunter, C. Jacobsen, D. J. D. Adam, A. N. Boelsche, M. E. Haggard, H. F. Wiese and A. E. Hansen 1958 Influence of dietary linoleic acid on histology of skin in premature infants. Federation Proc., *17:* 457 (abstract).

epidermal flakes other than the saturated and unsaturated fatty acids identifiable by gas-liquid chromatography of known standards.

The young puppy was chosen as the experimental animal because it is particularly susceptible to a dietary deficiency of linoleic acid, and serial skin biopsies can be taken from the same animal for histology and lipid analysis during the development and during the curative stages of linoleic acid deficiency. Also, periodic changes in distribution of fatty acids in blood serum which reflect changes in linoleic acid intake can be followed.

EXPERIMENTAL CONDITIONS AND METHODS

Experimental conditions. Beagle puppies from the time of weaning were fed isocaloric diets which were considered fully adequate in calories, protein, vitamins, and minerals, but differed in the kind and amount of fat (17). Five diets were deficient in linoleic acid, one having 30% of the calories as hydrogenated coconut oil, one low in fat (1% of the calories), and 3 diets low in fat to which sufficient linolenic, oleic, or arachidonic acid was added to supply 2% of the calories. Diets which contained linoleic acid were prepared by isocaloric substitution of sucrose in the low fat diet with butter, lard, soy bean lecithin,[3] corn oil, or safflower oil[4] to provide one to 16% of the calories as linoleic acid. Likewise, during the period of recovery from the deficient state, trilinolein,[5] lard, soy bean lecithin, corn oil or safflower oil was substituted isocalorically for sucrose to provide 2% or more of the calories as linoleic acid. During recovery, ethyl arachidonate[6] was fed in place of linoleic acid to 2 dogs. The fats and oils chosen as dietary sources of linoleic acid represent types consumed in American households.

Diets were fed at the normal caloric level for growing puppies (17). Immunization and care of the animals also were the same as previously described (17).

Skin biopsies. To determine the most feasible site for detecting early signs of deficiency, skin biopsies were taken from the abdomen, interscapular and thigh areas. Under local anesthesia, crescent shaped biopsies were dissected along the fascial layer and any subcutaneous fat was carefully removed. One-half of each specimen was placed in 10% formalin with 1% calcium acetate for histologic examination and the other half was frozen immediately in a tightly stoppered vial and stored at $-10°$ until analyzed. Histologic sections of skin were stained with hematoxylin and eosin for general characterization. Staining with Sudan black for possible appearance of phospholipids, aldehyde fuchsin for characterization of elastin tissue, toluidine blue for pigment content and Hale's dialyzed iron preparation for mucopolysaccharides did not provide additional information.

Young dogs show the earliest gross signs of linoleic acid deficiency on the abdomen, then on the thigh and last in the interscapular area. Histologic examination of the skin from these 3 areas for 5 animals confirmed the differences in degree of deficiency signs that were observed grossly. Extraction of lipids from whole skin showed the interscapular area to be highest and the abdominal area to be lowest in total fatty acid content. Abdominal skin tended to be highest in saturated and lowest in monoene and linoleic acids. Interscapular skin was consistently highest in linoleic acid. The observations correlated well with the degree of gross and histologic changes for the 3 areas. Since sequential biopsies could be taken alternately from the dorsal surface of each thigh, and gross appearance, histology and fatty acid composition of the skin were typical for the nutritional state of the animal, all of the following data represent results for skin biopsies taken from the dorsal surface of the thigh.

Skin fractions. Although the most marked histopathologic changes in skin occur in the epidermal layer during the development and during the curative stages of linoleic acid deficiency, attempts to separate epidermis and dermis in dog skin by a combination of 2 methods (18, 19) were very unsatisfactory. By histologic examination of the remaining dermis, it was estimated that 70 to 95% of the epidermis was removed in only 10 of 24 skins. Compari-

[3] Generously supplied by Central Soya, Chicago, through the courtesy of Mr. H. T. Iveson.
[4] Generously supplied by Pacific Vegetable Oil Products, Richmond, California, through the courtesy of Mr. Richard Purdy.
[5] The Hormel Institute, Austin, Minnesota.
[6] Generously supplied by Hoffmann-La Roche, Inc., Nutley, New Jersey.

son of the results from analysis of the saponifiable components in epidermis and dermis of these 10 specimens with those from silicic acid fractions of whole skin showed fatty acid patterns in epidermis to be similar to those of cholesterol esters in whole skin and patterns in dermis to be similar to those of triglycerides in whole skin. Also, epidermis and cholesterol esters of whole skin contained high percentages of unidentified components which were not noted in dermis or triglycerides of whole skin. Carbon numbers of these components calculated from retention times were in the same range as those observed in epidermal flakes removed from linoleic acid-deficient dogs and conformed to those reported by other workers (15) in human surface lipids as $C_{13.5}$ to $C_{21.5}$ straight-chain or branched-chain fatty acids. Characterization of fatty acids in dog skin in relation to dietary fat, therefore, has been confined to the total fatty acids and fatty acids obtained by silicic acid separations of lipid extracts from whole skin.

Chemical analyses. In preparation of whole skin for analysis, hair was carefully removed with fine scissors, and the skin was cut into small bits, weighed, and then ground by hand in a porcelain mortar with washed, ignited sea sand. Analyses included total cholesterol (20), total fatty acids (21), and, when possible, silicic acid separation of the triglyceride, cholesterol ester, and phospholipid fractions (22). Fatty acids from each of the above fractions were methylated at 70° with 2% sulfuric acid in methanol. The methyl esters were chromatographed in a Beckman GC-2A instrument using 183-cm diethylene glycol succinate columns at 220° and helium as the carrier gas. Peaks were identified by comparison with retention times of known methyl esters.[7] The 5,8,11-eicosatrienoic acid was identified by its conformity to the theoretical retention time of an eicosatrienoic acid which appeared in serum from linoleic acid-deficient animals and subsequently disappeared from serum of the animals after feeding linoleic acid. This retention time also conformed to that of 5,8,11-eicosatrienoic acid reported by Ackman (23). Peak areas were quantitated both by triangulation and by measurement of peak heights and relative retention times. Correction factors were applied to compensate for differencs in detector response to individual fatty acid components. Dectector response was linear within a given instrument sensitivity setting and injected sample sizes were chosen to allow use of a single sensitivity setting.

Fasted blood specimens were drawn at intervals of 2 months when skin biopsies were taken and the serum fatty acids prepared and chromatographed by the same procedures used for skin.

RESULTS

The data represent observations and analysis of skin and blood serum for 85 dogs over a period of 6 to 8 months from the time of weaning. Most dogs reach maturity at approximately 8 months of age; hence the data were obtained during the entire growth period when the requirement for linoleic acid appears to be the greatest.

Total cholesterol and fatty acids in whole skin and blood serum. Since fatty acids in blood serum readily reflect either the absence of fat in the diet or the composition of the dietary fat and since nutrients for the skin are derived from serum, comparisons were made between fatty acid components in serum and those of the skin. Figure 1 demonstrates the relative differences in the amount of cholesterol and fatty acids derived from the various lipid fractions in skin and serum under 3 dietary conditions: when the diet was low in fat and when 30% of the calories was provided as hydrogenated coconut oil or corn oil. Total cholesterol in skin was very low (1 to 2% of dry weight) for all puppies. Total fatty acid content of skin varied considerably, but mean values for newborns and puppies fed the low fat diet were lower than when hydrogenated coconut oil or corn oil diets were fed. Silicic acid separation of the triglycerides, phospholipids, and cholesterol esters in skin showed that triglycerides constituted the major fraction of lipids in whole skin for all puppies. No blood serum was available for the newborns; however, in blood serum of the other 3 groups, over 40% of the total fatty acids was derived from phospholipids. In skin of newborns, phospholipid fatty acids made

[7] Applied Science Laboratories, Inc., State College, Pennsylvania.

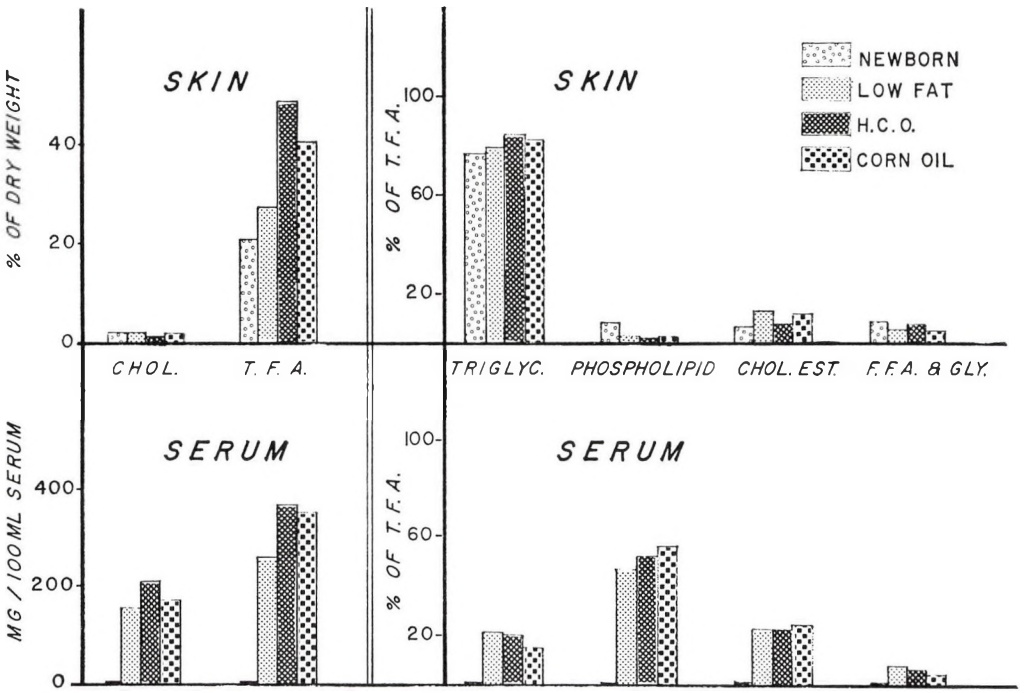

Fig. 1 Lipid fractions in skin of newborns and in skin and serum after feeding weanling puppies diets with and without fat for 4 months.

up 8% of the total fatty acids but after a 4-month feeding period, this decreased to 1 to 2%. Cholesterol ester fatty acids were consistently lower in skin than in serum. The amount of the fraction containing free fatty acids, mono- and diglycerides was approximately the same in skin and serum.

Saturated and unsaturated fatty acids in skin and serum of puppies fed diets deficient in linoleic acid. Fatty acids in skin of newborn puppies were compared with those in skin of puppies fed linoleic acid-deficient diets for 2 months at which time minimal gross and histologic changes usually become evident. Figure 2 illustrates mean values for distribution of saturated and unsaturated fatty acids in skin of 5 puppies at birth, five fed the diet low in fat, three fed the diet containing 30% of the calories as hydrogenated coconut oil, and each of 3 puppies fed the low fat diet with 2% of the calories as methyl linolenate, triolein or ethyl arachidonate. Expressed as the percentage of the total fatty acids in skin, monoene fatty acids, principally oleic, greatly exceeded the level for saturated acids and in some instances were much higher than in blood serum. Skin of newborns contained more linoleic and arachidonic acid than did the skin of puppies fed the linoleic acid-deficient diets for 2 months. The extremely low level of linoleic acid in skin and serum of puppies fed 2% of the calories as methyl linolenate, triolein, and ethyl arachidonate was of special interest because the mother of these puppies was fed the low fat diet during her pregnancy and period of lactation. Mean serum levels of linoleic and arachidonic acids for these puppies at weaning were very low — 3.4 and 3.0% of the total fatty acids, respectively — whereas, 5,8,11-eicosatrienoic acid made up 10.4% of the total fatty acids. Also of special interest was the identification, by retention time, of linolenic acid in skin when methyl linolenate or triolein was fed. The puppy fed triolein had previously received methyl linolenate in his diet. Although the latter fatty acid had disappeared from blood serum, it was still present in the skin. The 5,8,11-eicosatrienoic acid which is characteristic of a dietary deficiency of linoleic acid was not identified in the total fatty acids of skin at this age, although serum levels indicated synthesis of this metabolite by all of the puppies. Arachi-

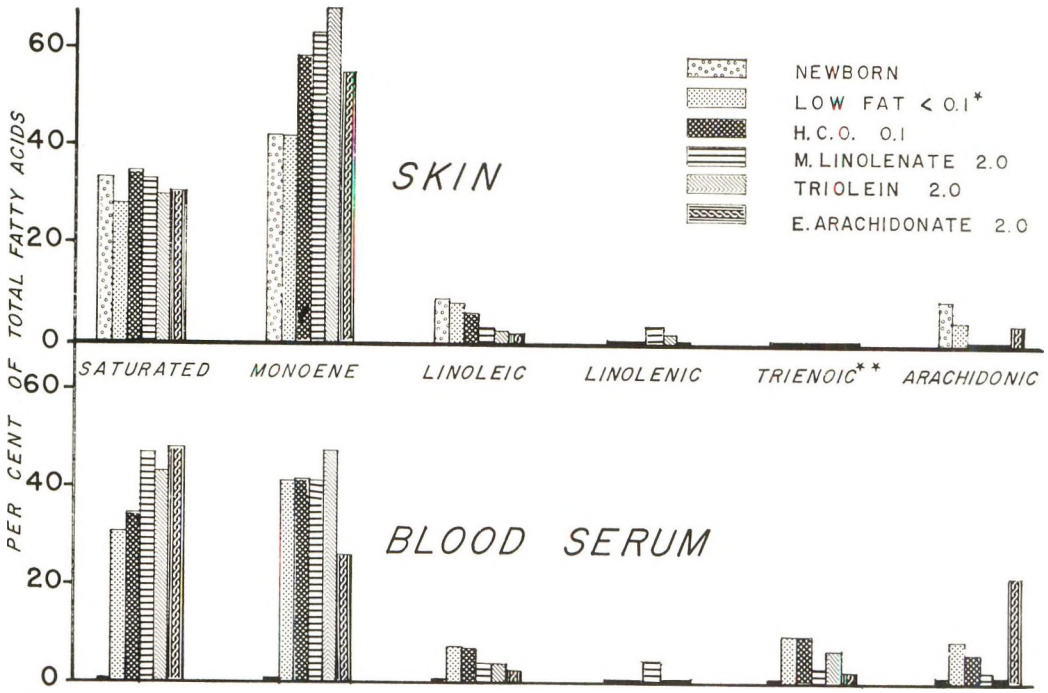

Fig. 2 Saturated and unsaturated fatty acids in the total fatty acids in skin of newborns and in skin and serum after feeding weanling puppies diets deficient in linoleic acid for 2 months. * dietary linoleic acid as % of total calories; ** 5,8,11-eicosatrienoic acid.

donic acid was present in the total fatty acids in skin of newborns, of 3 puppies fed the low fat diet (3/5) and of the puppy fed ethyl arachidonate. In the latter instance the arachidonate level in serum was 21% of the total fatty acids. Gross and histologic appearance of the skin was normal for this animal. Gross and histologic deficiency changes were minimal for the other young puppies.

Saturated and unsaturated fatty acids in skin and serum of puppies fed diets with and without linoleic acid for 4 months. When weanling puppies are fed diets deficient in linoleic acid for 4 months, definite and often marked gross and histologic alterations in skin are evident (24). Fatty acid composition of these grossly abnormal skins was compared with healthy skin at the time of birth and skin of puppies fed diets which provided low, moderate and generous amounts of linoleic acid. Figure 3 demonstrates mean values for saturated and unsaturated fatty acids in skin for 5 newborns and in skin and serum after feeding the following diets for 4 months: 11 puppies, low fat; 12 puppies, hydrogenated coconut oil; 4 puppies, butter fat; 4 puppies, fresh lard; 5 puppies, corn oil. Fat in the latter 4 diets provided 30% of the calories with linoleate contributing 0.1, 1.0, 4, and 16% of the calories, respectively. Saturated fatty acids in skin of puppies fed the low fat diet remained relatively unchanged from the 2- to 4-month period. Saturated fatty acids increased in skin of animals fed hydrogenated coconut oil as a result of increased deposition of lauric and myristic acids during the 2- to 4-month feeding period. Except for puppies fed the hydrogenated coconut oil diet, the level of monoene fatty acids in skin decreased step-by-step with increasing levels of dietary linoleate just as it did in blood serum. When the diet was low in fat or contained hydrogenated coconut oil, linoleic acid in skin and serum were lower after 4 months than after the 2-month period. It increased both in skin and serum with step-by-step increases in dietary linoleate. Small amounts of octadecatrienoic acid identified by retention time as linolenic acid were

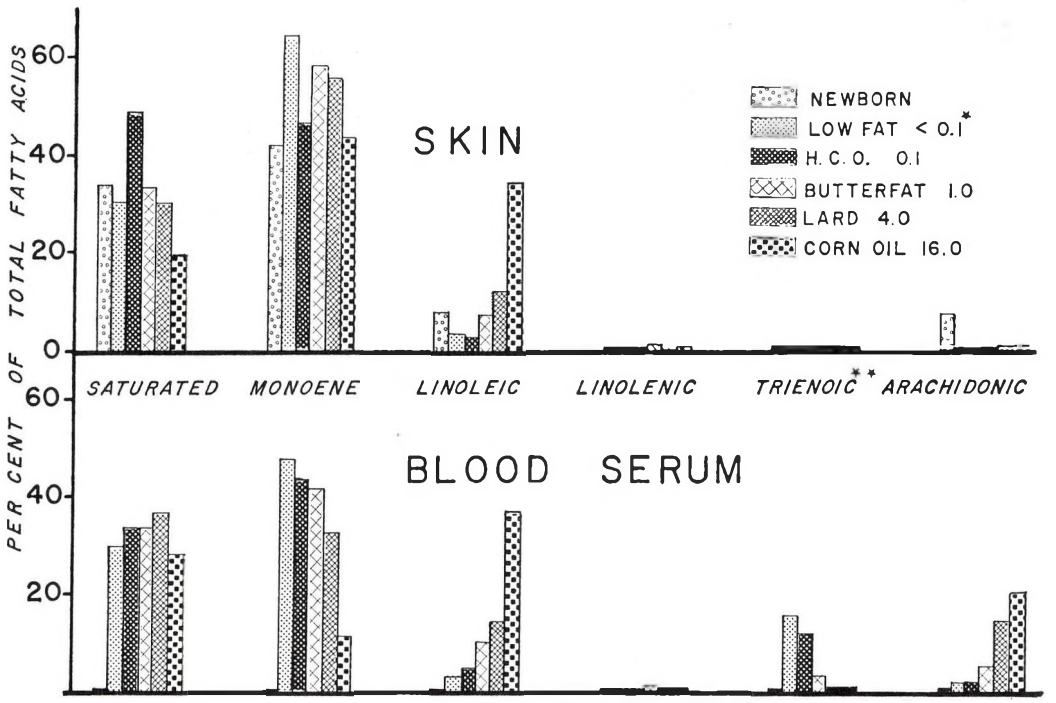

Fig. 3 Saturated and unsaturated fatty acids in the total fatty acids in skin of newborns and in skin and serum after feeding weanling puppies diets with and without fat for 4 months. * dietary linoleic acid as % of total calories; ** 5,8,11- eicosatrienoic acid.

noted in the skin of puppies fed butter fat or corn oil. The 5,8,11-eicosatrienoic acid which appeared in serum of animals fed the low fat, hydrogenated coconut oil or butter fat diets was observed in the skin of only one puppy fed the low fat diet (1/11). Arachidonic acid, which is present in relatively large amounts in skin at the time of birth, was not identified in the total fatty acids of skin from puppies fed deficient diets for 4 months. Only trace amounts of this fatty acid were noted occasionally in skin of the puppies fed lard or corn oil. Arachidonic acid was still present in the serum of all animals.

Figure 4 illustrates the distribution of linoleic, linolenic, 5,8,11-eicosatrienoic and arachidonic acids in the total, triglyceride, phospholipid and cholesterol ester fatty acids of skin from the same 6 groups of dogs after a 4-month feeding period. Linoleic acid was found in all silicic acid fractions. However, in contrast with the usual observation in serum, cholesterol esters in skin did not consistently show the highest percentage of linoleic acid. Also as previously discussed, cholesterol esters differed from other silicic acid fractions in skin in having many unidentified components. Octadecatrienoic acid, which appeared in the total fatty acids of skin from puppies fed butter fat or corn oil, was found in small amounts quite consistently in the triglyceride fraction of skin. The 5,8,11-eicosatrienoic acid was found only in the phospholipids from skin of 2 animals (2/11) fed the low fat diet. Archidonic acid which was prominent only in the total fatty acids in skin of newborns was present in the phospholipids of skin from each group of animals but not always for each animal. In addition, the silicic acid fraction which was composed of free fatty acids, mono- and diglycerides also showed linoleic acid for all animals but arachidonic acid was found infrequently in this fraction.

Fatty acid composition of skin after feeding periods of 6 to 8 months. When the feeding period was continued from 4 to 6 or 8 months with the same diet, there

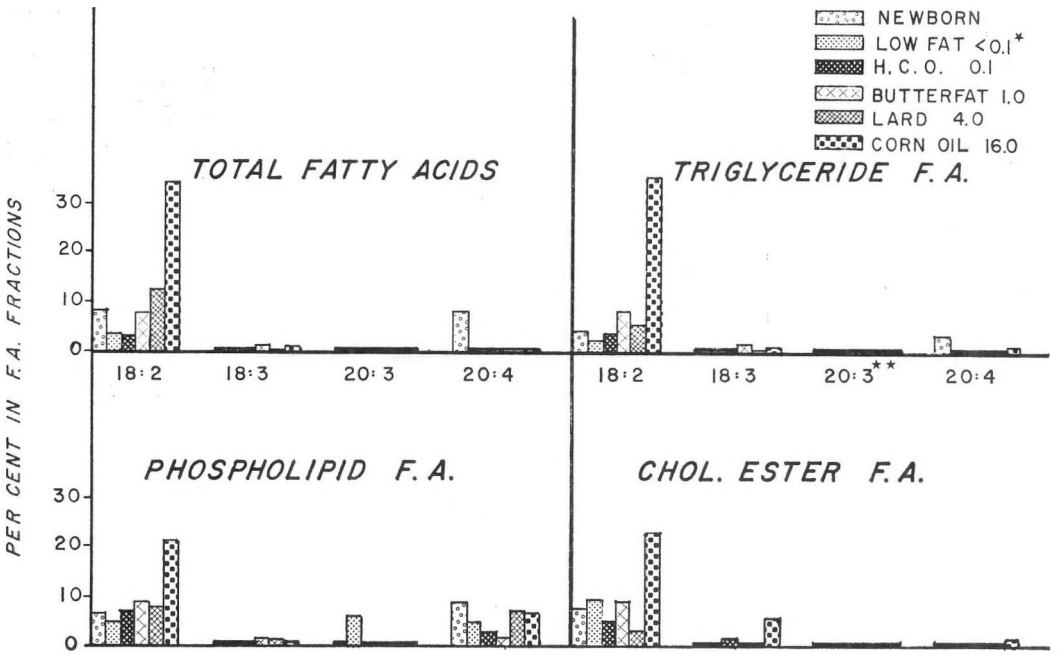

Fig. 4 Unsaturated fatty acids in the total fatty acids, glyceride, phospholipid, and cholesterol ester fatty acids in skin of newborns and after feeding weanling puppies diets with and without fat for 4 months. * dietary linoleic acid as % of total calories; ** 5,8,11-eicosatrienoic acid.

were no major changes in distribution of saturated or unsaturated fatty acids in skin. With diets low in fat, linoleic acid decreased slightly but at no time did it disappear completely from the total fatty acids in skin. Arachidonic acid, however, was found infrequently only in phospholipids of skin after prolonged feeding of a diet lacking linoleic acid. Small amounts of both linoleic and arachidonic acid were present in the total fatty acids of blood serum under all dietary conditions and at all times.

Although there were no marked changes in fatty acid composition of skin during the 4- to 8-month feeding periods, the general condition of the dogs fed diets deficient in linoleic acid became progressively poor after 4 months. After 6 to 8 months there was loss of hair, extensive desquamation, an unkempt appearance and tremulousness. Histologically, the skin showed variable degrees of acanthosis, abnormal keratin, slight-to-moderate epidermal thickening, moderate to numerous sebaceous glands, and round cell infiltration in the upper dermis. Most of the puppies fed butter fat remained in good condition throughout the period of study, but showed some degree of dryness of the skin with very fine desquamation. Histologic changes were minimal with slightly increased density of the keratin and very slight thickening of the epidermis. General health and appearance of the skin and hair were excellent for most of animals fed diets containing lard, soybean lecithin, corn oil or safflower oil which provided 4% or more of the calories as linoleic acid.

Saturated and unsaturated fatty acids in skin and serum after feeding linoleic or arachidonic acids to dogs showing severe signs of linoleic deficiency. To detect early changes in distribution of fatty acids in skin and serum during the period of recovery from essential fatty acid deficiency, linoleic or arachidonic acid was incorporated into the diet of 14 dogs at a level of 2% of the calories for a period of 2 months. Groups of 2 dogs were fed ethyl arachidonate, corn oil, safflower oil, or fresh lard; and groups of 3 dogs were fed trilinolein or soybean lecithin. Figure 5 illustrates the mean levels for saturated, monoene, linoleic, 5,8,11-eicosatrienoic and arachidonic

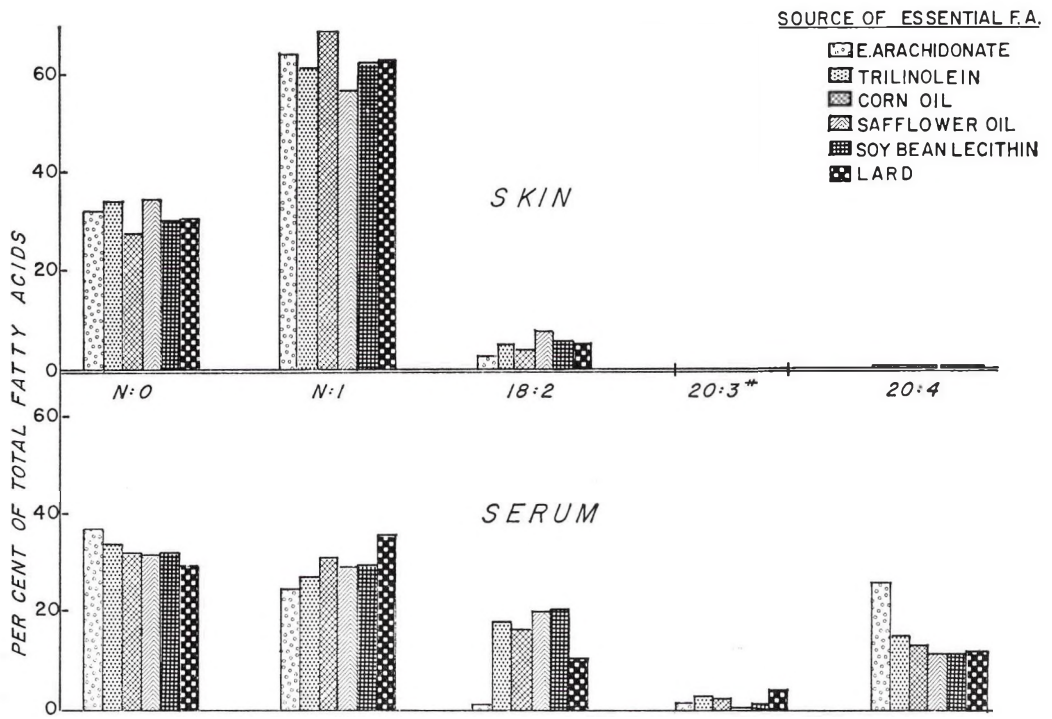

Fig. 5 Saturated and unsaturated fatty acids in the total fatty acids in skin and serum after feeding linoleic acid deficient puppies diets containing ethyl arachidonate, trilinolein, corn oil, safflower oil, soybean lecithin, or lard to provide 2% of the calories as essential fatty acid for 2 months. ++ 5,8,11-eicosatrienoic acid.

acids in the total fatty acids of skin and serum. Levels for monoene fatty acids in skin exceeded those in serum. The relatively low levels for linoleic acid in skin were of the same order of magnitude as those for skin after feeding deficient diets for 4 to 6 months. Except for the 2 dogs fed ethyl arachidonate, serum levels for linoleic acid were much higher than those observed during the deficient state. No 5,8,11-eicosatrienoic or arachidonic acids were identified in the total fatty acids of skin. The presence of small amounts of 5,8,11-eicosatrienoic acid in serum of all animals indicated incomplete recovery from the deficient state. Silicic acid separation of triglycerides, cholesterol esters, and phospholipids in skin showed linoleic acid in all fractions, but 5,8,11-eicosatrienoic acid was not identified in any fraction. Arachidonic acid was noted only occasionally in the phospholipids for animals fed corn oil, soybean lecithin or lard. Interpretation of the presence of arachidonic acid in the phospholipids of skin from these animals as an indication of superior curative effects of corn oil, soybean lecithin and lard over those of safflower oil, trilinolein, and ethyl arachidonate does not appear justified at this time. Gross appearance of all animals showed considerable improvement. Skin in the abdominal area was smooth, soft, and pliable; but extensive desquamation and some loss of hair were still evident in the interscapular area. Histologically, skin from the thigh showed variable degrees of recovery with improved keratin, thinning of the epidermis, and mild acanthosis. Keratin plugs were still evident in many hair follicles. There was little change in the number and size of the sebaceous glands.

Recovery from the deficient state was notably more rapid for animals fed corn oil, safflower oil, or soybean lecithin when linoleate intakes were 5, 8, or 12% of the calories than when the intake was 2% of the calories. Under these conditions, lin-

oleic acid levels in skin were 20 to 30% of the total fatty acids. Arachidonic acid was found occasionally in the phospholipids of skin. It is not clear from the data whether differences in fatty acid composition of the dietary fats and oils other than their linoleic acid content influenced the deposition of linoleic or arachidonic acid in skin.

DISCUSSION

This study was designed to compare fatty acid constituents in healthy skin of growing puppies fed diets containing linoleic or arachidonic acid with those in abnormal skin of puppies fed diets deficient in these essential fatty acids. No attempt was made to follow metabolites of cholesterol synthesis or of the keratinization process. The data represent fatty acids derived from the dermis, the epidermis, and the sebum which also may be affected by the diet.

Linoleic acid levels in skin decreased markedly when the dogs were fed diets deficient in this fatty acid; however, it did not disappear completely from skin or serum even after prolonged deficiency (6 to 8 months). Arachidonic acid, which was present in the total fatty acids of skin from all puppies at the time of birth, was still present in the serum but was not detected in the total fatty acids of skin after the puppies had been fed a diet deficient in linoleate for a period of 4 months. The small amount of arachidonic acid observed occasionally in phospholipids of these skin biopsies was not detectable in the total fatty acids. The relatively high level of arachidonic acid in skin of newborn puppies was of special interest in view of reports for significantly higher levels of this fatty acid in serum of normal infants at birth than in the serum of their mothers during the third trimester of pregnancy (25, 26). Although no correlations were demonstrated by Hansen and co-workers (25) between dietary intakes of 30 mothers during the third trimester and serum lipids in their neonates, arachidonic acid in serum of the mothers was significantly lower in the third trimester than 2 to 9 weeks postpartum. Effects of dietary fat on arachidonic acid metabolism during pregnancy and the significance of high arachidonate levels in tissues of newborns require further investigation.

Triglycerides make up the major lipid fraction in whole skin and reflect the composition of dietary fat to the greatest extent. Constituents of cholesterol esters are similar to those of epidermis and show a high content of unidentified components which are not found in dermis or in the triglyceride fraction of whole skin. Some of these components are observed in normal and in abnormal skin and may represent metabolites of the keratinization process. Phospholipids which make up the smallest fraction in skin reflect fatty acid metabolites that are considered characteristic of the intake of linoleic acid. Both arachidonic acid which normally is synthesized in the body from linoleic acid (27) and 5,8,11-eicosatrienoic acid which is synthesized when the diet is lacking linoleic acid (28) are observed principally in phospholipids of skin. Changes in fatty acid composition in this fraction of skin correlate well with histologic alterations which occur in epidermal cells during a dietary deficiency of linoleic acid. Other silicic acid fractions may reveal intermediate metabolites of linoleic acid. For example, the octadecatrienoic acid found in the triglyceride and cholesterol ester fractions of skin from animals fed butter fat, corn oil, and soybean lecithin may prove to be, in part, an intermediate metabolite in the conversion of linoleic to arachidonic acid (29). Hence, future studies on the role of linoleic acid in the maintenance of healthy skin should include distribution of fatty acid metabolites in the phospholipid, triglyceride, and cholesterol ester fractions of whole skin.

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