THE JOURNAL OF NUTRITION®

OFFICIAL ORGAN OF THE
AMERICAN INSTITUTE OF NUTRITION

VOLUME 91

JANUARY – APRIL 1967

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

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LUCIE RANDOIN

(1885 — 1960)

LUCIE RANDOIN

Lucie Randoin

— A Biographical Sketch

(May 11, 1885 — September 13, 1960)

In spite of the rapid passage of time, the memory of Lucie Randoin remains quite alive and vivid in the minds of her associates and admirers. And for those who did not know her, a brief sketch of her vital work in nutrition will serve to extend this appreciation of her many contributions in a field to which she devoted her life.

In a small village in the middle of a wooded, lonely region of Othe on the border of Bourgogne and Champagne Counties in France, a fair and healthy little girl was born on May 11, 1885, to a well-to-do forester family. Her name was Gabrielle Fandard. The material wealth of her family did not seem to have any influence on her intellect or ambitions. Nor, could anyone foresee during her childhood that it was she who was going to become the renowned Lucie Randoin.

To what measure, exactly, her healthy and fresh environment influenced her, as she often claimed, and to what extent it conditioned her creative mind and temperament, no one knows. However, one thing is certain; she intensely loved the free life of her childhood, the wild and healthy life of the woods and forest. That kind of life was reflected in her facial features. She deliberately used to call it back to her memories by climbing a tree and sitting there for hours reading, studying and relaxing.

When she was seven, however, she moved to Paris, where her parents opened a bookstore at Passy, one of the prettiest boroughs of the Capital. There, later on, Lucie Fandard (Lucie was her middle name, the only one she used later on) prepared outstanding classical studies of the humanities and successfully completed the *Baccalauréat* degree. Next she decided to prepare for the degree *Licence-ès-Sciences* (M.S.) at the Sorbonne and the diploma of *Agrégation*,[1] while providing for her needs by a teaching assistantship. It was during those years of her studies that Lucie Randoin lost her relatively young parents and was obliged to earn her own livelihood.

Because of her brilliant scholarly achievements, she was allowed to attend as a free auditor the *Ecole Normale Supérieure* on Ulm Street. From its founding during the French Revolution this institution had prepared only male *professeurs agrégés*. However, due to the vivid intelligence of Lucie Randoin and her inflexible determination, she became the first woman to be received at the same level on this competitive and most arduous examination of higher education — a meritorious fact which the press of that day did not fail to report.

She continued to be a pioneer until her death — first, by adventuring into the unexplored territories of nutrition, then by effectively initiating projects in order that all could enjoy the benefits of a correct diet.

While she was preparing for her examination of *Agrégation*, she became acquainted with a young geologist, Arthur Randoin. They were married the last day of July in 1914. Two days later general mobilization began and World War I started. Arthur left to defend the country, and Lucie committed herself entirely to science.

In 1918, Arthur returned from the war. He had contracted on the battlefield a serious pulmonary disease, which obliged him for the rest of his life to take cautious care of his health. After a few years, however,

[1] Competitive examination conducted by the State for admission to the post of teaching staff of the Lycées.

he was able to re-enter the field of geology, the profession which he pursued with great conscientiousness. Although the Randoins had no children of their own, they engaged actively in the education of their nephews and grand-nephews.

However, the studies and scientific research of Lucie Randoin did not isolate her from the exterior world. Nevertheless, these pursuits obliged her to turn away from other areas in which she could easily have distinguished herself also. For example, she practiced the art of painting with delicacy. Her literary talents and even poetic ones found their expression in the art of telling anecdotes and short stories, or in her mimicking daily life, or in the writing of her publications, which have high literary value due to the beauty of her style. At various events, such as family or social festivities, her fine literary talents enabled her to compose sensitive poems suited to the occasion.

Although the limits are certainly not sharply defined, three periods are discernible in the life of Lucie Randoin. Each period approximates 25 years' duration.

The first period covers the time from her birth up to 1910. This was the period of her childhood and studies.

The second period could be from 1910 until 1935. This was one of scientific engagement, in which she did basic research. Lucie Randoin analyzed problems and made important contributions to her chosen field.

The last period extended from 1935 to 1960. During this time she taught and disseminated what she had learned. Lucie Randoin communicated to others the enlightenment she had received.

Period of basic research

Unity is certainly one of the outstanding features in the life and work of Lucie Randoin.

First, we shall discuss her unity of environment. For nearly two decades she worked assiduously as a student, then as a teacher in the heart of *Quartier Latin* at the Sorbonne. From 1925 until her death in 1960 — 35 full years — she animated various departments of the *Société Scientifique d'Hygiène Alimentaire* which is located only a few paces from Panthéon. If one considers that she worked also at the Institute of Ocenography with Professor Paul Portier and attended the famous *Ecole Normale Supérieure* in order to prepare for the competitive examination (Diploma of *Agrégation*), it is evident that her arduous toil was performed entirely within a radius of 100 yards.

Then, with respect to the unity of her scientific work, Lucie Randoin devoted her main efforts to the study of vitamins and nutritional imbalances.

From 1909 until 1919 the young graduate worked in the Laboratory of Physiology of the Faculty of Sciences. This Department of Physiology of the Sorbonne was at that time still under the influence of its renowned founder, Claude Bernard. By 1909, however, this great physiologist had long since passed away; nevertheless, the chairman of the Department was one of his last students, Albert Dastre. It was this estimable scientist who guided the young student in the preparation of her graduate studies of the *Diplôme d'Etudes Supérieures*. It was also the same Dastre who advised her to prepare for the diploma of *Agrégation* while she was working on her *Doctorat-ès-Sciences* (Ph.D.) dissertation, which was entitled "Experimental Research on Free Sugar and Sugar Bound to Proteins in the Blood." By the nature of the laboratory where she worked as well as the nature of her research, she should be included in the spiritual descendants of the great Claude Bernard. This kind of rating, which might have been premature in 1911, became justifiable a few years later when her discoveries of the utilization of sugars were recognized as valid in scientific circles.

From the very beginning of her work, Lucie Randoin was so well-established that neither marriage, nor the war, which impaired or even destroyed so many scientific careers, were able to interfere with her scientific endeavors.

In August 1914 the experimental part of her dissertation was almost achieved, but her work here had been interrupted for several years because of her immensely demanding teaching duties at the Sorbonne. Because of the mass departure of men to military service, the laboratories became deserted. The young graduate,

Madame Randoin, gladly accepted the request to assist her aging Master Dastre. By this avenue she successively attained the position of *Préparateur* (Instructor), *Chef de Travaux* (Assistant Professor) and *Maître de Conférence* (Associate Professor).

In 1917 she completed her Ph.D. dissertation and received specific advice from her Master which influenced her entire scientific career. Dastre suggested that she initiate research on vitamins which he predicted would revolutionize the biological sciences. However, it was not with Dastre, who died shortly after having given her that valuable recommendation, but with Portier that Lucie Randoin initiated the line of research which was to make her famous. In 1919, in conjunction with Dr. Portier, she published a short communication of the technique of experimental avitaminosis (vitamin deficiencies) by sterilization of the diet. Her first studies met with general indifference; they were sometimes even the object of mockery in scientific circles. This was because the notion itself of a vitamin was not acceptable to the customary reasoning of that time. With what skepticism and reluctancy, indeed, was the term of "vitamin" accepted — factors detectable only by their absence in the diet!

Because of the criticism she encountered from the most respected scientific circles she began a different branch of research, that pertaining to nutritional balance, although she did not abandon the study of vitamins completely. She defined the principal lines of this second branch of research in terms as follows: "It would be ideal to maintain an approximate balance among the various mineral elements necessary for everyday nutrition . . . the maternal milk is valuable because it contains various useful principles in certain proportions forming a convenient chemical and physical-chemical balance, which is an essential condition of good nutrition. . . ."

She was convinced that the study of vitamins and of nutrition equilibria were the main fields where nutritional research would progress most rapidly.

Studies of vitamins and nutritional balances required the proper selection of experimental animals. This Lucie Randoin handled capably. Beginning in 1922, she developed artificial diets — one devoid of factor C for guinea pigs, the other one lacking factor B only, for pigeons.

These experimental conditions which she patiently established allowed her to detect, and very soon even determine, various vitamin activities in a great number of foods. For example, the study of vitamins in the Mollusques led her to prove the presence of an antiscorbutic factor in the oyster in 1923.

However, Lucie Randoin did not satisfy herself with a simple detection of different principles of the vitamins in various foods, but she sought to perceive their mode of action. She was at this time still very much interested in blood sugars. She questioned whether there could be a relationship between the utilization of sugars and the action of vitamins. From her profound observations on the deficiencies of vitamins C and B she determined that the utilization of sugars was in relationship with the deficiency in factor B.

So, Mme. Randoin, in collaboration with H. Simonet, studied the effect of sugars on the precocity of the appearance of a polyneuritic syndrome, using diets deficient in vitamin B. From this study they concluded that the requirement of vitamin B was not absolute but relative. They indeed showed that its requirement was not only dependent upon the animal itself (species, weight), but was relative to the proportions of one or several nutrients in the diet, particularly to the amount of assimilated sugar.

Today, if one glances backward 40 years and considers the efforts of nutritionists, and dietitians, as well as of housewives, in establishing balanced diets, the undeniable value of the theories published by Mme. Randoin and H. Simonet in 1924 must be recognized. In one of their papers presented at the Academy of Sciences the following reflections appear:

"It is well known that the problem of isodynamics is slightly related by the necessity of a minimum of essential aminoacids and probably also by minimal amounts of fat and carbohydrates. Isn't it limited also in a much narrower measure by the quantitative changes of various

basic principles: some minerals, some vitamins, etc. . . . In other words, is it possible to increase or to reduce the proportions of energetic compounds without changing at the same time one or several non-energetic nutrients of the diet?

"As far as the sugars and the vitamin B are concerned, our answer is no. It is for sure that vitamin B plays a role in the metabolism of sugars . . . We go here beyond the classical definition of the minimal amounts of essential nutrients, and we arrive at the definition of a nutritional balance which is related to the proportions among various basic elementary compounds and those compounds which are sources of energy."

From the observations of deficiency symptoms, Lucie Randoin predicted the existence of unknown factors and described their physiological function long before those factors were chemically defined. It seems appropriate to quote at this time her communication presented to the session of the *Société de Chimie Biologique* where, in 1924, during this initial era on the knowledge of vitamins, she revealed the existence of several vitamins of group B by stating the following:

"The factor B strictly speaking would prevent all the disorders and would allow the utilization of sugars. This is the factor of utilization of one of the energetic substances, but it is only preventive and when it is absent a series of disorders occur, particularly the accumulation of a toxic substance which would cause in a certain time sudden nervous complications.

"When the effects of this disorder are evident, it is too late to administer factor B. An entirely different, antineuritic factor, essentially curative, is needed. It acts rapidly, emptying in some way the body of toxic substances which accumulated in the organism, after which the nervous disturbances do not occur any more, at least for a certain time."

One immediately recognizes in these statements, in the following order, riboflavin, pyruvic acid, and thiamine. The fact that she detected these compounds by their function and relationship is indeed noteworthy. Without being aware of the importance of the problem, she partly explained the mode of action of factor B, utilization of sugars, defined nutritional balance, and demonstrated the plurality of factor B.

Mme. Randoin also carried out a great deal of work on the problem of scurvy and the physico-chemical properties of the antiscorbutic factor. In 1927 with M. R. Lecocq she observed two forms of vitamin C, both active as antiscorbutic factors — today known as reduced and oxidized forms of ascorbic acid.

Lucie Randoin, by her competent animal experimentation, as well as by penetration and the finesse of her observation, successfully worked for a number of years in the field of various vitamin factors which were successively individualized and defined.

For nearly a half century her unceasing scientific activity was crowned with great success. Dr. Randoin was author or co-author of more than 500 papers, abstracts and notes pertaining to the physiology of nutrition and vitaminology. Her studies on vitamins date as far back as 1918.

Her two main works deserve to be mentioned here. The first, "Les Données et les Inconnues du Problème Alimentaire" (in two volumes, with H. Simonet as co-author), was published by Presses Universitaires de France in 1927. The second is entitled "Les Vitamines" (with the same co-author) and the first edition was published by Armand Colin in Paris in 1932; the second edition was issued in 1942 by the same publisher.

However, her scientific production, although still prolific, began to diminish after 1935 as a result of her growing interest in applying her research to the daily problems of social welfare.

Period of practical application

For the last 25 years of her life Lucie Randoin devoted herself to the teaching and dissemination of the nutritional knowledge which she had acquired and unceasingly extended through continuous study and laboratory research.

During this practical application of her knowledge she succeeded in compiling valuable information on the nutritional conditions of various classes of the French population and defining requirements nec-

essary for them to enjoy the benefits of proper nutrition.

What were the circumstances that made such a talented investigator engage herself in a social enterprise? First of all, undoubtedly because she was a woman, she engrossed herself with practical as well as theoretical problems of nutrition. Her work and world-renowned reputation (she represented France at the International Conferences of Standardization of Vitamins held in London both in 1931 and 1934) assured her a sympathetic and powerful support for such a role. Because of her kindness and graciousness, and her bonds of friendship with colleagues whose rise in scientific careers were similar to hers, such as Dean Réné Fabre, she was able to enlist the aid and devotion of these colleagues for her difficult but noble task. The political situation helped also. Due mainly to her efforts, the League of Nations showed interest in the problems of nutrition. In France the situation was also favorable; in 1936 the era of socialism had begun. From 1940, Lucie Randoin encountered the worst nutritional conditions one can imagine and for which she tried to find a remedy.

She established three goals. They were 1) to determine the composition of aliments in order to be able to calculate the nutritional value of various diets; 2) to know the requirements of a population in order to establish limits that available stocks of food could satisfy; and 3) to teach the general public the rules of proper nutrition and how to observe them.

To accomplish each part of this ambitious program, Lucie Randoin forged the necessary tools: tables of chemical composition of the various aliments, nutritional inquiries, teaching and disseminating the knowledge of nutrition.

In order to establish the tables of composition of aliments, Lucie Randoin spent much time on determining the chemical composition of various foods. The broad studies conducted for this goal under her direction received the support of the *Centre National de la Recherche Scientifique* and that of the *Institut National de la Recherche Agronomique*. Since the publication of these tables in 1937, they have contained the most up-to-date material on the composition of foods with respect to various vitamins, minerals, and trace elements.

To investigate the nutritional conditions of various peoples in the world, the *Bureau d'Hygiène* of the League of Nations recommended, in 1935, the foundation of national institutes of nutrition in various member-countries of the League. In France, it was Professor André Mayer, renowned physiologist, who was charged with founding such an institute. It was also decided to organize a nutritional survey for all of France, to begin in 1937. The direction of this enterprise was entrusted to Lucie Randoin.

The goal of the survey was to identify the poor nutritional habits prevalent in various regions of France. It was necessary to know the mistakes made in preparation of food, as well as the nutritional imbalances in order to improve the health of the general public.

Lucie Randoin charged herself with the formation of a survey team which made nutritional inquiries of the French population. These inquiries revealed the nature and the quantity of food consumed. From the results of this survey in Paris, the Central Service calculated the relative ratios of nutrients with the aid of data recorded in the Tables on the Composition of Aliments.

This important and expensive enterprise was interrupted at the height of its activity in 1939 by the war and later by the occupation of French territory. In spite of that fact, Lucie Randoin, with considerably reduced funds and personnel, continued during the war to organize the inquiries, but this time in order to detect the poor food supply of larger cities. She tried to remedy the lack of food by the use of substitutes.

At all times, and not less in war circumstances, it is useful to disclose to the public the physiological and economical benefits of proper nutrition. She not only instructed home economics teachers, but also presented a great number of lectures, conferences, radio broadcasts and, during the last years of her life, telecasts. She produced films, photographic documents and numerous brochures pertaining to the food rations and principles of nutrition.

In her enterprises, she advantageously applied all of her skills and honors. Important were her valuable scientific works, her membership in scientific societies, particularly in the *Académie Nationale de Médecine*, a title held previously by one other woman only, Marie Curie Sklodowska.[2] She also held the high-ranking position of Secretary General of the *Société Scientifique d'Hygiène Alimentaire*, as well as offices in the *Société de Chimie Biologique*. She was Editor-in-Chief of the Bulletin of the *Société de Chimie Biologique* from 1932 to 1942, and in 1945 was elected the President of the Society. A year before her death she was nominated by the President of the Republic for the *Commandeur de la Légion d'Honneur*, an award given only for exceptionally distinguished work.

In 1938, Dr. Randoin founded, in Paris, an institute of nutrition of higher education (*Institut Supérieur de l'Alimentation*) for the training of professors of home economics. Fifteen thousand students have attended the courses of the Institute since its founding. She recommended that nutrition be included in the teaching program of all elementary and secondary schools. In collaboration with Professor Tremolières, she founded, also in Paris, the only French school of dietetics (*Ecole de Diétetique*), which has produced more than 500 dietitians since its founding in 1951. The personal satisfaction for her numerous endeavors, never discouraged and never abandoned, was derived from the founding of this school of dietetics. This school in her eye embodied the best way to advance nutritional science which she served indefatigably for more than half a century with her imagination, experience, word and writings.

Lucie Randoin's death was due to an incurable illness from which she suffered for more than two years. Nevertheless, because of her extraordinary energy and will-power, she continued to assume her professional tasks until three months before her death. She was survived by her husband, who died less than three years later.

One can ask what remains today of the work of Lucie Randoin. So much was taken and applied from her ideas without giving credit to source. Many roads in research which she opened, and their usage, which she stimulated, have been so much broadened that one cannot recognize her as their founder or discoverer. After all, none had lived more than Lucie Randoin the challenging adventure of nutrition.

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Comparative Studies of the Metabolism of ^{14}C-Pyrimidine-labeled Thiamine, ^{14}C-Thiazole-labeled Thiamine and ^{35}S-labeled Thiamine in the Rat [1]

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ABSTRACT The nature of the urinary products of thiamine catabolism was studied in rats, using 3 species of radioactive thiamine. Adult rats were injected with ^{14}C-pyrimidine-labeled thiamine, ^{14}C-thiazole-labeled thiamine, or ^{35}S-thiazole-labeled thiamine, and the chromatographic patterns of urinary radioactive metabolites were compared. Of the 4 major unidentified radioactive metabolites found in urine (more than 20 were detected), all contained the pyrimidine ring and at least a portion of the thiazole ring. One compound contained the 2-carbon of thiazole but not the sulfur atom. One compound (designated as V-1) contained both moieties of the molecule, did not support the growth of *Lactobacillus viridescens*, was not converted to thiochrome, and was not cleaved by the bisulfite reaction. There was no evidence for the presence of free thiazole or thiazole-like compounds in the urine. A speculative sequence for the catabolism of thiamine by the rat based on these data is presented.

The detection of more than 20 different metabolites of thiamine in the urine of rats receiving daily intraperitoneal injections of thiamine labeled with ^{14}C in the pyrimidine ring or with ^{14}C-thiazole-labeled thiamine has been reported in previous publications (1–3). In the present study the urinary metabolic excretion patterns obtained with ^{14}C-pyrimidine-labeled thiamine, ^{14}C-thiazole-labeled thiamine and ^{35}S-thiazole-labeled thiamine were compared. This procedure permitted the classification of the numerous urinary thiamine metabolites into 3 groups, those containing the pyrimidine moiety only, those containing the thiazole moiety only and those containing both moieties of the molecule. Furthermore, the use of 2 species of thiamine labeled in the thiazole moiety permitted certain generalizations to be made about the metabolism of this portion of the thiamine molecule. Further attempts to elucidate the structure of one of the major urinary metabolites of thiamine are also described.

MATERIALS AND METHODS

The techniques used in the fractionation of urine into 5 radioactive peaks by column chromatography on Amberlite CG-50 [4] and subsequent paper chromatography of each peak have been detailed in our previous publications (1–3). Pyrimidine-labeled thiamine was synthesized as described by Neal and Pearson (1) and had a specific activity of 1200 cpm/μg. The ^{14}C-thiazole-labeled thiamine was obtained commercially [5] and had a specific activity of 5600 cpm/μg. The ^{35}S-thiazole-labeled-thiamine was also obtained commercially [6] and on receipt had a specific activity of 15,000 cpm/μg. All radioactivity measurements were corrected for quenching by use of an internal standard. In general, weanling rats were fed a thiamine-deficient diet for 2 weeks, after which time the daily injections of one species of radioactive thiamine were begun. These injections were contin-

Received for publication June 24, 1966.
[1] This study was supported by Public Health Service Research Grants no. AM-5441 and AM-07709 from the National Institute of Arthritis and Metabolic Diseases.
[2] A portion of these data was taken from a thesis submitted by Mesbaheddin Balaghi to the graduate faculty of Vanderbilt University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry.
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[4] Mallinckrodt Chemical Company, Milwaukee, Wisconsin.
[5] Nuclear Chicago, Inc., Chicago.
[6] See footnote 5.

ued for at least 2 weeks to ensure reasonable saturation of the tissues with radioactive thiamine and then the comparative studies were commenced. The daily dosage level (intraperitoneal injection) of the ^{14}C-thiazole-labeled thiamine and the ^{35}S-thiazole-labeled thiamine was 40 μg/day. The dosage level of the ^{14}C-pyrimidine-labeled thiamine was 50 μg/day.

RESULTS AND DISCUSSION

The overall metabolism of ^{14}C-pyrimidine and ^{14}C-thiazole-labeled thiamine by the rat have been described previously (1, 3). Only that of ^{35}S-thiazole-labeled thiamine will be discussed here.

In the studies with ^{35}S-thiamine, 6 adult rats of the Sprague-Dawley strain were injected daily with 40 μg of the labeled compound after a 2-week depletion period. Three and one-half per cent of the daily injected radioactivity appeared in the urine during the first 24 hours after injection. This increased to 21.9% by the end of the first week and to 33.3% by the end of the second week. The mean daily group excretion for the last 18 days of a 4-week injection period was 41.7 ± 6.4% of the daily dose. The fecal radioactivity for the entire group was measured twice during the study — each time for a one-week period. The daily group excretions of radioactivity during these 2 periods were 15.3 and 12.7% of the daily dose, respectively. Although a certain error in the collection of urine and feces is unavoidable (about 10%), these rats were retaining at least 30% of their daily dose.

Figure 1 shows typical chromatographic patterns obtained by Amberlite CG-50 fractionation of urine obtained from rats injected with ^{35}S-thiamine, ^{14}C-pyrimidine-labeled thiamine or ^{14}C-thiazole-labeled thiamine. Comparison of the 3 elution patterns shows a single major difference. That is, peak II is completely absent from the urine of rats injected with ^{35}S-thiazole thiamine. It is also evident that peak I from the urine of the rats treated with ^{14}C-pyrimidine-labeled thiamine may actually consist of 2 components which contain more of the total radioactivity than the same peak from the urine of rats injected with ^{14}C-thiazole-labeled thiamine. Peak I from the urine of rats injected with ^{35}S-thiazole-labeled thiamine contains a higher proportion of the total radioactivity than the same peak isolated from the urine of rats injected with the ^{14}C-labeled thiamine. Except for these differences, the distribution of radioactivity in the other peaks (III, IV, V) obtained with the 3 forms of radioactive thiamine is remarkably similar.

Paper chromatography of peaks II, IV and V obtained from the urine of animals injected with ^{35}S-thiamine gave radioautographs that were similar to those obtained in the corresponding experiment with ^{14}C-thiazole-labeled thiamine, i.e., 10 radioactive bands were found in peak II, 2 in peak IV and 4 in peak V. These bands had similar shapes, R_F values and film densities. Paper chromatography of peaks II, III, IV and V from animals injected with ^{14}C-pyrimidine-labeled thiamine also gave radioautographic patterns that were virtually identical with those obtained with ^{14}C-thiazole-labeled thiamine.

In order to perform the radioautographic studies in as precise a manner as possible, the corresponding thiazole-labeled and pyrimidine-labeled peaks were chromatographed side by side on the same sheet of paper and a radioautograph was made. Figure 2 shows the result of such an experiment with peak II. In this study, an amount of ^{14}C-thiazole-labeled peak II containing about 30,000 cpm was spotted next to a spot containing a mixture of the same sample (15,000 cpm) and an equivalent number of counts of ^{14}C-pyrimidine-labeled peak II. Ascending paper chromatography was then carried out (*n*-propanol/1 M acetate buffer, pH 5.0/H_2O, 70/10/20) for 18 hours, the paper was dried and a radioautograph was made. The 3 spots which appeared have identical R_F values in both samples and are of similar shape. This shows a quantitative and qualitative similarity between the thiazole-labeled and pyrimidine-labeled compounds in peak II and indicates that modified forms of the entire molecule are present.

Figure 3 shows a similar experiment which compares the radioactive bands found in peak III for rats given pyrimidine- and thiazole-labeled thiamine. Although there are some differences in the minor components of these peaks, the bands

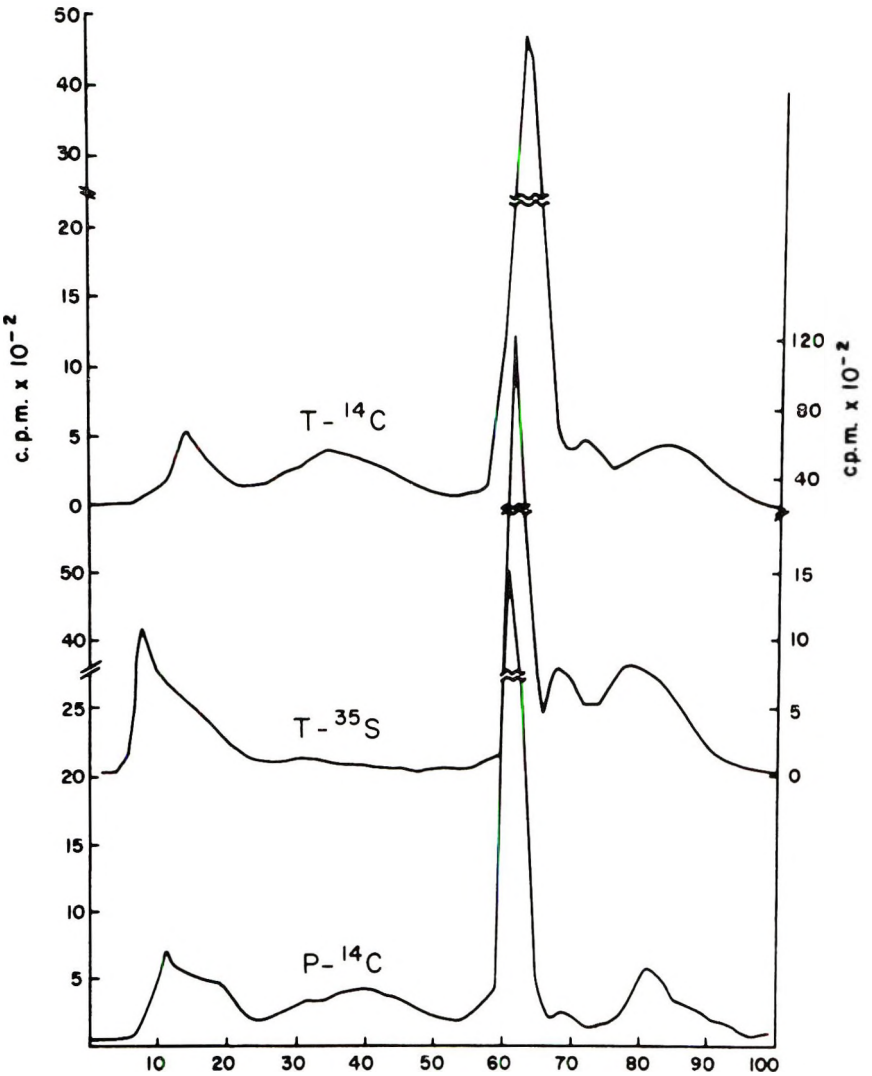

Fig. 1 Comparison of the chromatographic behavior of the urinary metabolites of thiamine with 3 different forms of radioactive thiamine. Exchanger, Amberlite CG-50 (200–400 mesh), 1.0 × 4.0 cm, in the H^+ form. Flow rate 60 ml/hour, fraction size 5 ml.
T-^{14}C: Elution pattern of urinary metabolites of thiazole-^{14}C-thiamine.
T-^{35}S: Elution pattern of urinary metabolites of thiazole-^{35}S-thiamine.
P-^{14}C: Elution pattern of urinary metabolites of pyrimidine-^{14}C-thiamine.

which contain the bulk of the radioactivity have the same approximate R_F values in peaks for thiazole-labeled and pyrimidine-labeled thiamine. At this point in our studies, however, only a gross similarity can be claimed. The reduced number of radiometabolites visualized in this particular radioautogram can be ascribed to the relatively low radioactivity and short film-exposure time.

A similar comparison of the radioactive bands in peak V is shown in figure 4. An aliquot of thiazole-labeled peak V containing 70,000 cpm was spotted on the left, and a similar amount of pyrimidine labeled peak V was spotted on the right side of the chromatogram paper. In the middle, a mixture containing 35,000 cpm of each of the 2 preparations was spotted. The chromatography was carried out in the

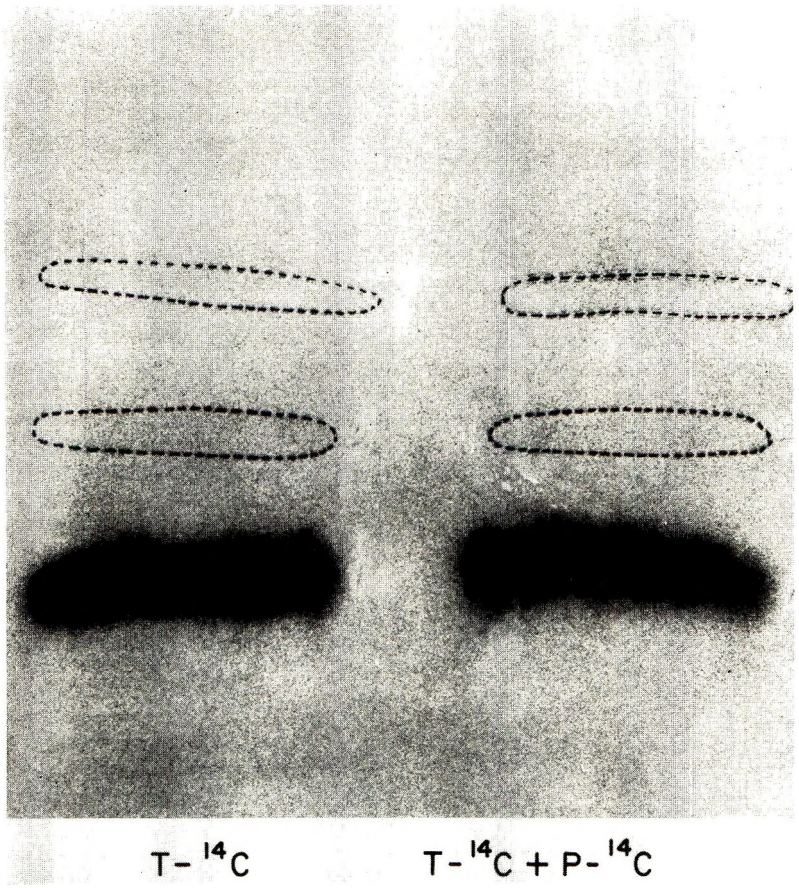

Fig. 2 A photograph of a radioautogram showing the chromatography of peak II from the Amberlite CG-50 chromatography of urinary metabolites of ^{14}C-thiazole-labeled thiamine (T-^{14}C), and a mixture of equal amounts of the same preparation and the corresponding peak obtained with ^{14}C-pyrimidine-labeled thiamine (T-^{14}C + P-^{14}C). The chromatogram was developed for 18 hours in a mixture of *n*-propanol/1 M acetate buffer, pH 5.0/water (70/10/20).

n-propanol system described previously. Although one of the minor spots in the pyrimidine-labeled peak shows a different R_F, the fast-moving compound which accounted for the major part of the radioactivity shows the same behavior and has the same quantitative importance in all 3 samples. This compound will be designated as V-1 during the remainder of this publication.

Some properties of compound V-1. Because this compound was quantitatively important, was cleanly separated on paper chromatography and was accompanied by very small amounts of colored extraneous material, some of its properties were studied as follows:

(a) Ultraviolet absorption spectrum. An amount of this compound equal in radioactivity to 20 μg of thiamine was purified by column chromatography, and by paper chromatography first in the *n*-propanol system and then in a system composed of isopropanol/0.2 M acetate buffer/water (65/15/20). The compound was visualized by radioautography, eluted, lyophilized, dissolved in 1 ml of water and extracted with 1.0 ml of dry ethyl ether to remove impurities absorbing in the ultraviolet region. The pH was then adjusted to 6.0, and the com-

pound was washed again with another volume of dry ethyl ether. The sample was then diluted to 2.0 ml with distilled water and transferred to a 3-ml quartz cuvette. A piece of filter paper of the same size and shape was cut from an adjacent area of the same chromatogram and was carried through the same procedure as the sample to serve as the photometric blank. An ultraviolet spectrum was obtained in a Cary continuous recording spectrophotometer at pH 6.0, then 0.2 ml of 2 N hydrochloric acid was added and a second spectrum was recorded. These spectra are shown in figures 5 and 6 along with the spectra of authentic thiamine obtained under the same conditions. The "shoulder" at approximately 260 mμ is characteristic of the thiazole moiety of the molecule, and is more pronounced at pH 6.0 because of the marked shift of the major pyrimidine absorption peak.

(b) Bisulfite cleavage. One of the properties of thiamine and some of its related compounds is susceptibility to bisulfite cleavage. This reaction breaks the molecule at the methylene bridge and yields information concerning the type of linkage connecting the thiazole and pyrimidine rings because its success depends upon the presence of an electron-deficient methylene carbon susceptible to nucleophilic attack by bisulfite. An amount of compound V-1 equivalent to 50 μg of thiamine was

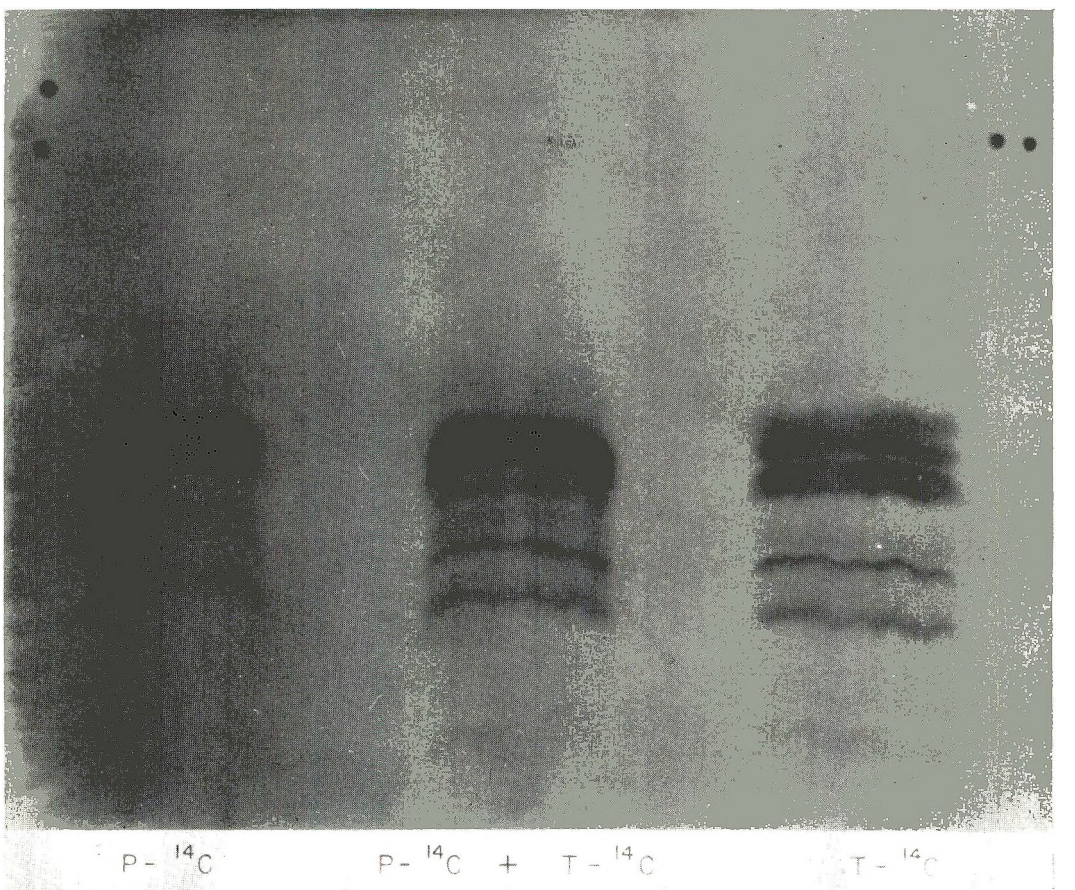

Fig. 3 A photograph of the radioautogram showing the co-chromatography of peak III from the Amberlite CG-50 chromatography of urinary metabolites of ^{14}C-thiazole-labeled thiamine (T-^{14}C), ^{14}C-pyrimidine-labeled thiamine (P-^{14}C), and a mixture of equal amounts of these 2 preparations (P-^{14}C + T-^{14}C). The chromatogram was developed for 18 hours in a mixture of *n*-propanol/1 M acetate buffer, pH 5.0/water (70/10/20).

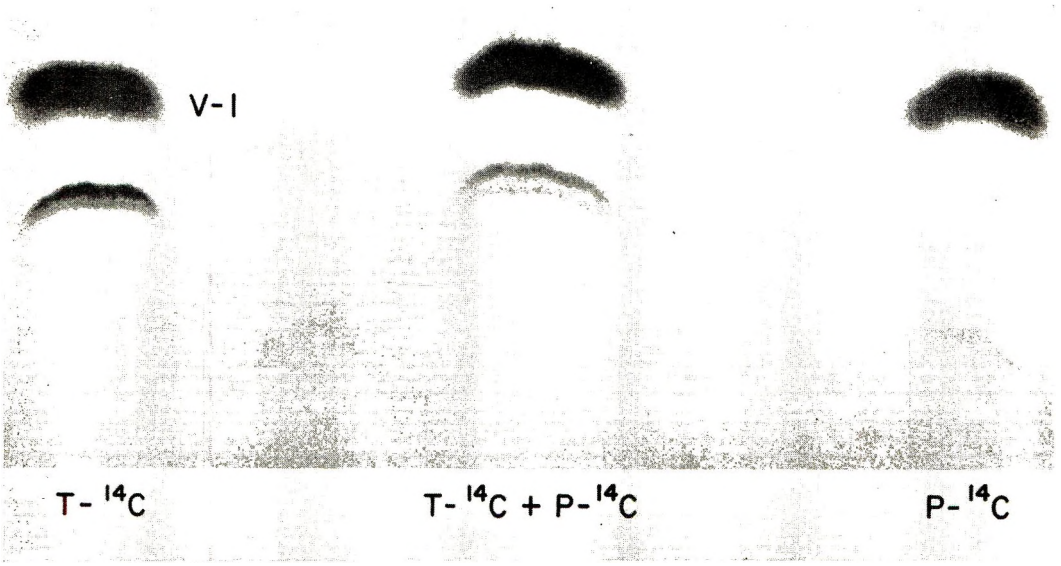

Fig. 4 A photograph of a radioautogram showing the co-chromatography of peak V from the Amberlite CG-50 chromatography of ^{14}C-thiazole-labeled thiamine (T-^{14}C), ^{14}C-pyrimidine-labeled thiamine (P-^{14}C), and a mixture of equal amounts of these 2 preparations (T-^{14}C + P-^{14}C). The chromatogram was developed for 18 hours in a mixture of *n*-propanol/1 M acetate buffer, pH 5.0/water (70/10/20).

treated with bisulfite according to the method of Williams et al. (4) and the reaction mixture was subjected to ascending paper chromatography in isopropanol/ammonium hydroxide/water (225/3.9/45). The same amount of the untreated compound was chromatographed as a control. Radioautography revealed that the R_F values of the bisulfite treated and untreated fractions of compound V-1 were identical (0.81). Observation of the chromatogram under an ultraviolet light showed a single ultraviolet absorbing spot in both the bisulfite treated sample and the control sample which coincided with the radioactive spot located by radioautography. A similar experiment with ^{14}C-pyrimidine-labeled compound V-1 gave identical results. The R_F values of the bisulfite-treated compound labeled in either the thiazole or pyrimidine moiety and the untreated compound were also similar in the *n*-propanol solvent system. It was thus concluded that compound V-1 was not susceptible to conventional bisulfite cleavage since under similar conditions thiamine was readily cleaved and was easily resolved into 2 ultraviolet absorbing spots on a paper chromatogram.

(c) Thiochrome reaction. Since the formation of thiochrome from thiamine requires certain structural properties (presence of both the pyrimidine and thiazole rings, free amino group on C-4 of the pyrimidine ring, unsubstituted C-2 in the thiazole ring), it seemed useful to learn whether compound V-1 could be converted to a thiochrome type compound. A chromatogram containing a spot of this compound equivalent in radioactivity to approximately 10 μg of thiamine was cut so that the spot was bisected. One-half of the chromatogram was sprayed with alkaline ferricyanide solution (95% ethanol/10% sodium hydroxide/2.5% potassium ferricyanide, 40/20/1 by volume) and dried. When the 2 parts of the paper were reconstituted and the restored halves of the spot were compared under ultraviolet light, no fluorescent increase in the sprayed half was observed. One microgram of thiamine hydrochloride spotted on the same paper and sprayed with the same solution gave the characteristic blue fluorescence of thiochrome.

(d) Ninhydrin reaction. The ultraviolet absorption spectrum of compound V-1,

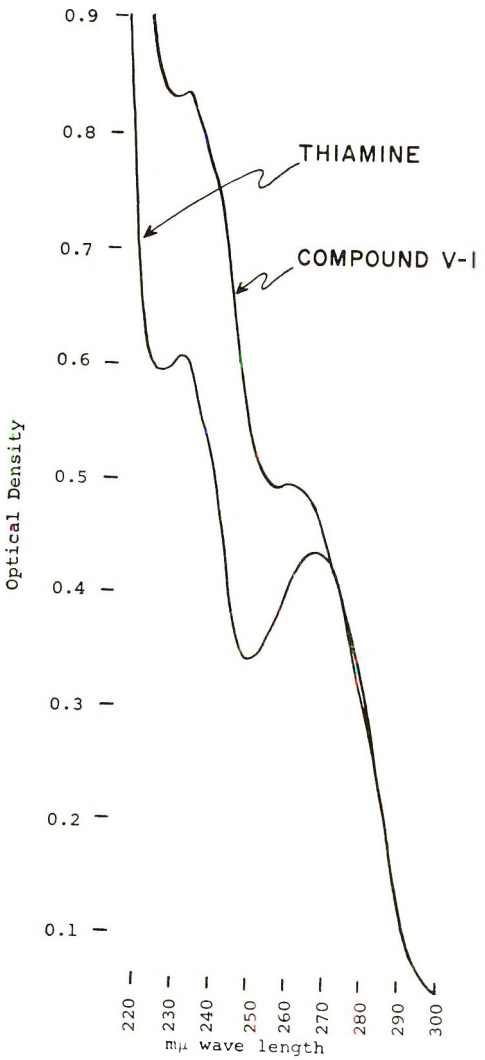

Fig. 5 A comparison of the ultraviolet absorption spectra of metabolite V-1 and thiamine at pH 6.0.

(figs. 5 and 6) suggested the presence of a thiazole ring. Although it appeared unlikely that the compound was a thiol form of thiamine conjugated to an amino acid through a disulfide linkage this possibility was investigated by spraying with ninhydrin reagent a chromatogram containing an amount of this compound equivalent to 50 μg of thiamine. The chromatogram was heated at 80° for 10 minutes, but no ninhydrin-positive reaction was detected.

(e) Paper chromatographic comparison with known thiamine derivatives. The R_F value of a number of thiamine derivatives was compared with that of compound V-1 by paper chromatography in the *n*-propanol solvent system. Table 1 shows the R_F values obtained. None of the compounds tested had an R_F value comparable to that of compound V-1.

DISCUSSION

The greater percentage of radioactivity found in peak I isolated from the urine of rats injected with ^{14}C-pyrimidine thiamine

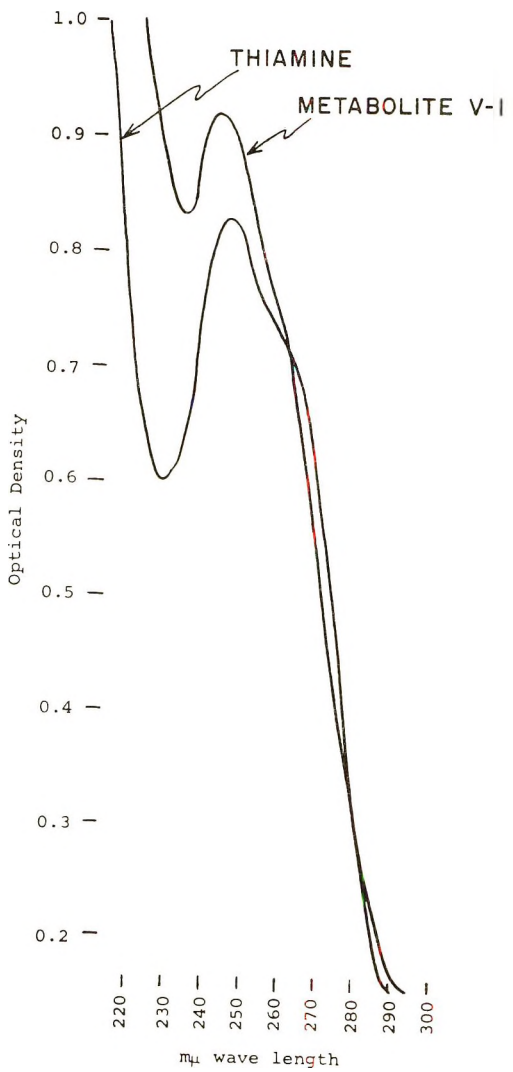

Fig. 6 A comparison of the ultraviolet absorption spectra of metabolite V-1 and thiamine hydrochloride, after acidification.

TABLE 1

R_F values of some thiamine compounds and of metabolite V-1 in an n-propanol/water/1 M acetate buffer, pH 5.0 (70/20/10) solvent system

Compound [1]	R_F
Metabolite V-1	0.64
Thiamine monophosphate	0.16
Thiamine pyrophosphate	0.08
Thiochrome	0.56
Thiamine disulfide	0.38

[1] Each compound was subjected to ascending paper chromatography for 18 hours and the spots were localized by observation of the quenched areas under ultraviolet light.

than that found in the urine of rats injected with ^{14}C-thiazole thiamine was not unexpected. One of the principal components of peak I, ^{14}C-pyrimidinecarboxylic acid, (2) is not radioactive if the ^{14}C is located in the thiazole ring. The observation of a greater percentage of radioactivity in this peak in rats injected with ^{35}S-thiazole thiamine is unexplained. Perhaps inorganic ^{35}S compounds or ^{35}S-labeled thiamine derivatives devoid of the thiazole 2-carbon appear in this peak, but we have not examined this possibility.

The complete absence of peak II from the urine of rats injected with ^{35}S-thiazole thiamine deserves special comment. The radioactivity in this peak is contained primarily in a single well-defined spot when urine from rats injected with ^{14}C-pyrimidine or ^{14}C-thiazole-labeled thiamine is fractionated. There are several possible explanations for its absence in ^{35}S-thiazole-labeled thiamine rat urine. The first possibility is that ^{35}S-labeled compounds of peak II were either not adsorbed in the charcoal adsorption step or not eluted from the charcoal by the pyridine-ethanol elution mixture. We consider this unlikely, because approximately 95% of the radioactivity originally present in the urine could be accounted for in the pyridine-ethanol eluate. The second possibility is that the compounds in peak II appear in the urine later because of a lower turnover rate. This explanation was also discarded because re-examination of the data obtained with ^{14}C-thiazole-labeled thiamine showed this peak to be present in the urine collected during the first week of the injection. The third possibility, and the one we favor, is that the main component of this peak is a thiamine metabolite which lacks the sulfur atom. Desulfation is a well-known mammalian mechanism for the catabolism of organic sulfur compounds (5) and Borsook et al. (6) have found that up to 25% of the sulfur contained in ^{35}S-labeled thiamine appears in human urine as inorganic sulfate. Thus the sulfur atom must be removed from the thiamine molecule somewhere in the catabolic sequence.

It was pointed out previously that the following 6 compounds account for most of the radioactivity found in urine: 1) pyrimidinecarboxylic acid (peak I); 2) the principal compound in peak II; 3) two compounds in peak III; 4) thiamine (peak IV); and 5) the principal compound in peak V ("V-1").

Thus, of these 6 metabolites, two have been identified and four are still unidentified. The close similarity between the column chromatographic patterns observed with pyrimidine-labeled and thiazole-labeled thiamine indicates that these 4 unidentified compounds (peaks II, III, IV and V) are thiamine derivatives having both pyrimidine and thiazole rings. This point is illustrated in a more convincing manner by the results of the chromatography experiments (figs. 2, 3 and 4). The two main compounds in peak III and V-1 contain the 2-carbon of both thiazole and thiazole sulfur. One main compound (peak II) lacks the sulfur atom but contains the thiazole 2-carbon.

All of the radioactive compounds found on paper chromatograms except for one compound in peak III and compound V-1 displayed traces of biological activity for *Lactobacillus viridescens*. This observation also supports the premise that the major thiamine metabolites in urine contain both moieties of thiamine because this organism is known to respond only to preformed thiamine, i.e., it will not respond to either moiety separately or together.

Of particular note was our failure to identify free thiazole as a principal metabolite of thiamine. Authentic thiazole appears in peak III in our column chromatographic system, but on paper chromatography its R_F value in the isopropanol system is 0.9. A radioactive band in this

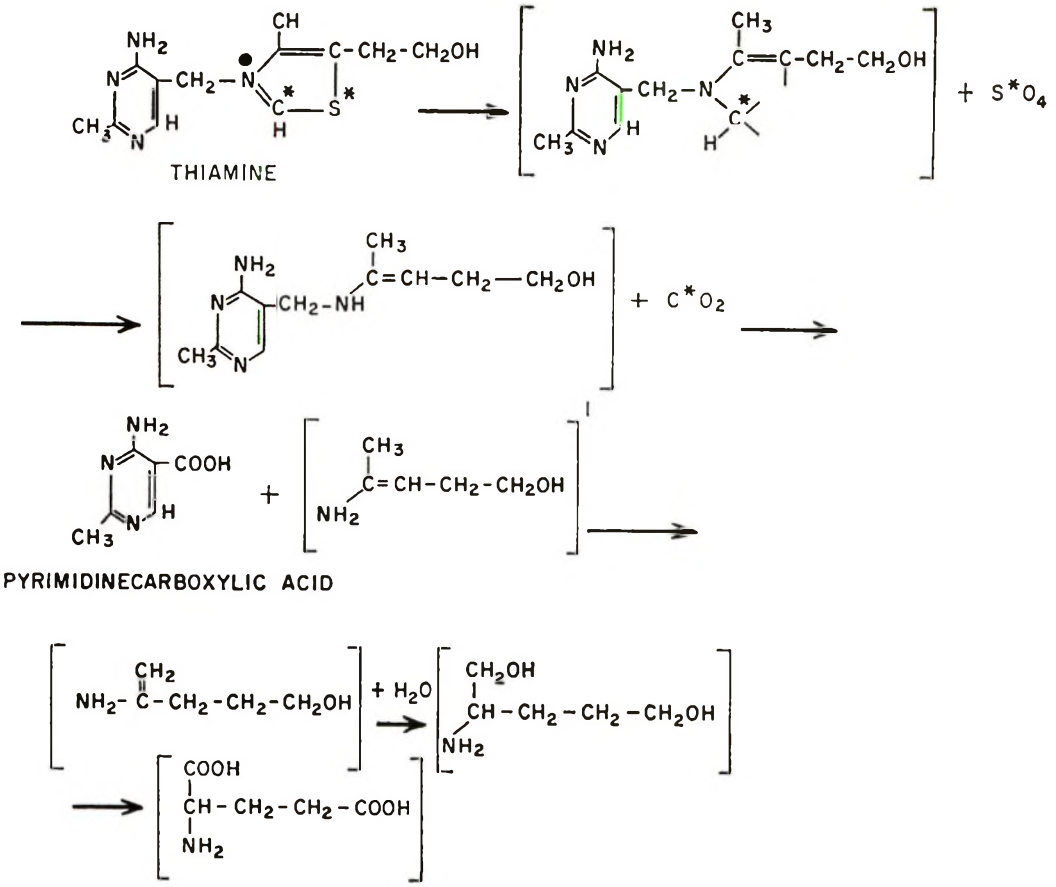

Fig. 7 A speculative sequence for thiamine catabolism in the rat. The compound indicated by superscript 1 is unstable and in addition to its proposed conversion in the above scheme, it is probable it can be converted to the corresponding imino and keto analogue.

area has never been observed. A few distinct metabolites of the thiazole-labeled compound, of low R_F value, that are not present when the pyrimidine labeled compound is used, do appear in rat urine (peak III) but they are present in negligible quantities.

The absence of an equivalent quantity of a thiazole counterpart for pyrimidinecarboxylic acid, when considered with the conversion of the sulfur atom to inorganic sulfate and the conversion of the 2-carbon of the thiazole ring to CO_2 (3) suggests that these fragments are derived from the same molecule. The quantitative aspects are roughly comparable. Pyrimidinecarboxylic acid may account for up to 20% of the intake (7) and carbon dioxide accounts for 7 to 24% of the intake.[7] In the human ^{35}S-sulfate is known to account for up to 25% of thiamine intake (6). Thus, if about 20% of the thiamine intake is converted to pyrimidinecarboxylic acid after the breakdown of the thiazole ring, then the first step of the breakdown might logically be removal of the sulfur atom. The appearance of the principal compound in peak II which lacks the sulfur atom but still contains the 2-carbon of the thiazole ring may be considered as evidence for this suggested step. The next step might be the removal of the 2-carbon of thiazole to yield CO_2. After these 2 steps it may be postulated that the re-

[7] Unpublished observations.

mainder of the molecule is cleaved to 2-methyl-4-amino-5-hydroxymethyl pyrimidine and an aliphatic, unsaturated amino alcohol. The former is probably converted quantitatively to pyrimidinecarboxylic acid or to other compounds since it does not appear in urine (2). It is suggested that the amino alcohol formed might be converted to glutamic acid according to the speculative scheme shown in figure 7.

The observation that compound V-1 appears in urine when either pyrimidine or thiazole-labeled thiamine are used indicates that it contains both moieties of the molecule. This is confirmed by the ultraviolet absorption data which indicate the presence of an intact thiazole moiety. The resistance to bisulfite cleavage, however, suggests that the type of connection between these 2 rings in this compound differs from that found in thiamine or that the rings are so modified that cleavage will not occur. The behavior of the compound on chromatography on Amberlite CG-50 suggests that the primary amino group on the 4-carbon of the pyrimidine ring is intact. The ultraviolet absorption spectrum of the compound also favors this possibility. A substitution on the 2-position of the thiazole ring could result in a lack of biological activity, a negative thiochrome reaction, and perhaps in the failure of bisulfite cleavage, but we have no evidence indicating that such a substitution has taken place.

Whether the metabolic pathways for thiamine we have described in the rat also occur in the human remains to be learned. The nature of the thiamine metabolites found in human urine is still obscure despite the recent publications by Ziporin et al. (8, 9) who measured the excretion of thiamine and "thiamine metabolites" by men fed diets low in thiamine. These investigators consider "pyrimidine" and "thiazole" to be the principal urinary metabolites of thiamine found in urine and measure their presence by the "yeast coupling" procedure (10). In their studies the urinary excretion of thiamine decreased rapidly as the experiment progressed but, paradoxically, the excretion of metabolites increased. This raises serious misgivings about the nature of the substances being measured in urine by the yeast-coupling procedure. Rats whose body stores are uniformly labeled with radioactive thiamine do not continue to metabolize thiamine in an amount consistent with tissue saturation when fed a thiamine-deficient diet. Their excretion of urinary metabolites decreases rapidly beginning 24 hours after the dietary change (7). Moreover, if the thiamine needs of rats were calculated on the basis of the rate of metabolite excretion as used by Ziporin et al. (9) for the human, a different value would be obtained at every level of intake. It is possible that the metabolism of thiamine in the rat differs markedly from that in the human, but we consider this to be unlikely. Preliminary studies of the urinary excretion pattern in the primate reveal a marked resemblance to that in the rat,[8] and Baker et al.[9] have recently reported a similar pattern in man.

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Thiamine Deficiency, Infantile Manipulation and Startle Response in Rats [1]

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ABSTRACT Two experiments were designed to reveal changes in startle response to electric shock in preclinically thiamine-deficient rats. In experiment 1, it was demonstrated that increased D_S (specific displacement or vertical displacement following electric shock/100 g body weight) occurred in rats following thiamine depletion for 10 days. A similar effect was observed with infantile manipulation, but combining both factors ameliorated the effect of the vitamin B_1-deficiency. In experiment 2, the effect of electric shock on D_S was measured in normal animals and in animals depleted for 4, 7, 10, and 13 days. Although there were no significant differences in body weight, thereby suggesting a state of preclinical deficiency, the transketolase activity of erythrocytes was progressively diminished, and D_S progressively increased from the fourth day. The measurement of D_S may provide an additional behavioral parameter for marginal nutritional deficiency in rats.

Thiamine, vitamin B_1, is essential for the metabolism of carbohydrates. It is the coenzyme of cocarboxylase, in the decarboxylation of pyruvic acid (1), and is therefore needed for the liberation of energy. Physiologically it is an essential factor for maintaining nerve function (2), and in rats, thiamine deficiency produces various neurological symptoms, such as ataxia and polyneuritis after periods of depletion of from 21 to 28 days. Preclinical biochemical effects have also been demonstrated, however. Although growth continues for 10 to 14 days in rats, it has been shown by Brin et al. (3–5) that a biochemical lesion occurs in the activity of the transketolase enzyme after as short a period as 5 days of complete thiamine deprivation in rats, and after 10 days of partial deprivation in man.

Earlier studies on rat behavior in nutritional deficiency revealed increased spontaneous activity in thiamine-deficient rats, at about the time growth ceased (6, 7), suggesting possible behavioral correlates of early thiamine deficiency, just as the transketolase defect appeared preclinically. Also, infantile manipulation of the neonatal rat has been shown to have profound physiological and behavioral effects in the adult animal by Levine et al. (8–10) and by Newton (11, 12). These animals gained more weight, consumed greater quantities of food, had shorter latencies in movement initiation, responded more readily to stress than controls, and showed a significant delay in mortality when tumor-bearing.

The present study was devised to test the responsiveness of preclinically thiamine-deficient rats, when infantile manipulated (IM) and nonmanipulated, to mild electric shock, and to measure the effects as the deficiency became more severe.[5]

MATERIALS AND METHODS

The startle response was measured by the use of a device which both shocked the rat and recorded its response. A clear plastic box was mounted on the platform of a Hanson 2.3-kg (5-pound) scale. Nine 0.6-cm brass rods constituted an electric floor grid which was wired in experiment 1 so that alternate bars were of opposite polarity. In experiment 2, the apparatus

Received for publication July 19, 1966.
[1] Supported in part by Public Health Service Grant no. AM-03127 from the National Institute of Arthritis and Metabolic Diseases, and Mead Johnson Laboratories, Inc., Evansville, Indiana.
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[5] Preliminary report presented at the annual meeting of the American Institute of Nutrition, Atlantic City, New Jersey, 1966 (Federation Proc., 17: 245, 1966, abstract).

was altered so that the grid and a tail clip were of opposite polarity. The power source was a Grass D.C. stimulator. The platform of the scale was connected to a force displacement transducer for which the readout was a Grass polygraph. The apparatus was calibrated so that a deflection of 1 mm on the polygraph recording paper corresponded to a force displacement of 12 g.

Rats were chosen at random, weighed, the paws cleaned of feces and urine, and then the animals were placed in the testing box. Following a 2-minute adaptation period, the rat was given 100-v pulsating monophasic D.C. shocks of 0.1-second duration. Thirty seconds later it then received the first of six 34-v shocks of the same duration. These were separated to avoid anticipation by intervals of 15, 45, 30, 15 and 45 seconds, respectively. The response which was measured, therefore, was the downward force in grams exerted by the rat when shocked. Data are expressed as specific displacement (D_S), which is calculated to be equal to the average vertical displacement per shock per 100 g body weight.

In experiment 1, pregnant, albino rats of Cesarean-derived stock were isolated in standard nesting cages. One day after birth the litters were culled to 9 pups each. Pups of half of the litters were removed from the warm nest with a pair of covered tweezers and placed in separate cardboard containers at ambient temperature for 3 minutes, when they were returned to their nest. This procedure was carried out daily for days 2 through 7 inclusively. The nonmanipulated litters remained undisturbed in their warm nests until weaning at 21 days of age. The manipulation procedure was a modification of the method of Levine (8). Sixty males, one-half of which had received early manipulation were then chosen at random. Fifteen groups of 4 pups were each placed in a long metal cage and given water and a complete rat purified diet as previously described (13), ad libitum.

At 35 days of age, half of the sub-groups were continued with the complete rat purified diet and half were given the same formula, from which thiamine was omitted. Four groups of 15 rats each were then assigned as follows: group A: infantile manipulated (IM) + thiamine-deficient; group B: IM + complete diet; group C: nonmanipulated (non-IM) + thiamine-deficient; and group D: non-IM + complete diet.

The assignment to groups was staggered over a 4-day period to facilitate uniformity in the shocking procedures. Starting at 45 days of age and for 4 consecutive days, 4 rats from each of the experimental groups were tested on the shock apparatus. Individual rats were chosen at random for the testing sequence.

In experiment 2, male albino rats weighing an average of 110 g were divided into 4 groups of 10 rats each, and a control group of 8 rats. The rats were placed in small metal cages with water and complete rat purified diet, ad libitum. The experimental groups were fed the thiamine-deficient diet for periods of 13, 10, 7, and 4 days, and the control group continued to be fed the complete diet. The assignment of rats was staggered over a

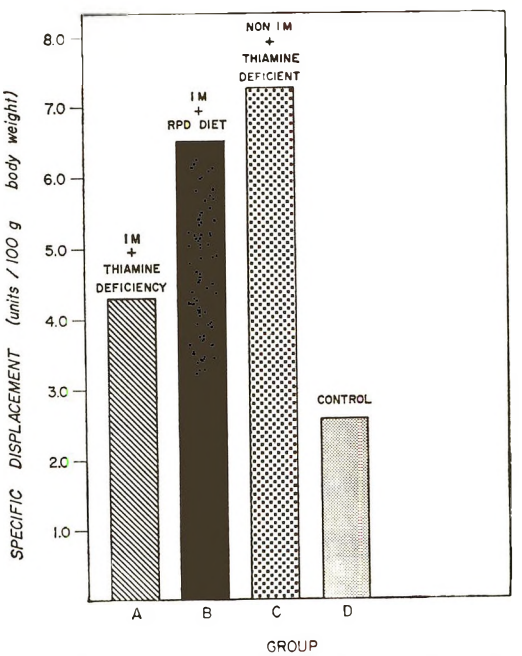

Fig. 1 The effects of infantile manipulation (IM), thiamine deficiency, and both of these, on the startle response to electric shock, in rats. The startle response is represented as D_S (specific displacement), which is equal to the vertical displacement of the rat/100 g body weight, upon being shocked under controlled conditions.

4-day period so that rats from each experimental group could be tested on the same day. Testing was carried out on the shock apparatus using the same procedure as in experiment 1, except for placing of the electrodes, as previously mentioned. Immediately following testing, each rat was killed and the blood was analyzed for erythrocyte transketolase activity by the method of Brin (14). Lactate was determined by the method of Barker and Summerson (15).

RESULTS

Experiment 1. The final average weights of the rats were 132.0, 135.5, 123.3, and 147.9 g for groups A, B, C, and D, respectively. There were no significant differences from the controls for either thiamine deficiency or for IM after the 10-day test period.

As presented in figure 1, the D_s values for groups A, B, and C were greater than D_s value of the control group. Mann Whitney U-Test values (16) for significance for each group, when compared with the value for group D were significantly different at $P = < 0.02$, $P = < 0.002$, and $P = < 0.002$, respectively. Thus the effects of thiamine deficiency alone, or of early manipulation alone, was to increase the specific displacement or startle response of rats. The effect of combining these variables, however, was to decrease the responsiveness, when compared with that which was produced by the individual variables. Group A was found to be significantly less responsive than group C ($P = < 0.05$), although group A was not significantly less responsive than group B. The difference in responsiveness between A and C, then appeared to be due to the effects of IM alone.

Experiment 2. The effect of vitamin B_1-deficiency alone on responsiveness in experiment 1 was so highly significant in as few as 10 days, which was before any clinical signs of vitamin B_1 deficiency appeared, that we were stimulated to design the second study to correlate the responsiveness effect with that of transketolase activity, at varying time periods.

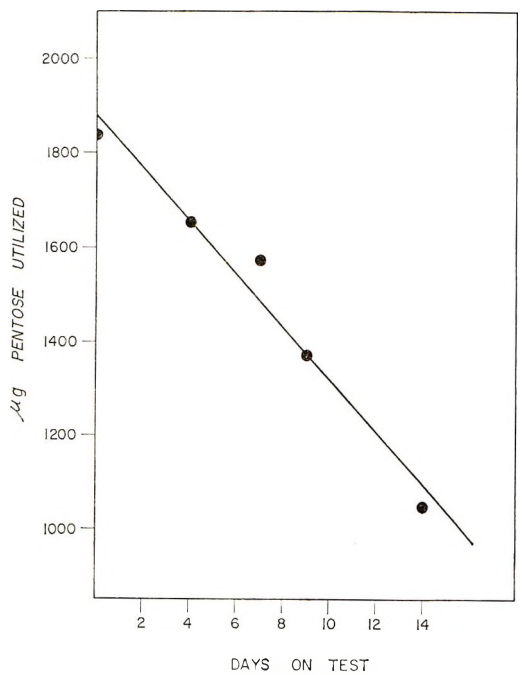

Fig. 2 Pentose utilization, as measured in the transketolase assay, decreased progressively as the rats were fed the vitamin B_1-deficient diet for between zero and 14 days.

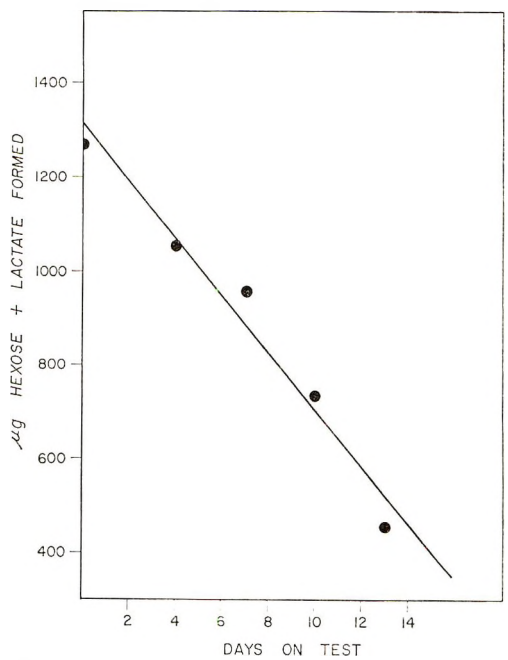

Fig. 3 The formation of hexose, in the transketolase assay decreased progressively as the rats were fed the vitamin B_1-deficient diet for between zero and 14 days.

To assure progressive thiamine depletion in these rats, transketolase assays were made on erythrocytes obtained at the time of killing. As presented in figure 2, pentose utilization decreased in as few as 4 days of depletion, and decreased progressively in a linear fashion as the experiment continued through 13 days. Similarly the formation of hexose plus lactate from ribose phosphate decreased progressively as the deficiency developed (fig. 3).

The average weights of the groups were 182.4, 172.1, 172.7, 170.9, and 170.8 g for the controls and for the rats depleted for 4, 7, 10, and 13 days, respectively. Although the depleted groups weighed less than the controls, the differences were not statistically significant, supporting a preclinical vitamin B_1-deficiency in these rats. Appearance and gross behavior were normal.

The data for D_S in figure 4 indicate that D_S values were higher at 4 days and increased progressively in a linear fashion as the deficiency became more severe.

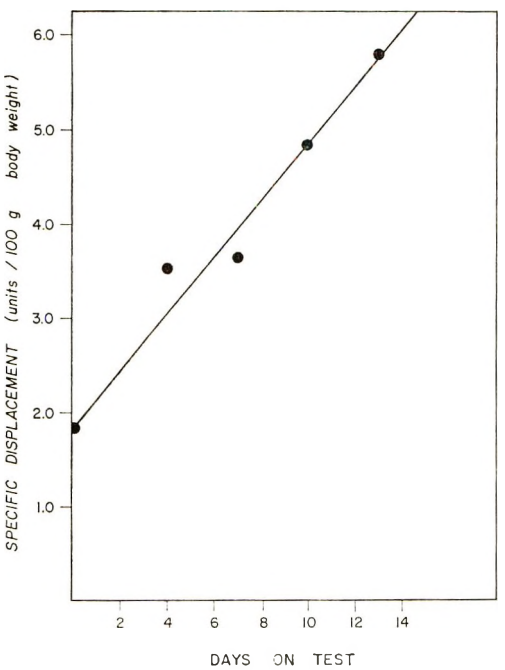

Fig. 4 Specific displacement (D_S) to electric shock increased regularly in rats that were maintained with vitamin B_1-deficient diets between zero and 14 days.

DISCUSSION

Increased responsiveness of IM rodents to environmental stress had been shown by Levine, as shorter latency periods to initiate movement on runways (8) and shorter fighting-response latencies (10). The observation of increased D_S values following electric shock, is not without precedent, therefore.

The difference in response to thiamine deficiency between IM and non-IM rats was also not unexpected. Levine had shown that in IM rats significantly more rapid rates of growth occurred and also lower mortality rates, when rats were totally deprived of food and water for long periods of time (9). Thus it appeared that early manipulation favorably affected both the rate of metabolism and the ability to withstand food and water deprivation. Possibly, then, the differences which were observed in D_S between groups A and C in experiment 1, may have been due to amelioration of the deficiency state by IM.

Brozek and Vaes (17) have recently reviewed experimental studies on the effects of dietary deficiencies on animal and human behavior. It was pointed out by them and by others (18) that a large number of studies were made before the advent of nutritionally complete purified diets, suggesting that pure deficiencies or complete deficiencies may not have been attained. Also, where studies of pure vitamin B_1-deficiency states were made, the results were generally similar to those obtained in probable multiple vitamin B-complex deficiency states. Thiamine was probably a very significant factor in maintaining normal behavior, therefore (17). Also, most early studies on maze learning, memory, and the development of conditioned responses, etc., were made with clinically deficient subjects, after growth had ceased, or polyneuritis had developed. One such study proved to have a practical application (19). Two studies in marginal deficiency states were particularly noteworthy, however.

Guerrant and Dutcher (6) observed that normal growth was maintained for 14 days in thiamine-deficient rats. However, significantly increased activity was noted by the end of the first week of the study (and before growth was affected) and

twice normal activity at 21 days. Beyond this, activity decreased as the deficiency became more severe. Another study, by Wald and Jackson (7) showed cessation of growth on the fourth day, and increased activity on the sixth day, following the initial feeding of diets deficient in thiamine or riboflavin, or deprivation of food, water, or both.

The observation of progressively increasing D_5 in vitamin B_1-deficiency in rats indicated progressively increasing hyperexcitability in the neurological responsiveness to electric shock. This appears to be at variance with the classical concepts of the neurological effects of advanced vitamin B_1-deficiency, which include polyneuropathy and nerve demyelination and degeneration (19), but it correlates well with the observation of increased spontaneous activity in preclinically thiamine-deficient rats, and with the early appearance of decreased tissue transketolase activity. The specificity for thiamine remains to be shown, nevertheless. This may provide us with an additional behavioral and neurological parameter in the evaluation of marginal malnutrition in rats.

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Influence of Dietary Fat and Protein on Metabolic and Enzymatic Activities in Adipose Tissue of Meal-fed Rats [1]

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ABSTRACT The influence of dietary protein and fat on the response of adipose tissue to meal-feeding (a single daily 2-hour meal) has been investigated in the rat. Meal-feeding stimulated the incorporation of pyruvate carbon into fatty acids and the oxidation of pyruvate by isolated adipose tissue. This response to meal-feeding was completely abolished by feeding a high fat diet. The activities of glucose 6-phosphate dehydrogenase and malic enzyme were higher in adipose tissue and liver of meal-fed rats consuming a high carbohydrate diet, but were unchanged in tissue of rats meal-fed a high fat diet. The activities of glucose 6-phosphate dehydrogenase and malic enzyme were depressed in adipose tissue of rats fed the high fat diet, whereas only glucose 6-phosphate dehydrogenase activity was depressed in liver of nibbling rats consuming the high fat diet. Adipose tissue from rats fed the high fat diet was able to convert significantly more pyruvate-2-^{14}C to glyceride-glycerol than tissue from rats fed the high carbohydrate diet. The possible significance of this observation is discussed. Dietary protein did not influence the response of adipose tissue to meal-feeding. Increasing the dietary protein level did increase hepatic glutamic-oxaloacetic (GOT) and glutamic-pyruvic (GPT) transaminase activities and decreased the ability of isolated adipose tissue to oxidize glucose-^{14}C and leucine-^{14}C and to incorporate these substrates into fatty acids. Adipose tissue from rats meal-fed diets containing 9, 18 or 36% casein oxidized and incorporated into fatty acids significantly more glucose and leucine than tissue from animals fed the same diets ad libitum.

Meal-feeding, restricting the consumption of food to a short daily period, has been shown to induce dramatic metabolic alterations in the rat. The ability of liver and adipose tissue to convert ^{14}C-labeled substrates to fatty acids is markedly enhanced as a consequence of meal-feeding (1–4). Accompanying this increased lipogenesis is an elevation in the activity of several dehydrogenase enzymes, presumably related to fatty acid synthesis. Tepperman and Tepperman (1) and Cohn and Joseph (5) observed that the activity of the pentose pathway dehydrogenases, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, and of malic enzyme was higher in livers of meal-fed as compared with nibbling (ad libitum-fed) rats. Hollifield and Parson (2) observed an increase in the combined activity of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in adipose tissue of meal-fed rats. Leveille and Hanson (6) showed increased activities of glucose 6-phosphate dehydrogenase, malic enzyme and citrate cleavage enzyme in rat adipose tissue as a consequence of meal-feeding.

The adaptive response to meal-feeding as observed in rat adipose tissue appears to be dependent upon the ingestion of carbohydrate. Meal-feeding a high fat, "carbohydrate-free" diet does not result in the typical meal-feeding response (6). Adipose tissue from rats meal-fed such a diet shows neither an increased lipogenic capacity nor an elevated activity of enzymes. The effect of meal-feeding a high fat diet on hepatic enzyme activity has not been determined, nor has the ability of adipose tissue from rats meal-fed such a diet to metabolize substrates other than glucose or acetate been evaluated.

Received for publication June 6, 1966.

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

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The influence of dietary protein on the adaptive response to meal-feeding has received little attention. Cohn et al. (7) have reported that protein metabolism is impaired in meal-fed rats. This conclusion was based on the observations that 1) urea N excretion was higher for meal-fed rats; 2) more of a dietary dose of ^{15}N-labeled protein was excreted as urea-^{15}N in the meal-fed animals; and 3) the hepatic arginine synthetase activity was increased as a consequence of meal-feeding. The lipogenic capacity of adipose tissue from meal-fed rats consuming a low protein diet is apparently not altered (8). The ability of isolated adipose tissue to incorporate acetate-^{14}C into fatty acids was increased to a similar degree by meal-feeding diets containing 5 or 20% casein. Adipose tissue of meal-fed rats has the ability to utilize significantly greater quantities of glutamate and aspartate carbon than similar tissue from nibbling animals (6, 9). One of the features of the adaptive response to meal-feeding may be an enhanced capability to utilize amino acids, particularly to incorporate amino acid carbon into lipid. Such metabolism would require deamination or transamination of the amino acids, and since rat adipose tissue possesses substantial transaminase activity (10), a higher activity of these enzymes might be expected in tissue of meal-fed animals.

The present report describes an attempt to evaluate the influence of dietary fat and protein on the response to meal-feeding. The ability of adipose tissue, isolated from rats meal-eating or nibbling diets varying in fat or protein, to utilize various ^{14}C-labeled substrates was studied, as well as the activity of a number of enzymes.

MATERIALS AND METHODS

Experimental animals and diets. Male rats of the Holtzman strain weighing approximately 140 g were used for all studies. The animals were housed in stainless steel cages having raised wire floors and in a temperature and humidity controlled room (21° and 50% relative humidity). The animals were fed the experimental diets for 3 or 4 weeks, a period shown previously to be of sufficient length to induce the adaptive changes to meal-feeding (4). Food consumption and body weight were determined weekly. Each of the various diet groups was subdivided into 2 regimen groups; one was fed ad libitum (nibblers) and the other was allowed access to food from 8 to 10 AM only (meal-eaters). The composition of the diets fed in these studies is shown in table 1. The levels of casein, vitamins and minerals were increased in the high fat diet to compensate for the reduced food intake resulting from the higher caloric density of this diet. The hydrogen-

TABLE 1

Composition of experimental diets

	Exp. 1		Exp. 2		
	High carbohydrate diet	High fat diet	Diet 1	Diet 2	Diet 3
	g/100 g diet		*g/100 g diet*		
Casein (vitamin-free)	18.0	30.0	9.0	18.0	36.0
L-Cystine	0.3	0.45	0.15	0.3	0.6
Non-nutritive fiber [1]	4.0	4.0	4.0	4.0	4.0
Vitamin mixture [2]	2.2	3.0	2.2	2.2	2.2
Salt mixture [3]	4.0	6.0	4.0	4.0	4.0
Corn oil	5.0	—	5.0	5.0	5.0
Hydrogenated vegetable oil [4]	—	56.55	—	—	—
Glucose	66.5	—	75.65	66.5	48.2

[1] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

[2] The vitamin mixture, when fed at a level of 2.2 g/100 g diet, supplied the following per 100 g of diet: vitamin A, 1980 units; vitamin D, 220 units; and (in mg) α-tocopherol, 11; ascorbic acid, 99; inositol, 11; choline Cl, 165; menadione, 5; *p*-aminobenzoic acid, 11; niacin, 10; riboflavin, 2.2; pyridoxine·HCl, 2.2; thiamine·HCl, 2.2; Ca pantothenate, 6.6; and (in μg) biotin, 4.4; folic acid, 198; and vitamin B_{12}, 3 (Vitamin Diet Fortification Mixture in Dextrose, Nutritional Biochemicals Corporation).

[3] Salt Mixture USP XIV (Nutritional Biochemicals Corporation).

[4] Crisco, Procter and Gamble, Cincinnati.

ated vegetable oil used in the high fat diet was determined by gas chromatographic analysis to contain approximately 30% linoleate and, therefore, would have supplied the requirement for essential fatty acids.

Metabolic studies. The meal-fed animals were killed immediately after their daily meal; nibbling rats had access to food until the time of killing. The animals were decapitated; the epididymal adipose tissue and, where appropriate, liver tissue were quickly excised. Pieces of adipose tissue weighing approximately 100 mg were taken from the thin peripheral portion of the epididymal pad, weighed on a torsion balance and quickly transferred to 25-ml Erlenmeyer flasks containing 3.0 ml of the appropriate buffer. Calcium-free Krebs-Ringer bicarbonate buffer (11) was used in all these studies. The flasks were gassed with 95% O_2–5% CO_2 and stoppered with rubber serum stoppers from which was suspended a polyethylene well containing a 2 × 2 cm piece of Whatman no. 1 filter paper. The flasks were shaken in a reciprocating water bath (90 strokes/minute) at 38° for 3 hours. At the end of the incubation, 0.1 ml of 25% KOH was introduced through the rubber stopper with a syringe and hypodermic needle onto the filter paper. To ensure complete liberation of CO_2, 0.5 ml of 0.2 N H_2SO_4 was injected into the buffer and shaking was continued for 20 minutes. The $^{14}CO_2$ was trapped and counted essentially as described by Buhler (12). The filter paper containing the $^{14}CO_2$ was transferred to a liquid scintillation vial, allowed to dry, then was flattened in the bottom of the vial and 10 ml of toluene scintillation solution was added. The overall efficiency of the method was established to be 20% using buffer containing $NaH^{14}CO_3$. The adipose tissue was treated essentially as described by Cahill et al. (13). The tissue was rinsed 3 times in 0.9% NaCl solution and was extracted in 15 ml of chloroform:methanol (2:1) with constant shaking for 6 hours. The lipid extract was washed 3 times by the "salty wash" method of Folch et al. (14) to remove non-lipid radioactivity. The solvent was evaporated under a stream of O_2-free nitrogen, and the lipids were saponified by refluxing at 80 to 85° in 3% methanolic KOH. The non-saponifiable lipids were removed by three 5-ml extractions with petroleum ether (BP 30–60°). The fraction containing the saponifiable lipids was acidified with HCl, and the fatty acids were extracted with three 5-ml portions of petroleum ether. The fatty acid extracts were combined, washed with water and transferred to a liquid scintillation vial. The solvent was evaporated under a stream of O_2-free nitrogen and the fatty acids were dissolved in 10 ml of toluene scintillation solution. The volume of the aqueous phase containing glyceride-glycerol was measured and radioactivity determined in an aliquot. The toluene scintillation solution used contained per liter: 4 g 2,5-diphenyloxazole (PPO), 0.015 g 1,4-bis-2-(5-phenyloxazolyl) benzene (POPOP), 230 ml ethanol and toluene to one liter. All samples were counted in a Nuclear-Chicago Model 722 ambient temperature liquid scintillation spectrometer.

Enzyme studies. Adipose tissue or liver was homogenized in cold 0.15 M KCl. The homogenate was centrifuged at 1000 × *g* at 5° for 15 minutes. The clear intermediate layer of the adipose tissue homogenate was used for assay. The supernatant from the liver homogenates was recentrifuged for 30 minutes at 15,000 × *g* at 5°, and the resulting clear supernatant was used for enzyme assay. Glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) were assayed separately in adipose tissue, as described by Horecker and Smyrniotis (15). In liver homogenates these enzymes were assayed by the procedure of Glock and McLean (16), assuming that 50% of the total activity measured, with glucose 6-phosphate as substrate and in the presence of excess 6-phosphogluconate dehydrogenase activity, represented glucose 6-phosphate dehydrogenase activity. Malic enzyme (EC 1.1.1.40) was assayed by the spectrophotometric method of Ochoa (17). Glutamic-oxaloacetic transaminase (GOT) (EC 2.6.1.1) and glutamic-pyruvic transaminase (GPT) (EC 2.6.1.2) activities were assayed by a spectrophotometric method similar to that described by Wroblewski and LaDue (18). Each cuvette contained, in a final volume

TABLE 2

Body weight and food consumption of meal-eating and nibbling rats fed a high fat or a high carbohydrate diet

Diet	Regimen	Week of experiment			
		Initial	1	2	3
		Body weight, g			
High carbohydrate	Meal-eating	241 ± 15 [1]	196 ± 13	204 ± 19	226 ± 24
	Nibbling	238 ± 10	255 ± 19	270 ± 27	304 ± 13
High fat	Meal-eating	236 ± 11	246 ± 11	274 ± 12	302 ± 10
	Nibbling	240 ± 11	275 ± 7	303 ± 8	329 ± 12
		Food consumption, g/rat/week			
High carbohydrate	Meal-eating	—	59 ± 9	84 ± 10	104 ± 13
	Nibbling	—	112 ± 17	143 ± 18	147 ± 8
High fat	Meal-eating	—	76 ± 4	74 ± 5	78 ± 7
	Nibbling	—	88 ± 6	98 ± 5	83 ± 13

[1] Mean for 6 rats ± SD.

of 2.8 ml, the following: (μmoles) phosphate buffer, pH 7.4, 200; NADH, 0.18; α-ketoglutarate, 20; and L-alanine, 200 (GPT) or L-aspartic acid, 100 (GOT). The homogenate volumes used for the transaminase assay contained 10 to 100 μg of nitrogen. The reaction was started by the addition of α-ketoglutarate, and the oxidation of NADH was followed at 340 mμ. NADH was omitted from the blank cell.

Nitrogen content of the homogenates was determined by micro-Kjeldahl digestion followed by Nesslerization (19). The enzyme activities are expressed as units per milligram of nitrogen, where a unit is defined as the transformation of 1 μmole of substrate/minute at 30°.

The data were evaluated statistically by the *t* test or by analysis of variance as indicated.

RESULTS

Experiment 1. The results of this experiment are presented in tables 2–4. The effects of feeding the high fat or high carbohydrate diet and of meal-eating as compared with nibbling on body weight and food consumption are shown in table 2. Rats meal-fed the high carbohydrate diet lost 18% of their initial body weight during the first week, then proceeded to gain weight but at a slower rate than rats fed the same diet ad libitum. Animals meal-fed the high fat diet gained weight throughout the 3-week experimental period, but more slowly during the first week than ad libitum-fed rats receiving the same diet. The observed changes in body weight paralleled the food consumption data (table 2). Food consumption was depressed by meal-feeding to a greater extent in rats ingesting the high carbohydrate as compared with animals fed the high fat diet.

Adipose tissue from rats meal-fed the high carbohydrate diet utilized more pyruvate-2-^{14}C for oxidation $^{14}CO_2$ and incorporation into fatty acids than comparable tissue from animals consuming this diet ad libitum, but meal-feeding did not influence the incorporation of pyruvate carbon into glyceride-glycerol (table 3). The addition of unlabeled glucose and insulin [3] to the incubation medium increased the incorporation of pyruvate into fatty acids and decreased the conversion to glyceride-glycerol by isolated adipose tissue of rats ingesting the high carbohydrate diet, but did not alter the relative differences between tissue from meal-fed and nibbling animals (table 3). Meal-feeding did not enhance pyruvate utilization by isolated adipose tissue when rats were fed the high fat diet (table 3), but the oxidation of pyruvate-2-^{14}C and the incorporation of this substrate into fatty acids was diminished as a consequence of ingesting the high fat diet. Adipose tissue from rats fed the high fat diet converted significantly greater quantities of pyruvate to glyceride-glycerol than did tissue from rats ingesting the high carbohydrate diet. Adi-

[3] The insulin used in these studies was generously supplied by Dr. W. Bromer, Eli Lilly and Company, Indianapolis, Indiana.

TABLE 3

Utilization in vitro of pyruvate-2-^{14}C by adipose tissue of meal-eating and nibbling rats fed a high fat or a high carbohydrate diet [1]

Diet	Regimen	$^{14}CO_2$		Fatty acid		Glyceride-glycerol	
		Buffer A [1]	Buffer B [1]	Buffer A	Buffer B	Buffer A	Buffer B
		mμmoles pyruvate-2-^{14}C converted/100 mg tissue/3 hours					
High carbohydrate	Meal-eating	888 ± 223 [2]	750 ± 143	1519 ± 432	3975 ± 342	95 ± 25	30 ± 20
	Nibbling	587 ± 96	381 ± 55	766 ± 68	1974 ± 454	116 ± 39	26 ± 18
	P (ME vs. nib.) [3]	< 0.025	< 0.001	< 0.005	< 0.001	ns	ns
High fat	Meal-eating	214 ± 40	193 ± 33	31 ± 29	177 ± 36	140 ± 34	40 ± 18
	Nibbling	240 ± 29	208 ± 40	19 ± 9	150 ± 48	170 ± 31	36 ± 24
	P (ME vs. nib.)	ns	ns	ns	ns	ns	ns
	P (ME-high CHO vs. fat)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.050	ns
	P (Nib.-high CHO)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.050	ns

[1] Incubation conditions as described in Methods section. Both buffer A and B contained 5 μmoles pyruvate and 0.167 μCi pyruvate-2-^{14}C/ml; buffer B also contained 5 μmoles glucose and 0.1 unit insulin/ml.
[2] Mean for 6 rats ± SD.
[3] Probability of significance; ns = not significant.

TABLE 4

Dehydrogenase activity in adipose and liver tissue of meal-eating and nibbling rats fed a high fat or a high carbohydrate diet

Diet	Regimen	Adipose tissue			Liver tissue		
		G-6-P dehydrogenase	6-phosphogluconate dehydrogenase	Malic enzyme	G-6-P dehydrogenase	6-phosphogluconate dehydrogenase	Malic enzyme
		μmoles of substrate utilized/mg N/min					
High carbohydrate	Meal-eating	1.535 ± 0.272 [1]	0.272 ± 0.041	3.511 ± 0.428	0.232 ± 0.047	0.204 ± 0.024	0.288 ± 0.058
	Nibbling	0.622 ± 0.266	0.212 ± 0.054	1.151 ± 0.636	0.113 ± 0.042	0.172 ± 0.044	0.122 ± 0.034
	P (ME vs. nib.) [2]	< 0.001	ns	< 0.001	< 0.001	ns	< 0.001
High fat	Meal-eating	0.181 ± 0.063	0.166 ± 0.045	0.162 ± 0.038	0.032 ± 0.004	0.138 ± 0.037	0.121 ± 0.058
	Nibbling	0.166 ± 0.031	0.183 ± 0.014	0.176 ± 0.043	0.032 ± 0.008	0.137 ± 0.040	0.084 ± 0.026
	P (ME vs. nib.)	ns	ns	ns	ns	ns	ns
	P (ME-high CHO vs. fat)	< 0.001	< 0.005	< 0.001	< 0.001	< 0.005	< 0.001
	P (Nib.-high CHO vs. fat)	< 0.005	ns	< 0.005	< 0.001	ns	ns

[1] Mean for 6 rats ± SD.
[2] Probability of significance; ns = not significant.

pose tissue from rats fed the high fat diet also responded to the addition of glucose and insulin to the incubation medium by increased incorporation of pyruvate into fatty acids and decreased conversion to glyceride-glycerol.

The activity of glucose 6-phosphate dehydrogenase and malic enzyme was significantly increased in adipose tissue and liver of rats meal-fed the high carbohydrate diet (table 4), whereas in animals consuming the high fat diet, meal-eating did not influence the activity of any of the enzymes studied. The activities of glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and malic enzyme were significantly lower in adipose tissue and liver of rats meal-fed the high fat diet than in tissue of animals meal-eating the high carbohydrate diet. In nibbling rats the activities of glucose 6-phosphate dehydrogenase in adipose tissue and liver and of malic enzyme in adipose tissue were decreased by ingestion of the high fat as compared with the high carbohydrate diet.

Experiment 2. The influence of dietary protein level on the response to meal-feeding was investigated in experiment 2 by allowing rats to consume diets containing 9, 18 or 36% casein on a meal-feeding or ad libitum schedule. The meal-fed animals lost weight during the first week of the experiment; the greatest weight loss was observed for the group fed the 9% casein diet, whereas those animals receiving the 36% casein diet lost the least. Following the first week for the meal-fed animals and throughout the 4-week experimental period for nibbling rats, weight gain was essentially related to dietary protein levels (table 5). Food consumption was almost identical for all the nibbling groups, and for the meal-eating animals was highest for those fed the 36% casein diet.

Adipose tissue from meal-fed rats oxidized and incorporated into fatty acids more glucose-U-^{14}C and leucine-^{14}C than did tissue from nibbling animals (table 6). Significantly more glucose-^{14}C was converted to glyceride-glycerol by adipose tissue of meal-fed rats than by tissue from nibbling control animals. The oxidation of glucose and leucine by adipose tissue was decreased as the dietary protein level increased for both meal-fed and nibbling rats. A similar decrease in the ability of adipose tissue to incorporate glucose and leucine carbon into fatty acids and glucose carbon into glyceride-glycerol was observed as the level of dietary protein increased (table 6).

Meal-feeding resulted in a significant decrease in adipose tissue GPT activity, and a similar trend was suggested for GOT activity (table 7). The activity of malic enzyme in adipose tissue increased in re-

TABLE 5

Body weight and food consumption of meal-eating and nibbling rats fed diets containing different levels of protein

Dietary protein	Regimen	Week of experiment				
		Initial	1	2	3	4
% of diet						
			Body weight, g			
9	Meal-eating	237 ± 13 [1]	179 ± 15	180 ± 10	193 ± 12	208 ± 12
18		238 ± 13	184 ± 21	189 ± 27	201 ± 21	217 ± 19
36		241 ± 15	208 ± 18	220 ± 31	244 ± 27	264 ± 27
9	Nibbling	239 ± 16	251 ± 21	271 ± 27	291 ± 26	300 ± 31
18		238 ± 16	250 ± 24	271 ± 15	290 ± 14	303 ± 15
36		238 ± 14	263 ± 12	288 ± 13	302 ± 13	312 ± 17
			Food consumption, g/rat/week			
9	Meal-eating	—	54 ± 7	62 ± 25	101 ± 23	88 ± 8
18		—	59 ± 11	68 ± 10	85 ± 10	95 ± 18
36		—	82 ± 22	82 ± 23	113 ± 19	118 ± 24
9	Nibbling	—	134 ± 19	133 ± 20	143 ± 8	139 ± 17
18		—	131 ± 15	128 ± 12	129 ± 11	142 ± 27
36		—	131 ± 10	129 ± 17	124 ± 12	125 ± 13

[1] Mean for 7 rats ± SD.

TABLE 6

Utilization in vitro of glucose-U-^{14}C and L-leucine-U-^{14}C by adipose tissue of meal-eating and nibbling rats fed diets containing different levels of protein

Protein level	Regimen	Glucose-U-^{14}C [1]			L-Leucine-U-^{14}C [1]	
		$^{14}CO_2$	Fatty acid	Glyceride-glycerol	$^{14}CO_2$	Fatty acid
% of diet		*mμmoles of labeled substrate converted/100 mg tissue/3 hours*				
9	Meal-eating	1482±550(6) [2]	1969±305(6)	392±143(6)	182±68(6)	507±220(6)
	Nibbling	760±334(7)	1230±672(7)	170±102(7)	90±37(7)	304±138(7)
18	Meal-eating	1125±147(7)	1878±299(7)	380±61 (6)	170±82(7)	529±233(7)
	Nibbling	559±133(7)	950±147(7)	165±70 (7)	65±11(7)	220±40 (7)
36	Meal-eating	721±247(7)	1281±302(7)	211±120(7)	83±47(7)	297±96 (7)
	Nibbling	346±132(7)	473±253(3)	39±33 (3)	59±11(7)	203±70 (7)
F values: [3]						
	Protein level	12.86*	10.27*	8.60*	5.78*	3.91*
	Meal-eating vs. nibbling	35.59*	40.18*	36.4*	21.57*	18.28*
	Interaction	2.16	< 1	< 1	3.14	2.10

[1] Both buffers contained per ml 5 μmoles glucose and 0.1 unit insulin; the glucose buffer also contained 0.167 μCi/ml glucose-U-^{14}C; the leucine buffer contained 5 μmoles L-leucine and 0.167 μCi L-leucine-U-^{14}C/ml.
[2] Mean ± SD for number of animals shown in parentheses.
[3] F values from analysis of variance; correction for disproportionate numbers has been made where applicable.
* Denotes significance at the 5% probability level.

TABLE 7

Activity of glutamic oxaloacetic transaminase, glutamic-pyruvic transaminase and NADP malic dehydrogenase in liver and adipose tissue of meal-eating and nibbling rats fed diets containing different levels of protein

Dietary protein level	Regimen	Adipose tissue			Liver	
		GOT	GPT	NADP-MD	GOT	GPT
% of diet		*μmoles of substrate utilized/mg N/min*				
9	Meal-eating	0.302±0.044 [1]	1.813±0.435	3.512±0.603	1.780±0.672	1.023±0.206
	Nibbling	0.303±0.032	2.019±0.416	1.492±0.504	1.251±0.212	1.070±0.277
18	Meal-eating	0.280±0.078	1.963±0.372	3.398±0.811	2.030±0.450	1.900±0.430
	Nibbling	0.331±0.030	2.068±0.200	1.612±0.506	1.946±0.403	1.761±0.526
36	Meal-eating	0.295±0.055	1.646±0.526	2.858±0.669	2.392±0.435	2.267±0.530
	Nibbling	0.352±0.094	2.179±0.442	0.850±0.437	2.417±0.381	2.005±0.380
F values: [2]						
	Protein level	< 1	< 1	4.84*	14.52*	24.58*
	Meal-eating vs. nibbling	3.82	4.83*	104.99*	2.37	1.40
	Interaction	< 1	1.07	< 1	1.24	< 1

[1] Mean for 7 rats ± SD.
[2] F values from analysis of variance.
* Denotes significance at the 5% probability level.

sponse to meal-feeding, but was depressed by increasing dietary protein levels. The activity of liver GOT and GPT was increased by higher levels of dietary protein; however, no change in adipose tissue transaminase activity could be attributed to dietary protein level. Meal-feeding did not influence the activity of GOT or GPT in liver.

DISCUSSION

The observations on body weight and food consumption (tables 2 and 5), with the exception of animals fed the high fat diet, are in accord with a previous report (4). Rats appeared to adjust to a single daily meal more readily when the high fat diet was fed; this is indicated by the observations that these animals did not lose weight, as did rats fed the high carbohydrate diet, and consumed 84% of the amount consumed by nibbling controls, as compared with a value of 53% for rats meal-fed the high carbohydrate diet. This observation is in accord with the report of Smith et al. (20), demonstrating

a more rapid adjustment of meal-fed rats to a high fat than to a laboratory ration diet.

Numerous reports have demonstrated an inhibition of lipogenesis by dietary fat in rat liver slices (21–24), and Hausberger and Milstein (25) and DiGiorgio et al. (26) have reported similar observations for rat adipose tissue. In a previous report (6) it was shown that feeding a high fat diet completely abolished the adaptive increase in fatty acid synthesis by adipose tissue. The present report corroborates these results and extends them by demonstrating that the adaptive changes in liver enzymes resulting from meal-feeding (1) are prevented by the ingestion of a high fat diet. The increase in glyceride-glycerol synthesis from pyruvate in adipose tissue of rats fed the high fat diet is of significance. Adipose tissue does not possess glycerokinase activity (27) and must, therefore, synthesize α-glycerophosphate from dihydroxyacetone phosphate. This becomes an important metabolic pathway in adipose tissue because of the constant α-glycerophosphate requirement for fatty acid esterification (28). The data in this report demonstrate that pyruvate can serve as a precursor for α-glycerophosphate in rat adipose tissue and that the activity of the pathway involved is increased in animals fed a high fat diet. The incorporation of pyruvate carbon into glyceride-glycerol by adipose tissue has been reported previously by Christophe et al. (29) and Leveille (30), and suggests that phosphoenol pyruvate carboxykinase activity is present in adipose tissue despite reports to the contrary (31, 32). The increased activity of the pathways involved in the reversal of pyruvate to α-glycerophosphate in adipose tissue of animals fed a high fat diet is in accord with the increased gluconeogenic capacity of kidney cortex from rats fed a high fat diet (33). The increased fatty acid synthesis and decreased glyceride-glycerol formation from pyruvate in the presence of glucose supports the contention that pyruvate is converted to glyceride-glycerol by a reversal of glycolysis. In the presence of glucose, pyruvate is oxidized to acetyl-CoA and incorporated into fatty acids to a much greater extent, and glucose is presumably serving as a precursor of glyceride-glycerol, thereby decreasing the need for the conversion of pyruvate to glyceride-glycerol.

It has been suggested that dietary protein level does not influence lipogenesis in liver (21) or adipose tissue (25) of the rat. The data presented in this report suggest that the level of dietary protein may in fact influence lipogenesis by isolated adipose tissue. Fatty acid synthesis from glucose or leucine was depressed markedly by increased dietary protein, as was the oxidation of the ^{14}C-labeled substrates by adipose tissue. Alternatively, since protein was added to the diets at the expense of glucose, the interpretation that the observed response was due to decreased dietary glucose rather than to increasing dietary protein, must be considered. Actually this latter interpretation would be more compatible with previous studies, since increasing dietary glucose has been reported to stimulate hepatic lipogenesis (34) and to increase the activity of several hepatic enzymes including glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and malic enzyme (35). Consequently, the decrease in malic enzyme activity, observed in adipose tissue of animals fed higher protein levels, may be related to the decrease in dietary glucose. Dietary protein level did not influence the response to meal-feeding, an observation in accord with the report of Beaton et al. (8). GPT and GOT activities, in general, were not influenced by meal-feeding, with the exception of adipose tissue GPT activity which was lower in tissue of meal-fed animals.

Meal-feeding has been shown to increase the ability of isolated adipose tissue to convert several amino acids to lipids. Thus, aspartic and glutamic acids (6, 9) and leucine (present report) are converted to fatty acids to a much greater extent by adipose tissue of meal-fed rats as compared with nibbling rats. This increased utilization of amino acids occurs without an increase in transaminase activity, suggesting perhaps that the transaminase activity of adipose tissue is maintained at a level capable of handling greater quantities of amino acids than required under

normal conditions. This would be analogous to the activity of NAD malic dehydrogenase which, although presumably a key enzyme in the lipogenic response, is present in sufficient quantity that an increase in activity in response to meal-feeding is unnecessary (6).

Increasing the level of dietary protein enhanced hepatic GPT and GOT activity, an observation in agreement with numerous reports recently reviewed by Knox and Greengard (36); however, adipose tissue transaminase activity was not influenced by dietary protein. It is interesting to note the relative difference in GPT and GOT activities between liver and adipose tissue; in liver the GOT activity is slightly higher, whereas in adipose tissue the GPT activity is markedly higher than the GOT activity. These values are in agreement with previously reported transaminase activities for rat epididymal adipose tissue (10).

ACKNOWLEDGMENTS

The author wishes to thank J. Taubr, J. Heidker and L. Schiff for technical assistance; Gerhard Isaac for statistical evaluation of the data; B. James for care of the animals; and Mrs. M. Iverson and Mrs. G. Castor for assistance in the preparation of the manuscript.

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Nutritional Control of Arterial Lipid Composition in Squirrel Monkeys: Major ester classes and types of phospholipids [1]

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ABSTRACT The lipid composition of the intima and inner media of the aorta was studied in squirrel monkeys fed either a basal semipurified diet which did not produce arterial lesions or an analogous diet which induced atherosclerosis. The cholesterol ester and total cholesterol concentrations of one-half of the fresh aorta were shown in a preliminary study to correlate closely with the percentage of intimal sudanophilia in the corresponding fixed and stained half. Animals were killed at 2, 3, 6, and 8 months. In addition some animals were transferred from the lesion-inducing to the control diet at 3 months and some animals were killed at 6 and 8 months (after fed the basal diet 3 and 5 months). Variance analyses indicated that there was a difference between the arteries of the basal and atherogenic groups in concentrations of total, free, and esterified cholesterol, total phospholipid, and phosphatidylcholine; but not in triglycerides, sphingomyelin or total protein. The sphingomyelin and free cholesterol concentrations of aorta increased above those of natural diet controls for all groups fed the semipurified diets, and these components appeared to increase and stabilize at the higher level before increases in other lipid components. The arterial composition of the monkeys fed the atherosclerosis-inducing diet followed by the basal semipurified diet was intermediate between that observed in monkeys fed those 2 diets throughout the experiment. Correlation coefficients of the various aorta lipids were determined and their possible significance was discussed.

A variety of analytical procedures of great sensitivity are now available for the measurement and characterization of lipids. It is possible to make comprehensive measurements on the lipids of the intima and inner media of the aorta of experimental animals and to detect small changes in composition, to detect differences in the composition of anatomically separate portions of the artery, and to combine histological studies with measurements of lipid composition and lipid metabolism (with tracer techniques). We have recently described methods (1) which were applied to the characterization of aorta (intima plus inner media) lipids from rhesus monkey fetuses of different ages. The smallest of the arterial preparations weighed less than 20 mg (wet weight).

Although there are several reports (2–4) of the composition of normal arterial lining and of atheroma, particularly from humans, relatively few comprehensive data (5–7) are available about the sequence of changes in composition that occur with age or during the period immediately after the initiation of diets known to produce lesions with increased lipid compositions. This report describes studies of the sequence of early nutritionally induced changes in the composition of the major lipid classes and subclasses of phospholipids from aortas of squirrel monkeys.

We have previously compared 3 species of New World monkeys to determine their susceptibilities to nutritionally induced atherosclerosis (8, 9). Of the species tested, the squirrel monkey was the most susceptible to sudanophilic lesions. (Middleton et al. (10) presented the first detailed report of naturally occurring atherosclerosis in this species). In all 3 species

Received for publication August 9, 1966.

[1] This research was supported by a grant-in-aid from the National Institutes of Health (HE-09744), Bethesda, Maryland. Publication no. 205 from the Oregon Regional Primate Research Center, supported in part by grant FR-00163 of the National Institutes of Health.

and in chimpanzees (11), the extent of aortic sudanophilia for individual animals correlated extremely well with their mean serum cholesterol level during the experimental period. The serum cholesterol level was controlled in part by the composition of the diet.

The present study had several objectives: (a) establishing a measurement of arterial lipids to substitute for the measurement of sudanophilia as an index of severity of atherosclerotic or pre-atherosclerotic lesions (quantitation of sudanophilia is partially subjective; it perhaps does not reflect the earliest increase of lipids; it requires tissue fixation and staining, thus negating the possibility of using that tissue for metabolic studies and perhaps for tissue analyses); (b) observation of the sequence of changes in lipid composition with diets which are known to produce atherosclerosis; (c) determinations of the potential for regression of lipid-rich lesions with dietary alterations; and (d) study of the correlations between the concentrations of the various lipid classes and subclasses as a possible indication of the mechanisms involved in atherogenesis.

METHODS

Experimental animals used. Eighty-seven sexually mature female squirrel monkeys from 2 animal importers were used in these experiments. One importer obtained Brazilian squirrel monkeys (*Saimiri madeirae juruanus* (12)) from a Leticia, Colombia, collecting station; the other importer obtained squirrel monkeys (*Saimiri boliviensis nigriceps* (12)) from Iquitos, Peru. In all experiments, either the animals were entirely from one source or the animals from each source (subspecies?) were evenly distributed between groups. Although squirrel monkeys from both sources were similar in many of the measurements made, the Brazilian squirrels appeared to be somewhat more susceptible to dietary-induced increases in arterial lipids and to be healthier animals than the squirrel monkeys obtained from Peru. The mean weights of these monkeys at the time of initiation of the semipurified diets was 621.2 g ± 77.2 (± 1 SD). All of these animals had third molar eruptions and their mean weight was essentially stable with the natural foods diet.[2]

Experimental diets used. The semipurified diets were slightly modified from those described previously (8). The change was the substitution of vitamin D_3 for vitamin D_2 and a slight modification of the vitamin formula.[3] A fibrous dysplasia of bone has been frequently observed in New World monkeys. There are now several observations that this condition is preventable and reversible by relatively low doses of vitamin D_3 but is prevented or reversed only by high levels of oral vitamin D_2. The 2 diets used most extensively were the ones that produced the fewest and the most aortic sudanophilic lesions during our previous studies of squirrel monkeys. The first of the diets provided 8% (16% of calories) corn oil and contained no added cholesterol; the second contained 25.2% butter (45% of calories) and contained cholesterol at 0.1 g/100 kcal. In one experiment, two additional diets were included as close composition controls for the diet which produced lesions. These diets provided 45% of calories as butter or corn oil.

Animals were weighed every 2 weeks. The weight changes during the experiment for animals fed the principal nonpurified diets are shown on table 1.

Preparation of arterial tissue. The detailed procedures for recovery of the aortic intima plus inner one-third of the media have been described (1). Aortas were opened *in situ* with a ventral incision and the intimal surfaces were thoroughly flushed with iced saline. In a fraction of the aortas obtained, the aorta was divided with a dorsal incision; one half was transferred to iced fixative, and the inner layers of the other half were removed with fine forceps and a 10 × binocular dissecting microscope (the tissue being maintained on an iced petri dish moistened with 0.9% saline). After staining with Sudan IV and grading the fixed portion for the approxi-

[2] Purina Monkey Chow, Ralston Purina Company, St. Louis.

[3] The following quantities of vitamins were supplied per kg of diet: vitamin A acetate, 12,500 units; α-tocopherol, 0.1 g; crystalline vitamin D_3, 100 μg (4000 units); menadione, 0.04 g; ascorbic acid, 0.5 g; inositol, 1.0 g; choline chloride, 5.0 g; niacin, 0.049 g; riboflavin, 0.01 g; thiamine, 0.01 g; pyridoxine, 0.01 g; Ca pantothenate, 0.03 g; biotin, 0.2 mg; folic acid, 1.0 mg; and vitamin B_{12}, 0.02 mg.

TABLE 1

Effect of feeding semipurified diets on the body weight of female squirrel monkeys [1]

Diet and duration	Relative mean weight
Basal (840 C): 3 months	0.953
Atherogenic (butter + cholesterol): 3 months	0.964
Basal: 6 months	0.959
Atherogenic: 6 months	1.003
Atherogenic: 3 months; basal: 3 months	0.982
Basal: 8 months	1.036
Atherogenic: 8 months	0.993
Atherogenic: 3 months; basal: 5 months	1.017

[1] The means of the final 2 weighings are expressed as a proportion of the means of the 2 weighings prior to initiation of the semipurified diets. The mean starting weights were 621.2 g ± 77.2 (± 1 SD).

mate percentage of surface area covered with lesions, the inner layers of this tissue were also removed for chemical analyses and comparison with the fresh tissue. Two methods of fixation and staining were used: 1) tissues were fixed in 10% formalin and stained with Sudan IV in ethanol; 2) tissues were fixed in cacodylate buffered glutaraldehyde and stained with Sudan IV in propylene glycol. Chemical analyses of the lipids of fresh and fixed stained tissues indicated that neither staining technique could be used without some loss or alteration of compounds. The alcohol procedure removed most of the triglycerides and the more saturated cholesterol esters. The propylene glycol appeared not to alter appreciably the concentrations of free and ester cholesterol, phospholipids, and subclasses of phospholipids, or the fatty acid compositions of ester cholesterol and phospholipids. Twenty-five to 40% of the triglyceride was, however, removed by this latter staining procedure. Because of this limitation and because it became apparent that chemical determinations of certain of the lipids provided a good index of sudanophilia, the fixing and staining procedures were not continued for most specimens. Unstained aorta surfaces were examined under 10 × magnification for elevated lesions in the majority of animals.

Measurement of lipids. The inner layers from the fresh aorta were ground in 0.25 M sucrose. Small aliquots were taken for protein determinations by the micro-method of Lowry et al. (12). The main aliquots of the homogenates (generally equal ones) were used (a) for enzymatic measurements (to be described elsewhere), and (b) for transfer to chloroform:methanol (2:1) and lipid extraction.

The methods of aorta lipid analysis have been described (1). Using the equivalent of 40 to 60 mg of tissue for lipid analyses, we were able to determine concentrations of free and ester cholesterol, triglyceride, phospholipids, 8 subclasses of phospholipids, and the fatty acid compositions of some of the ester forms. The methods were based on purification and separation of molecular species by thin-layer chromatography and quantification with gas liquid chromatography.

RESULTS

Relationship of sudanophilia to arterial lipid composition. The extent of aortic sudanophilia was compared with several lipid measurements in order to find a more objective method and one which did not alter tissue composition for appraising the severity of atherosclerotic and preatherosclerotic lesions. There was a good rank correlation of sudanophilia with ester cholesterol and total cholesterol concentrations of those aortas in which one half was stained and one half taken for analysis. Figures 1 and 2 illustrate the relationship of sudanophilia to aortic total and ester cholesterol respectively. Sudanophilia increased slowly with the increase in total cholesterol until the concentration reached about 5 mg/g and then increased sharply. The increase in sudanophilia was essentially proportional to the increase in ester cholesterol concentration. The flat part of the total cholesterol vs. sudanophilia plot represented a range of increasing free cholesterol concentrations before a threshold level where the ester cholesterol appeared in significant quantities. Figures 1 and 2 as well as data from other studies (14, 15) suggest that sudanophilia may be related to ester cholesterol concentration. Nevertheless, there is an indication, discussed below, that the increase in free cholesterol concentration invariably precedes the increase of ester cholesterol concentration and the appearance of intimal sudanophilia. The triglyceride concentra-

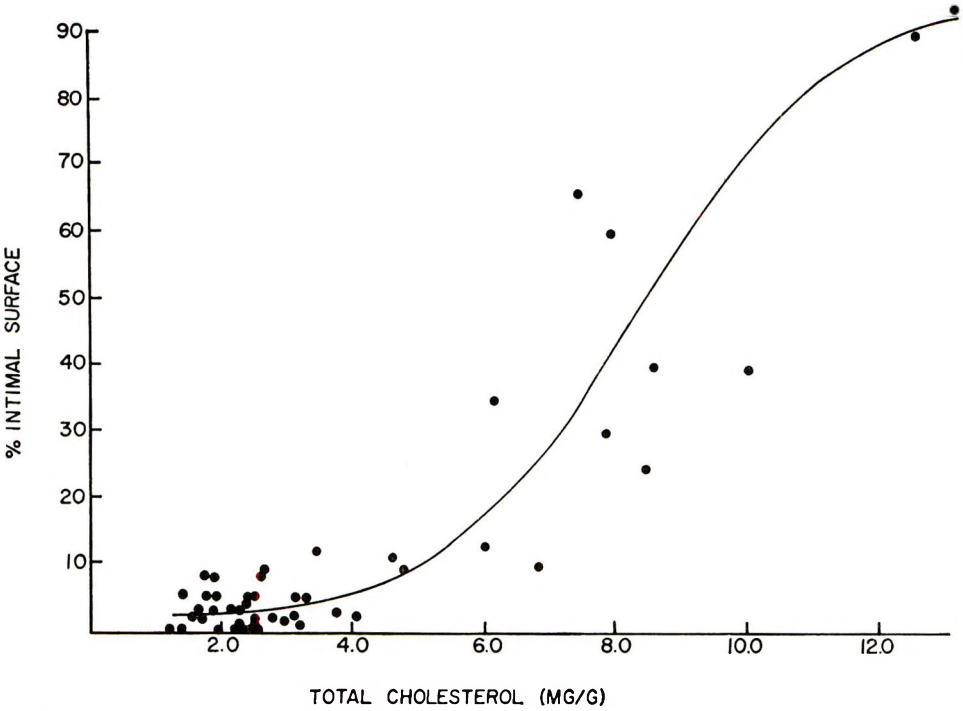

Fig. 1 Approximate percentage of the aortic intimal surface covered with sudanophilic or fibrotic lesions compared with the total cholesterol composition of the intima plus inner media of the aorta. One-half of the aorta was used for staining, the other half for lipid analysis. Correlation coefficient, $r = 0.635$, $P < 0.01$.

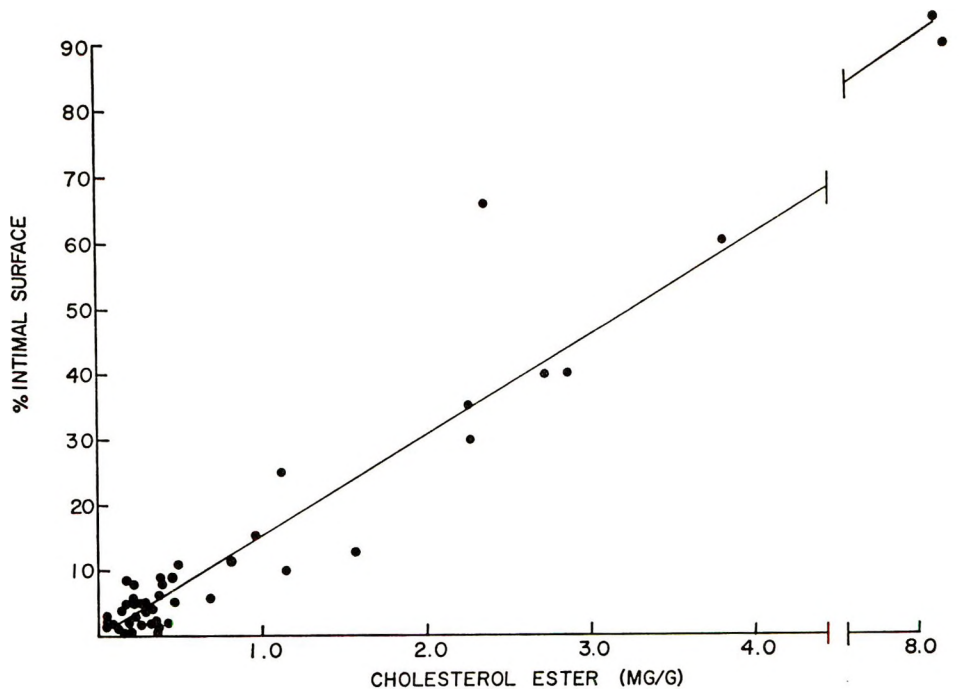

Fig. 2 Approximate percentage of the aortic intimal surface covered with sudanophilic lesions compared with the esterified cholesterol content of the intima plus inner media of the aorta. Correlation coefficient, $r = 0.936$, $P < 0.01$.

tion bore no relationship to aortic sudanophilia. Total phospholipids were increased in more severe atherosclerosis, but there was only an imperfect correlation of phospholipids with moderate intimal sudanophilia.

Changes in arterial lipids as a function of diet. Two series of squirrel monkeys were fed semipurified diets, one containing 16% of calories as corn oil (the basal, minimum lesion diet) and one with 45% of calories as butter and 0.1 g cholesterol per 100 kcal of diet. Some monkeys were killed at 2, 3, 6, and 8 months for chemical analysis of the aortic intima plus inner media and histological evaluation of the coronary arteries. A subseries from the high butter group was transferred to the corn oil diet at 3 months and animals were killed at 6 and 8 months of the total experimental period (3 and 5 months after the diet reversals). The statistical significance of the differences in arterial composition of the animals fed different diets was calculated by variance analysis (16). The F and P values are indicated on the figures illustrating mean aorta compositions. Because of the small sample which was changed from the lesion-producing to the basal diet ($n = 12$), this change did not have significant effects on arterial composition at the $P = 0.05$ level. Figure 3 shows the mean concentrations of total cholesterol in the aortic intima plus inner media and figure 4 the concentrations of aortic esterified cholesterol as a function of time on the experimental regimens. Both of these parameters, as well as the free cholesterol concentrations (fig. 5) were elevated by feeding the high butter diet (which also elevated the serum cholesterol level). There was also a lowering of the arterial concentrations of total and esterified cholesterol when the animals fed the high butter diet for 3 months were changed to the corn oil-containing diet. The cholesterol and phospholipid concentrations of the aortic preparations increased in all the animals after they were changed from the commercial monkey ration to either of the semipurified diets. There were higher concentrations of phospholipids in aortas of animals from the high butter group than in the aortas of the corn oil animals (fig. 6).

A further subfractionation of the aorta phospholipids into eight different subclasses showed that the two most prevalent fractions were sphingomyelin and phosphatidylcholine (lecithin). These fractions also increased most after the change from the commercial monkey ration to the

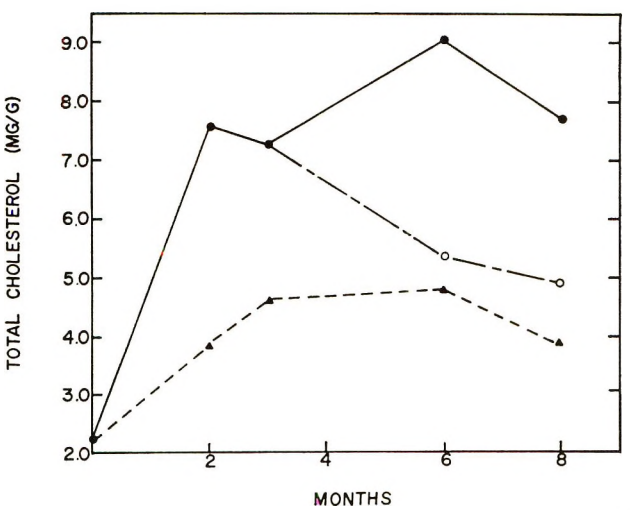

Fig. 3 Mean total cholesterol concentration of the aortic intima plus inner media from squirrel monkeys fed different diets for up to 8 months ($n = 53$). Semipurified diet regimens were instituted at time 0. ●——● High butter+ diet, ▲- - - - -▲ low corn oil diet (840 C), ○–·–·–·–○ animals changed from high butter+ to 840 C at 3 months. Difference of the high butter+ and 840 C groups, $F = 4.77$, $P < 0.05$.

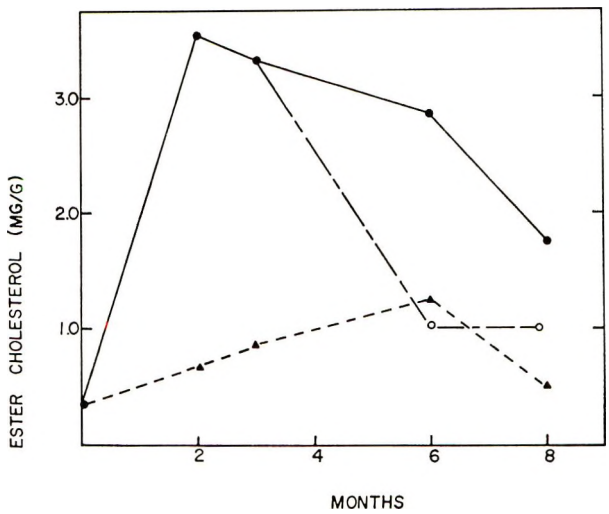

Fig. 4 Mean esterified cholesterol concentration of the aortic intima plus inner media from squirrel monkeys fed different diets for up to 8 months (n = 53). Semipurified diet regimens were instituted at time 0. ●——● High butter+ diet, ▲– – – –▲ low corn oil diet (840 C), ○–·–·–·–○ animals changed from high butter+ to 840 C at 3 months. Difference of the high butter+ and 840 C groups, $F = 8.38$, $P < 0.01$.

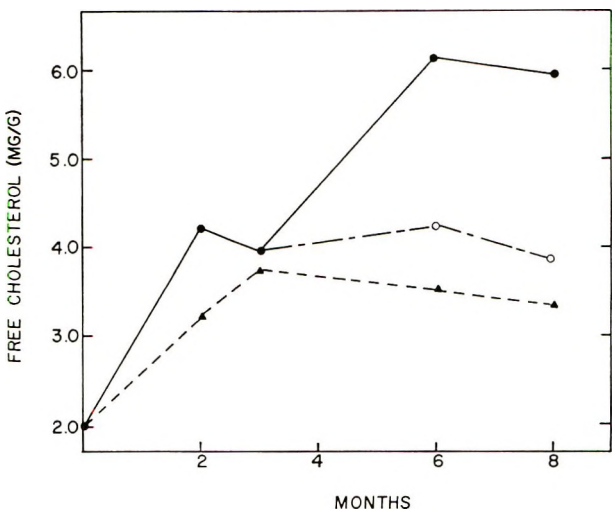

Fig. 5 Mean free cholesterol concentration of the aortic intima plus inner media from squirrel monkeys fed different diets up to 8 months (n = 53). Semipurified diet regimens were instituted at time 0. ●——● High butter+ diet, ▲– – – –▲ low corn oil diet (840 C), ○– – – –○ animals changed from high butter+ to 840 C at 3 months. Difference of the high butter+ and 840 C groups, $F = 8.39$, $P < 0.01$.

semipurified diet containing butter and added cholesterol. Figure 7 shows the mean aortic concentrations of phosphatidylcholine according to the diet fed. Figure 8 shows comparable data for sphingomyelin concentrations. This indicates that the mere change to a semipurified diet caused an increase in aortic sphingomyelin. There is also an indication that an increase in sphingomyelin concentrations

always was the first alteration in phospholipid composition (in comparisons presented in a subsequent section).

The concentrations of triglycerides (fig. 9) in the aortic intima plus inner media decreased, whereas the remainder of the lipid measurements increased during the feeding of the lesion-inducing diet. Thus, in aortas containing striking gross lesions the concentration of total glycerides seldom exceeded 0.30 mg/g of tissue.

The total protein content of the inner layers of the aortas of these monkeys was remarkably constant. The mean concen-

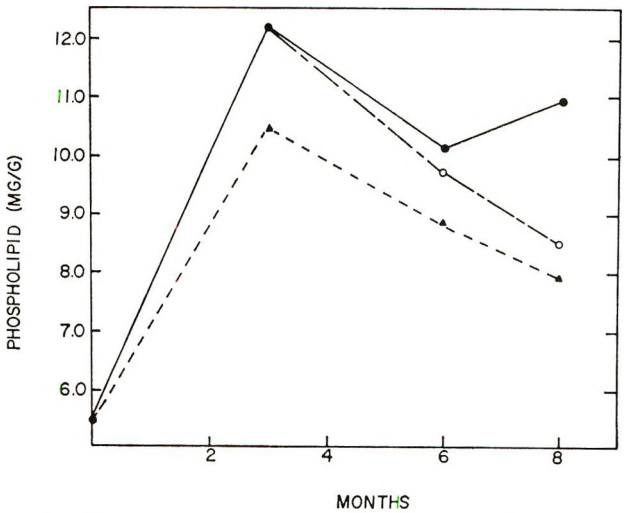

Fig. 6 Mean phospholipid concentration of the aortic intima plus inner media from squirrel monkeys fed the different diets for up to 8 months ($n = 53$). Semipurified diet regimens were instituted at time 0. ●——● High butter+ diet, ▲- - - - -▲ low corn oil diet (840 C), ○- - - -○ animals changed from high butter+ to 840 C at 3 months. Difference of the high butter+ and 840 C groups, $F = 4.85$, $P < 0.05$.

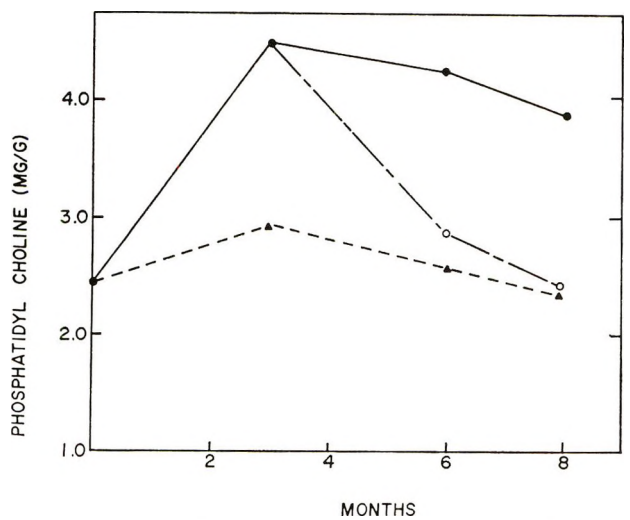

Fig. 7 Mean phosphatidylcholine concentration of the aortic intima plus inner media from squirrel monkeys fed different diets for up to 8 months ($n = 53$). Semipurified diet regimens were instituted at time 0. ●——● High butter+ diet, ▲——▲ low corn oil diet (840 C), ○- - - -○ animals changed from high butter+ to 840 C at 3 months. Difference of the high butter+ and 840 C groups, $F = 9.52$, $P < 0.01$.

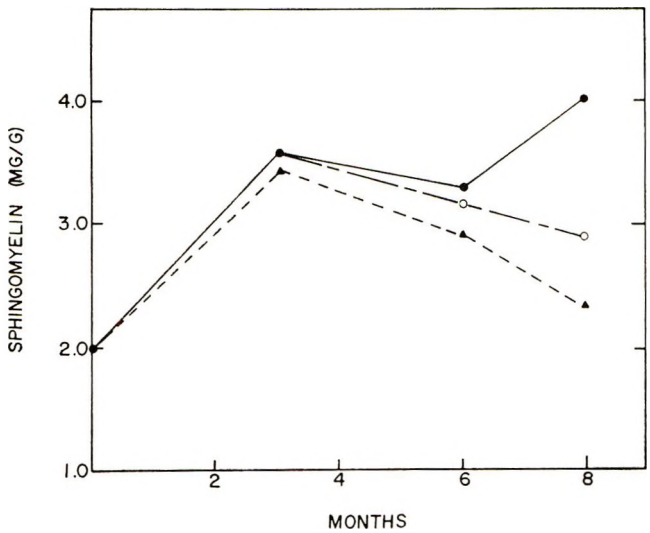

Fig. 8 Mean sphingomyelin concentration of the aortic intima plus inner media from squirrel monkeys fed different diets for up to 8 months ($n = 53$). Semipurified diet regimens were instituted at time 0. ●———● High butter+ diet, ▲- - - - -▲ low corn oil diet (840 C), ○- - - -○ animals changed from high butter+ to 840 C at 3 months. Difference of the high butter+ and 840 C groups, $F = 1.45$, $P > 0.05$.

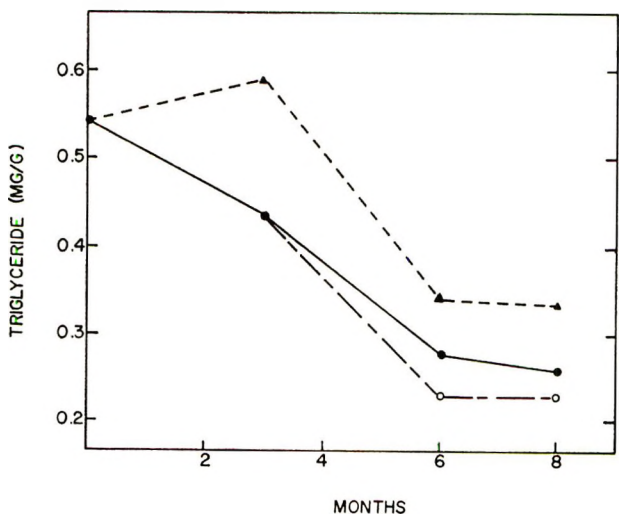

Fig. 9 Mean triglyceride concentration of the aortic intima plus inner media from squirrel monkeys fed different diets for up to 8 months ($n = 53$). Semipurified diet regimens were instituted at time 0. ●———● High butter+ diet, ▲- - - - -▲ low corn oil diet (840 C), ○- - - -○ animals changed from high butter+ to 840 C at 3 months. Difference of the high butter+ and 840 C groups, $F = 1.45$, $P > 0.05$.

tration for all groups was 116 mg/g tissue, and there were no significant differences between group means.

Interrelations of different lipid concentrations of aortas. When the aorta total cholesterol concentrations were plotted against the free and the esterified cholesterol concentrations (fig. 10 and table 2), it was apparent that the 2 subcategories increased in a very predictable manner as

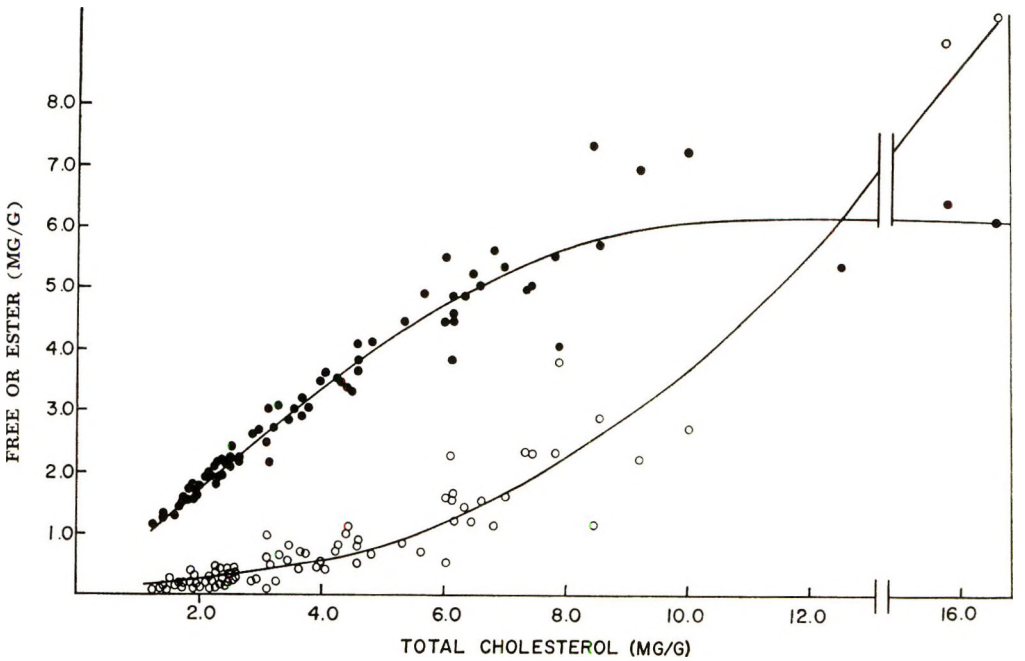

Fig. 10 Relationships of the free and of the esterified cholesterol concentrations to the total cholesterol in the aortas of squirrel monkeys. ● = free cholesterol, ○ = esterified cholesterol.

TABLE 2

Correlation coefficients (r (16)) for certain arterial lipids of 63 female squirrel monkeys [1]

Comparison	Value of *r*
Free cholesterol vs. sphingomyelin	0.744
Total cholesterol vs. sphingomyelin	0.654
Ester cholesterol vs. phosphatidylcholine	0.827
Total cholesterol vs. phosphatidylcholine	0.875
Total cholesterol vs. free cholesterol	0.926
Total cholesterol vs. ester cholesterol	0.944
Total cholesterol vs. triglycerides	−0.115
Total cholesterol vs. phospholipid	0.808
Total cholesterol vs. protein	0.070
Ester cholesterol vs. terminal plasma cholesterol	0.402

[1] N = 63 pairs of values; 1% level of significance for 61 df = ± 0.354.

the total cholesterol increased. The increase of free cholesterol was rapid during the initial rise of total cholesterol; subsequently, the ester cholesterol began to increase and increased sharply with the increase of total cholesterol.

The concentrations of two of the phospholipid fractions showed an interesting relationship to the total cholesterol concentration of the aortic inner layers. Figure 11 illustrates these relationships for sphingomyelin and phosphatidylcholine. The concentration of sphingomyelin correlated closely with the total cholesterol concentration until the latter value reached about 5 mg/g. The sphingomyelin increased only slightly at cholesterol concentrations above this value. The concentration of phosphatidylcholine was not correlated with the total cholesterol until the cholesterol reached about 5 mg/g, but at higher cholesterol concentrations, there was an obvious positive correlation of aortic total cholesterol and phosphatidylcholine. Thus the pattern of relationship of aortic sphingomyelin to total cholesterol resembled that of the free to total cholesterol, and the relationship of phosphatidylcholine to total cholesterol concentrations resembled the relationship of esterified cholesterol to total cholesterol.

Table 2 shows the correlation coefficients (16) for some of the measurements of aortic lipids.

DISCUSSION

This study indicates that the concentrations of several categories of lipids in the

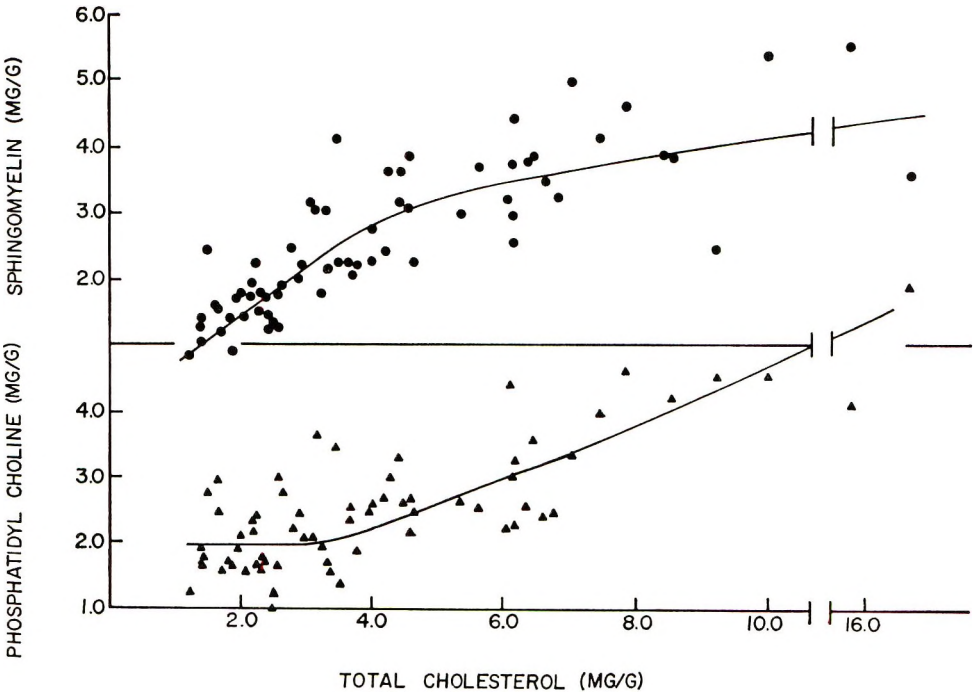

Fig. 11 Relationships of the phosphatidylcholine and of the sphingomyelin concentrations to the total cholesterol in the aortas of squirrel monkeys.

aortic intima plus inner media were influenced by the composition of the diet. When animals which had been fed an atherosclerosis-inducing diet for 3 months were changed to the basal corn oil diet, their aorta lipids tended to revert toward the "normal" or basal level. Thus the tendency noted in rabbits (7, 14) for lipid accumulation in aortas to be a self-sustaining process after an initial period of hypercholesterolemia was not evident in this study of squirrel monkeys.

The close correlations of the sphingomyelin and free cholesterol concentrations suggested that the increases in those 2 moieties were related. The continuum of increasing values for total cholesterol, free cholesterol, and sphingomyelin appeared to represent a sequence of changes between "normal" aorta and the initiation of sudanophilia.

We have shown previously (1) that the inner lining of aortas of rhesus monkey fetuses of various ages and of early neonatal animals had only about one-third the concentration of aortic sphingomyelin and substantially less aortic free cholesterol than adults, even though the adults were free of sudanophilic lesions. During this previous study of fetal and adult aortas, we pointed out that in other tissues sphingomyelin and free cholesterol made up higher proportions of the lipid of plasma membrane than of the lipid of intracellular organelles and cell sap. The increase of sphingomyelin and free cholesterol in the aorta with increasing age and, perhaps, in relation to early nutritionally induced hyperlipidemia might be a result of increased quantities of plasma membrane. McGill and Geer (17) have reported the consistent occurrence of dense osmophilic inclusions in otherwise apparently normal intimal cells of arteries. Weibel and Palade (18) have reported the occurrence of two cytoplasmic components of unknown functional significance in arterial endothelium. Parker and Odland (19) have reported an increase in endothelial vacuoles and in the prominence of endothelial cell organelles in rabbit aortas very early in experimental atherosclerosis. Quantita-

tive variations in these inclusions may be the morphological equivalent of the increased concentrations of free cholesterol and sphingomyelin that appear to occur prior to the appearance of sudanophilia or gross intimal lesions. Separations of normal aortic homogenates by differential and sucrose density gradient centrifugation [4] indicated a heterogeneous phospholipid distribution in different fractions with the "microsomes" the fraction richest in sphingomyelin.

The nature of the Sudan IV staining material in the earliest clear-cut lesions is still not clear. In this study, the triglyceride concentration of the aortic intima plus inner media was relatively constant, did not increase with nutritionally induced hypercholesterolemia, and did not correlate with those factors (esterified and total cholesterol) which did correlate closely with sudanophilia. Since esterified cholesterol did correlate with sudanophilia, appeared first in significant concentrations at the first appearance of sudanophilia, and had a known capacity (this is particularly true of unsaturated esters (20)) to incorporate limited amounts of Sudan IV, this class of lipids appeared to be the most likely cause of sudanophilia.

The relationship between the 2 phases of lipid increase in aorta (the first dominated by free cholesterol and sphingomyelin, the second by esterified cholesterol and phosphatidylcholine) during the induction of lesions is also uncertain. We suggest that the 2 phases of lipid alteration represent separate processes. The first phase might be the result of a small imbalance in the exchange of free cholesterol between plasma lipoproteins and cell membrane with the resulting formation of pinocytotic inclusions. The second phase characterized by increases in cholesterol ester and lecithin concentrations might result from a secondary tissue response to the first phase with alterations in endothelial permeability to intact lipoproteins. The cholesterol esters sequestered in the aorta are one of the least polar components of plasma lipoproteins and are a class of compounds which have limited potential to exchange with tissue lipoproteins. The increase in phosphatidylcholine could reflect an increase in overall membrane regeneration — in reparative and phagocytic processes (21). Our studies [5] of the incorporation of labeled fatty acids into phosphatidylcholine by cell-free preparations of normal and atherosclerotic aortas indicated that the level of endogenous lysophosphatidylcholine was an important factor in controlling the level of activity.

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Effect of Calcium and Gossypol on the Performance of Swine and on Certain Enzymes and Other Blood Constituents [1,2]

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ABSTRACT The effect of calcium and iron supplementation on gossypol toxicity, as measured by weight gains and changes in certain blood constituents, was studied in swine. Groups of 6 Duroc-Jersey pigs each were fed rations containing 2 levels of gossypol supplemented with 2 levels of calcium hydroxide. The protein source in the ration was cottonseed meal for the experimental groups and soybean meal for the control groups. Additional groups received the higher level of gossypol supplemented with ferrous sulfate, by itself or with the higher level of calcium used. Weight gains at 15 weeks showed that simultaneous supplementation with calcium and iron resulted in weight gains similar to those obtained with the control rations. Blood samples taken at zero, 7 and 15 weeks showed that hemoglobin and hematocrit values were significantly decreased, and glutamic-oxalacetic transminase was significantly increased by gossypol feeding. Lactic dehydrogenase, leucine amino peptidase, and aldolase in the serum were not significantly affected by gossypol.

Several workers (1, 2) have shown that the addition of ferrous ions to rations containing high levels of cottonseed meal decreased the toxicity of gossypol for monogastric animals. This is probably the result of a chelating action by the metal on the pigment. Jarquín et al. (3) reported that the addition of both calcium hydroxide and ferrous sulfate to a swine ration containing 42% of cottonseed meal resulted in good performance and in a complete protection from gossypol toxicity. However, they did not determine whether the addition of calcium alone could counteract the toxicity of the pigment.

The toxicity of gossypol in swine and other monogastric species is characterized by lesions of the heart, lungs, small intestine and liver (3, 4). The pathological changes in these organs might conceivably alter the levels of some of the blood-circulating enzymes, although the literature contains no specific report on the effect of gossypol in this respect. Ferguson et al. (5) showed that the levels of succinic dehydrogenase and cytochrome oxidase in the liver of chicks fed gossypol decreased in studies both in vitro and in vivo. No biochemical parameters, however, are available for the detection of early, asymptomatic, gossypol toxicity in sensitive animals.

We have attempted to determine the effect of 2 levels of calcium on the growth and on some of the blood constituents and enzymes of swine fed rations containing varying levels of gossypol.

MATERIALS AND METHODS

The experiment was carried out at the experimental farm of the Institute located in the Guatemalan highlands at 1,524 m over sea level with a minimal and maximal temperature of 7° and 27°, respectively.

Eleven experimental groups, of 6 purebred Duroc-Jersey weanling pigs each, were formed according to weight and sex and housed in concrete pens.

Experimental rations are shown in table 1. Control group 1 was fed a ration of only soybean meal; control groups 2 and 3 were fed rations supplemented with the 2 levels of calcium used in the experimental groups, respectively. These were fed a ration containing 42% cottonseed meal, prepared by the process of pre-press

Received for publication June 24, 1966.
[1] This investigation was supported by a grant from the W. K. Kellogg Foundation.
[2] INCAP Publication I-398.

TABLE 1

Composition of experimental diets

	Ration no.										
	1	2	3	4	5	6	7	8	9	10	11
Soybean flour	45.0	45.0	45.0	—	—	—	—	—	—	—	—
Cottonseed flour	—	—	—	42.0	42.0	42.0	42.0	42.0	42.0	42.0	42.0
Mineral mixture [1]	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Alfalfa leaf meal	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
$Ca(OH)_2$	—	0.5	1.0	—	0.5	1.0	—	0.5	1.0	1.0	—
$FeSO_4 \cdot 7H_2O$	—	—	—	—	—	—	—	—	—	0.1	0.1
Torula yeast	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Cottonseed kernel meal	—	—	—	—	—	—	1.3	1.3	1.3	1.3	1.3
Ground yellow corn	48.0	47.5	47.0	51.0	50.5	50.0	49.7	49.2	48.7	48.6	49.6
						mg/100 g					
Free gossypol	—	—	—	30.0	22.8	27.9	44.0	37.4	39.4	38.2	40.6
Total gossypol	—	—	—	396	405	426	405	381	474	446	449

[1] Salmina, Riverside Company, Guatemala, C. A. Contains: (in per cent) $CaCO_3$, 33; NaCl, 33; bone meal, 33, and minor elements, 1.

solvent extraction. The levels of free gossypol in the rations ranged from 22.8 to 44.0 mg/100 g, and total gossypol ranged from 381 to 474 mg, as determined by the AOCS official methods (6).

Levels of calcium hydroxide used were 0.5 and 1% of the ration. The gossypol content was increased by adding appropriate amounts of a cottonseed kernel meal with 1.13% g of free gossypol to the rations. Water and the experimental rations were available at all times. Weekly records of feed consumed and individual weight were kept.

The experimental period lasted 15 weeks. At the beginning, middle, and end of this period, all the animals were bled by jugular venipuncture after overnight fasting. Blood was collected, with and without anticoagulant, for the following determinations: hemoglobin by the method of Cannan (7), hematocrit by the method of Wintrobe (8), glucose by the method of Somogyi-Nelson (9), total protein according to Lowry and Hunter (10), albumin by the method of Lowry et al. (11), urea nitrogen by the method of Gentzkow and Mosen (12), lactic dehydrogenase by the method of Berger and Broida,[3] leucine amino peptidase by a modification of the Goldbarg and Rutenburg procedure,[4] glutamic-oxalacetic and glutamic-pyruvic transaminases by the Sigma-Frankel method [5] and aldolase by a modification of the Sibley and Lehninger procedure.[6] Aldolase was determined on the samples

TABLE 2

Effect of the addition of calcium and iron on the performance of swine fed rations containing 42% cottonseed meal

Ration no.[1]	Avg final wt [2]	Avg daily gain	Feed efficiency
	kg	*kg*	
1	53.8 ± 6.6 [3]	0.44 ± 0.04	3.0
2	47.5 ± 7.4	0.38 ± 0.05	3.2
3	44.9 ± 10.6	0.36 ± 0.08	3.2
4	44.6 ± 18.2	0.36 ± 0.16	3.2
5	40.4 [4] ± 11.7	0.32 ± 0.10	3.6
6	42.9 ± 5.0	0.34 ± 0.04	3.4
7	35.8 [4] ± 13.3	0.27 ± 0.12	3.8
8	43.8 ± 8.5	0.35 ± 0.07	3.6
9	34.0 [4] ± 7.8	0.25 ± 0.06	3.2
10	52.4 ± 5.0	0.43 ± 0.04	3.4
11	42.2 [4] ± 8.6	0.33 ± 0.07	3.0

[1] See table 1.
[2] Average initial weight, 7.4 kg.
[3] Avg ± SD.
[4] Difference between control and experimental groups significant at the 0.05 level of probability; least significant difference, 11.4 kg.

obtained during the middle and end of the experimental period.

Whole blood samples were analyzed for hemoglobin, hematocrit and glucose on the same day the animals were bled. After protein was determined, blood serum was immediately frozen, and unnecessary thawing was avoided until all enzymatic determinations were completed. The latter were

[3] Sigma Chemical Company 1964 Tech. Bull. no. 500 (April), St. Louis.
[4] Sigma Chemical Company 1963 Tech. Bull. no. 250 (March).
[5] Sigma Chemical Company 1964 Tech. Bull. no. 505 (May).
[6] Sigma Chemical Company 1963 Tech. Bull. no. 750 (April).

determined within 48 hours after the samples were received at the laboratory.

RESULTS

Table 2 shows the performance of the experimental animals. Final weight was significantly lower ($P < 0.05$) for rations 5, 7, 9, and 11, which contained 22.8, 44.0, 39.4, and 40.6 mg/100 g of free gossypol, respectively, as compared with the control ration. Separate addition of calcium and iron ions did not increase final weights, but the combination of both chemicals (group 10) resulted in final weights that did not differ significantly from those obtained with the soybean rations. During the whole experimental period, only one animal died in the group receiving the highest level of free gossypol (group 7). Postmortem examination revealed lesions that could be attributed to gossypol toxicity. Feed efficiency was not impaired by any of the treatments used.

Table 3 shows the values for protein, albumin and urea nitrogen in the blood serum of the experimental animals. Analysis of variance was applied for comparison among groups 1 to 9, and groups 10 and 11 were compared by Student's t test. The effect of time was highly significant ($P < 0.01$) for all determinations. There was a significant effect ($P < 0.01$) of gossypol on urea nitrogen. Table 4 shows the values for hemoglobin, hematocrit and glucose. The levels of gossypol, time and calcium in the ration each had a significant effect ($P < 0.01$) on hemoglobin, hematocrit and glucose values. The interaction between gossypol and time was highly significant ($P < 0.01$) for the 3 blood components.

Figures 1 to 3 show the results of the enzyme determinations. All enzymes studied were affected significantly ($P < 0.01$) by time, and only glutamic-oxalacetic transaminase was increased significantly ($P < 0.01$) by the level of gossypol fed in the ration. Calcium had a conditioning effect on the response to gossypol, as evidenced by a significant gossypol-calcium interaction. Furthermore, the conditioning effect was modified through time, as disclosed by a highly significant second-order interaction (Ca × gossypol × time). The interaction between gossypol and time was

TABLE 3

Effect of the addition of calcium and iron on some blood serum constituents of swine fed rations containing 42% cottonseed flour [1]

Ration no.[2]	Protein			Albumin			Urea nitrogen		
	Initial [3]	7 weeks	Final	Initial	7 weeks	Final	Initial	7 weeks	Final
	g/100 ml	*g/100 ml*	*g/100 ml*	*g/100 ml*	*g/100 ml*	*g/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*
1	5.27 ± 0.25 [4]	6.50 ± 0.19	6.66 ± 0.39	2.41 ± 0.13	2.39 ± 0.21	2.82 ± 0.03	20.4 ± 3.0	27.1 ± 4.1	30.2 ± 1.4
2	4.99 ± 0.68	6.36 ± 0.11	6.56 ± 0.61	2.48 ± 0.31	2.53 ± 0.14	2.85 ± 0.10	19.7 ± 3.1	25.2 ± 3.1	25.7 ± 1.1
3	5.46 ± 0.52	6.41 ± 0.28	6.90 ± 0.47	2.56 ± 0.31	2.46 ± 0.18	2.79 ± 0.14	21.7 ± 5.4	22.7 ± 2.7	25.8 ± 3.8
4	5.50 ± 0.21	6.25 ± 0.28	6.94 ± 0.50	2.51 ± 0.26	2.52 ± 0.30	2.79 ± 0.28	24.4 ± 3.0	20.6 ± 2.4	24.1 ± 1.6
5	5.66 ± 0.08	6.33 ± 0.23	6.75 ± 0.46	2.74 ± 0.15	2.55 ± 0.07	2.78 ± 0.17	23.2 ± 3.4	21.0 ± 2.8	24.0 ± 3.6
6	5.40 ± 0.16	5.94 ± 0.32	6.74 ± 0.30	2.49 ± 0.28	2.46 ± 0.18	2.85 ± 0.12	24.7 ± 3.6	18.5 ± 3.8	22.3 ± 1.2
7	5.41 ± 0.38	5.95 ± 0.50	6.87 ± 0.30	2.66 ± 0.29	2.44 ± 0.02	2.73 ± 0.22	20.3 ± 3.9	16.4 ± 2.0	25.6 ± 2.9
8	5.75 ± 0.38	6.47 ± 0.43	6.52 ± 0.68	2.68 ± 0.24	2.37 ± 0.14	2.66 ± 0.23	22.4 ± 5.2	18.5 ± 2.6	24.6 ± 3.1
9	5.73 ± 0.30	6.02 ± 0.60	6.70 ± 0.55	2.84 ± 0.26	2.14 ± 0.20	2.57 ± 0.12	20.0 ± 3.6	17.3 ± 5.2	23.2 ± 1.0
10	5.82 ± 0.26	6.09 ± 0.25	6.98 ± 0.38	2.66 ± 0.79	2.26 ± 0.02	2.81 ± 0.04	21.5 ± 2.7	23.3 ± 2.6	25.7 ± 2.7
11	5.53 ± 0.32	5.81 ± 0.69	6.74 ± 0.48	2.66 ± 0.36	2.23 ± 0.16	2.51 ± 0.34	21.6 ± 2.5	20.2 ± 2.6	23.2 ± 2.1

[1] Six pigs/group.
[2] See table 1.
[3] Initial sample; sample taken after 7 weeks on experiment; sample taken at the end of the experiment.
[4] ± SD.

TABLE 4

Effect of calcium and iron on some blood constituents of swine fed rations containing 42% cottonseed flour

Ration no.[1]	Hemoglobin			Hematocrit			Glucose		
	Initial [2]	7 weeks	Final	Initial	7 weeks	Final	Initial	7 weeks	Final
	g/100 ml	*g/100 ml*	*g/100 ml*	*%*	*%*	*%*	*mg/100 ml*		*mg/100 ml*
1	12.7±1.1 [3]	13.0±0.6	14.4±0.8	39±4	40±7	39±2	88±6	57±5	78±5
2	11.8±0.9	11.6±1.4	13.5±0.5	36±4	39±4	39±2	84±5	56±6	78±10
3	11.0±1.4	10.1±2.1	13.7±1.2	34±5	36±7	40±3	84±23	69±8	67±4
4	11.3±1.6	9.8±2.5	11.1±3.9	36±4	38±6	34±9	76±11	84±11	77±12
5	11.5±2.7	9.0±2.6	9.0±1.9	36±9	41±8	32±4	68±6	92±15	87±12
6	12.6±0.8	8.2±3.6	9.8±1.3	39±2	35±4	33±6	86±7	81±14	84±10
7	12.1±0.9	10.8±2.1	10.9±2.4	41±2	44±3	37±8	85±18	73±12	99±51
8	12.2±0.8	9.9±1.9	9.2±1.5	39±2	36±7	33±6	92±8	74±7	74±2
9	12.5±0.9	8.8±0.9	8.1±1.0	40±2	34±3	34±6	82±11	74±5	75±8
10	11.7±1.4	10.8±2.2	13.2±0.6	38±4	41±4	43±2	86±10	62±5	68±20
11	12.1±1.7	11.7±1.2	12.1±1.3	42±6	40±3	36±3	83±15	65±11	67±12

[1] See table 1.
[2] Initial sample; sample taken after 7 weeks on experiment; sample taken at the end of the experiment.
[3] ± SD.

highly significant on glutamic-pyruvic values, but no gossypol effect could be detected on the levels of this transaminase.

DISCUSSION

The results suggest that addition of both calcium and ferrous ions effectively counteracts gossypol toxicity in swine. Addition of one mineral at a time was not as effective and, in some cases, calcium supplementation alone resulted in decreased final weights even with the soybean meal rations. This is probably the result of alterations in the calcium-to-phosphorus ratio, or possibly of other mineral imbalances. Excess calcium in the ration might induce zinc deficiency (13). The mineral mixture used has been shown, in previous studies, to contain adequate amounts of zinc, so that a zinc deficiency in the study reported here appears unlikely.

Mortality did not occur in most of the groups, even though the animals were fed rations containing levels of free gossypol which should have been lethal for swine. This could be attributed to the calcium and iron added and to the high protein level of all rations, which has been reported to decrease gossypol toxicity (14). Protein quality of the cottonseed meal used was also high. Addition of iron alone did not cause greater weight gains than those obtained with the unsupplemented rations. A symptom not previously reported, concerning gossypol toxicity, was depigmentation of the hair of animals fed the cottonseed meal ration. This depigmentation was also evident, but to a lesser extent, in animals fed cottonseed meal with either calcium hydroxide or ferrous sulfate. A combination of both protected the animals from hair depigmentation. The mechanism(s) responsible for hair discoloration is now under study.

The results of Bressani et al.[7] showed that cottonseed meals treated with alkaline solutions resulted in lower values of free gossypol, and less toxicity, than those treated with acid solutions. Their results also indicated that the samples treated at low pH values resulted in increased toxicity of the meal when fed to laboratory animals. Since total gossypol remained constant under the whole range of pH tested, it can be assumed that free gossypol decreased at higher pH values, owing to increased binding by some of the constituents of the meal, especially protein. Furthermore, the mere contact of calcium ions and ferrous sulfate with cottonseed meal has been shown to decrease free gossypol (3). However, it has been suggested (1) that iron ions have a detoxifying effect on gossypol because iron binds the pigment into a chelated compound which is not absorbed. Our results suggest that the binding capacity of iron ions can be increased by the addition of alkaline ions, in the form of calcium hydroxide, a hypo-

[7] Bressani, R., L. G. Elías and J. E. Braham 1965 Effect of pH on the free gossypol level and nutritive value of cottonseed protein concentrates. Federation Proc., 24: 626 (abstract).

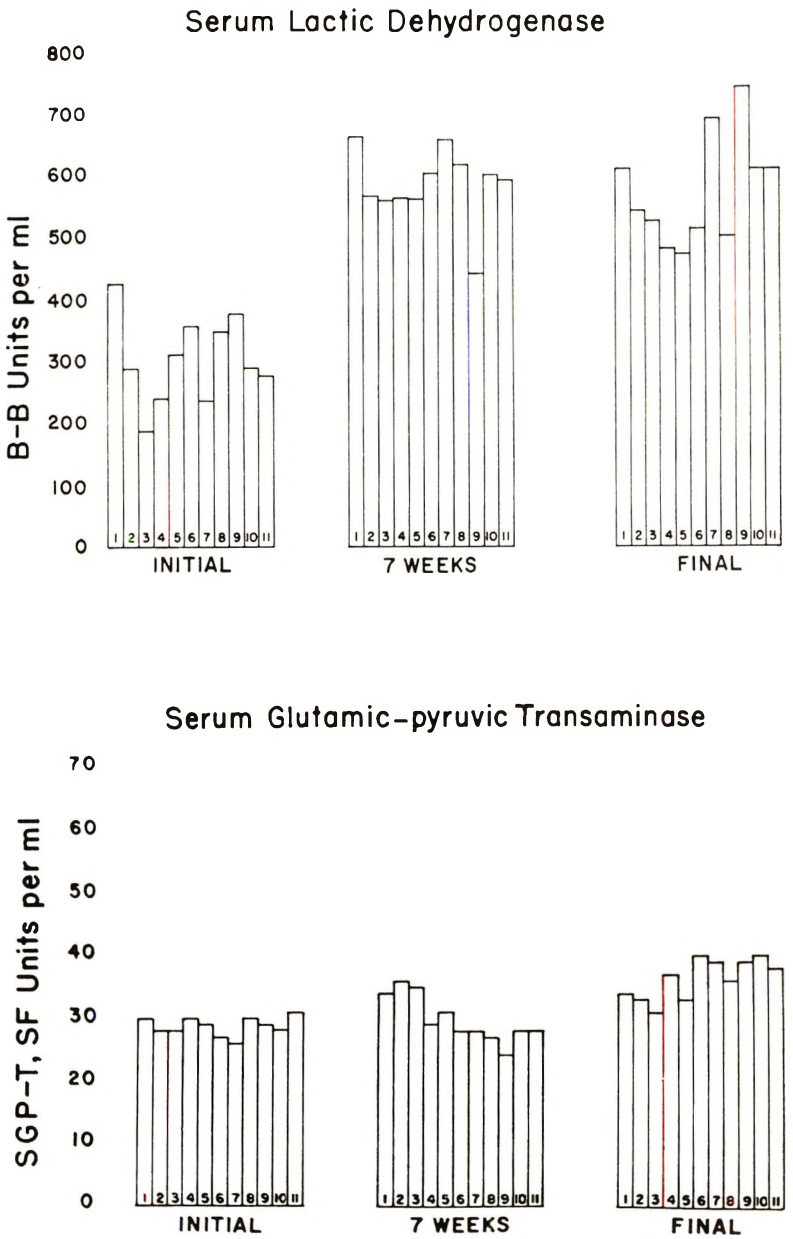

Fig. 1 Effect of gossypol and calcium and iron supplementation on serum enzymes of swine. Numbers within bars represent number of ration as described in table 1. B-B unit = one B-B unit will reduce 4.8×10^{-4} μmoles of pyruvate/minute at 25°. SF unit = one theoretical Sigma-Frankel unit of GO or GP-transaminase and will form 4.82×10^{-4} μmoles of glutamate/min at pH 7.5 and 25°.

thesis which is now under further investigation.

Hemoglobin and hematocrit values are inversely related to the gossypol content of the ration. These results corroborate those reported by Jarquín et al. (3). It is too early to state whether this effect is the result of a specific action of gossypol on hemoglobin synthesis, the binding of iron ions at the intestinal or at the tissue

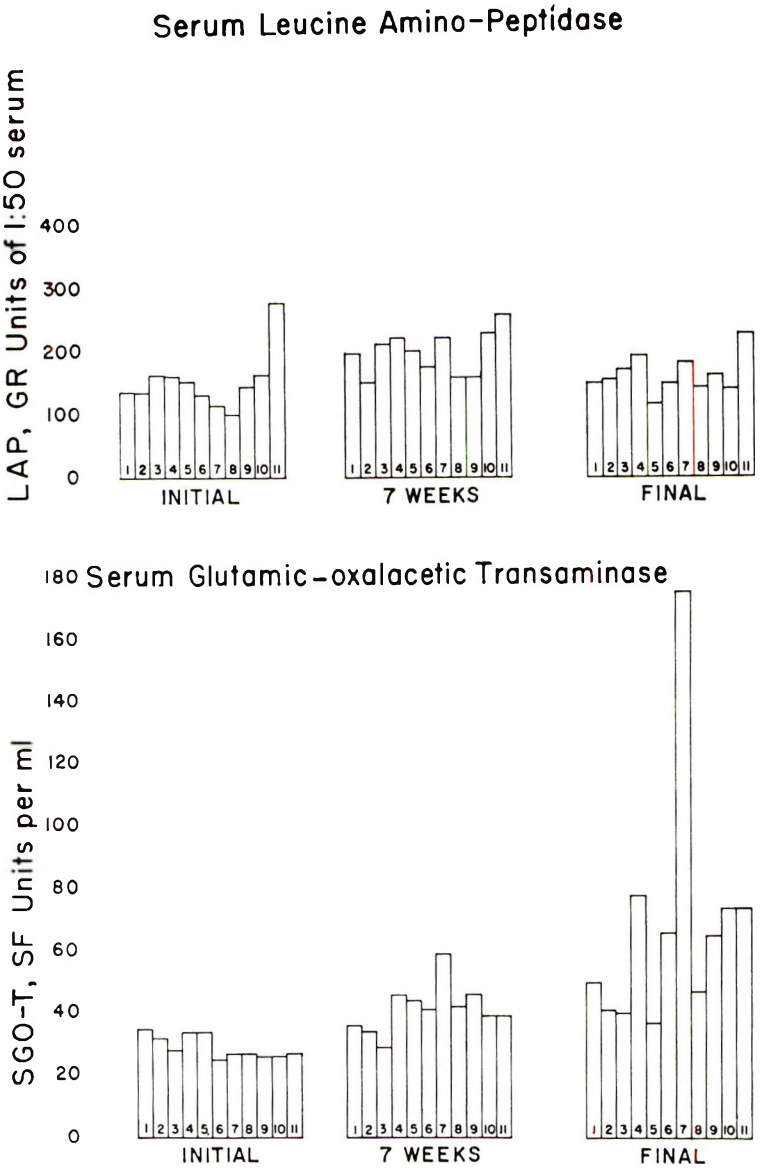

Fig. 2 Effect of gossypol and calcium and iron supplementation on the serum enzymes of swine. Numbers within bars represent number of ration as described in table 1. GR unit = 0.5 mg β-naphthol liberated/hour at 37°. SF unit = one theoretical Sigma-Frankel unit of GO or GP-transaminase and will form 4.82×10^{-4} μmoles of glutamate/min at pH 7.5 and 25[4].

level, or simply a reduction in food intake. The results of Smith (4) suggest, however, that gossypol intake results in a diminished capacity in the blood for oxygen-carrying. In turn, this could indicate an interrelationship between gossypol and hemoglobin levels.

Time was largely responsible for changes in the levels of blood-circulating enzymes. Lactic dehydrogenase increased until the seventh week of observation. After this, the levels appeared to reach a plateau where they remained until the fifteenth week. Heart changes associated with gossypol

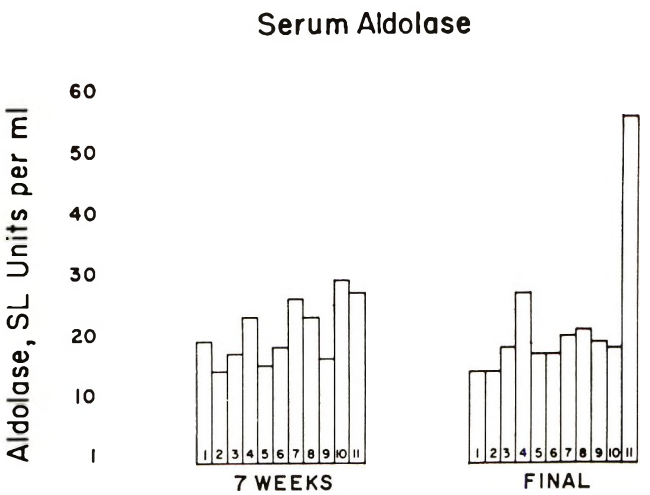

Fig. 3 Effect of gossypol and calcium and iron supplementation on serum aldolase of swine. Numbers within bars represent number of ration as described in table 1. SL unit = one Sibley-Lehninger unit of aldolase activity and is defined as the amount of enzyme which will split 1 mm^3 of fructose-1,6-diphosphate/hour at 37°, under the assay conditions described in the procedure.

toxicity (3) did not appear to affect lactic dehydrogenase levels. Probably the latter would increase shortly before death, thus having poor diagnostic value in the determination of early gossypol toxicity. Leucine amino peptidase, which has been reported to increase when there is pancreatic damage, was not increased by the feeding of gossypol. This corroborates the results of Jarquín et al. (3), who found atrophy of the acini of the pancreas in swine fed high levels of gossypol, but no evidence of the degenerative or necrotic lesions which have been reported to increase levels of lucine amino peptidase in human blood (15).

The transaminases, however, behaved differently. Glutamic-oxalacetic transaminase was significantly increased by gossypol. This is to be expected, since hepatic damage is one of the symptoms of gossypol toxicity in swine (3, 4). Glutamic-pyruvic transaminase was, however, not affected by gossypol feeding. As reported by De Ritis et al. (16), this enzyme increases specifically in cases of viral hepatitis in humans, whereas glutamic-oxalacetic transaminase increases in cases of cardiac infarction or hepatic necrosis.

The in vitro results of Grazi et al. (17) established that the active site of aldolase contains a lysine residue which reacts with the carbonyl group of the substrate to form a Schiff base intermediate. Since gossypol is known to bind the free ε-amino group of lysine, it was of interest to determine whether the feeding of the pigment affected the levels of circulating aldolase; they were not affected. However, the toxicity induced in these animals was rather mild, as evidenced by mortality rates.

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Thyroid and Pituitary Pathology in Iodine-deficient Rats Fed Fresh and Oxidized Fats and Oils [1]

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ABSTRACT To study the long-term effects of feeding fresh and mildly oxidized cottonseed oil, olive oil, corn oil, soybean oil, chicken fat, beef fat, butter, and lard on thyroid pathology in iodine deficiency, these fats were fed, at a level of 20% in an iodine-deficient diet to male rats of the Columbia-Sherman strain. The incidence of thyroids weighing more than 100 mg was significantly higher in the rats fed vegetable oils even though the iodine intakes of all groups were approximately the same. This was interpreted as being due to the presence of goiterogenic substances in vegetable oils rather than to increased iodine requirements in the rats fed these oils. Pituitary tumors occurred in approximately 30% of the rats dying spontaneously after 600 days of age. Among the rats fed animal fats, the percentage of pituitary tumors among rats with thyroids weighing 100 mg or more was significantly higher than in rats with smaller thyroids. The percentage of tumors associated with heavy thyroids was significantly lower in the animals fed vegetable oils, although the overall tumor incidence was the same as in the rats fed animal fats. Therefore, whatever the cause of the increased incidence of large thyroids among the animals fed vegetable oils, the materials responsible did not affect the incidence of pituitary tumors.

In the course of long-term studies (1,2) of the nutritional properties of fresh and mildly oxidized beef fat, chicken fat, butter, lard, cottonseed oil, olive oil, corn oil, and soybean oil fed to male rats in an iodine-low diet, some changes in the thyroid were observed which appeared to be associated with certain dietary fats rather than with the iodine level of the diet. Pituitary tumors were also frequently seen. The present report summarizes thyroid and pituitary data and discusses their relation to the possible existence of goiterogenic substances in vegetable oils.

EXPERIMENTAL PROCEDURE

These investigations were carried out in 2 parts. The first, carried out from late 1961 through early 1964, involved the feeding of fresh and mildly oxidized cottonseed and olive oils and chicken and beef fats (1). The second study began in the fall of 1963 (2). Both studies were carried out in the same animal room, with the same strain of rats, after the same treatment prior to the start of the experiments. The same personnel were involved in both studies.

The dietary fats had been prepared for human consumption. The portions to be oxidized were aerated at 60° for 40 hours. All fats were incorporated at a level of 20% in a purified diet containing 30% casein,[2] 44% glucose monohydrate,[3] 2% cellulose,[4] 4% salt mixture USP XIII no. 2,[5] which contains almost no iodine; and the following vitamin supplements;[6] (in mg/kg) choline dihydrogen citrate, 1000; inositol, 1000; nicotinamide, 100; *p*-aminobenzoic acid, 300; thiamine·HCl, 2; pyridoxine·HCl, 4; riboflavin, 4; Ca pantothenate, 10; folic acid, 2.5; biotin, 0.025; ascorbic acid, 25; vitamin K, 10; vitamin B_{12} (0.1% trituration in mannose), 5; crystalline β-carotene, 5; α-tocopheryl acetate, 50; free α-tocopherol, 10; and crystalline vitamin D_2, 0.5. To ensure an adequate vitamin intake despite the oxidized fat in some of the diets, each rat was given a weekly oral supplement of a multivitamin suspension. The diets were fed to groups of 20 to 40 male rats of the Colum-

Received for publication July 15, 1966.
[1] Supported in part by the Human Nutrition Research Division, ARS, USDA and by Grant U-1347 from the Health Research Council of the City of New York.
[2] Obtained from Nutritional Biochemicals Corporation, Cleveland.
[3] Cerelose, Corn Products Company, Argo, Illinois.
[4] Alphacel, Nutritional Biochemicals Corporation.
[5] See footnote 2.
[6] Hoffmann-LaRoche Inc., Nutley, New Jersey, supplied us with all the vitamins used in these studies.

bia-Sherman strain from the time of weaning. The groups were matched with respect to average body weight ± standard error and average growth tendency as determined by weight increase over a period of 3 days prior to the making up of the groups. The arrangement of the cages on the racks in an attempt to equalize environmental factors has been described previously (2).

In view of the absence of zinc, copper, and manganese from USP XIII salt mixture no. 2, the diet was analyzed for these elements. The results of the analyses together with food intake data showed that the rats had a minimal daily intake of about 20 μg of manganese, 30 μg of copper, and 200 μg of zinc (1,2). In addition, the rats may have consumed unspecified amounts from their drinking water and from the wood shavings used as bedding.

Iodine analyses of thyroids and dietary fats were carried out according to Barker et al. (3) as modified by Ware et al. (4).

In the calculation of the chi-square values, a correction suggested by Yates (5) was introduced which reduces the value considerably but increases its reliability.

RESULTS AND DISCUSSION

Table 1 gives the thyroid weights of animals dying spontaneously after 200 days of age. They are arranged according to age at death, and those dying at approximately the same age are on the same level in the table. Control data were obtained from 12 male rats of the same strain maintained with a commercial ration [7] and killed at 18 months of age. Their thyroids weighed from 23 to 31 mg. Among the experimental animals dying spontaneously, thyroids weighed from 34 to 1217 mg. There were significantly more rats with thyroid weights below 100 mg among the groups fed animal fats and dying after 600 days of age (chi-square = 29.7; $P < 0.001$) and significantly more rats with thyroids weighing over 300 mg among the rats fed vegetable oils (chi-square = 11.85; $P = 0.001$). The group fed fresh chicken fat was an exception. Although there was a tendency toward heavier thyroids with advancing age, the differences between groups were not due to differences in life span. In all age groups, there were more thyroids weighing less than 100 mg in the groups fed animal fats.

Histologically, the thyroids showed signs of iodine deficiency (fig. 1). There was essentially no colloid and the cells were cuboidal or low columnar, as is usual with highly stimulated thyroid cells. The thyroids contained the psammoma bodies described by Follis (6), although they appeared to be less frequent in the older animals. There were varying amounts of connective tissue and few lymphocytes. Histologically, there was little difference in the appearance of thyroids of the same weight from different groups. The thyroids of the control rats fed the commercial ration showed normal epithelium and colloid content.

Table 2 shows the average iodine content of the thyroids of a group of the original rats which was killed at about 700 days of age. Also shown are data for the rats fed the stock ration. The average iodine content of the thyroids of the latter was 15.7 ± 0.98 μg/specimen. The enlarged thyroids of the experimental groups contained much less iodine, and there were no significant differences among the groups.

As is true of older rats in general, pituitary tumors occurred frequently (7,8). A typical histological picture is shown in figure 2. The tumors were hemorrhagic chromophobe adenomas weighing at least 50 mg; many of them weighed over 200 mg. It was often possible to make a diagnosis before the animal died because many of them ate more than the average for their groups despite their having lost as much as 50% of their body weight.

Table 1 shows the occurrence of pituitary tumors in relation to age and thyroid weight. The occurrence of pituitary tumors is indicated in parentheses. The overall incidence of pituitary tumors among rats dying after 600 days of age was 28 out of 98 rats fed animal fats and 20 out of 83 animals fed vegetable oils, which is not a significant difference. Among the rats fed animal fats, there were 15 pituitary tumors among 29 rats with thyroids weigh-

[7] Rockland Rat Diet, obtained from Teklad Inc., Monmouth, Illinois.

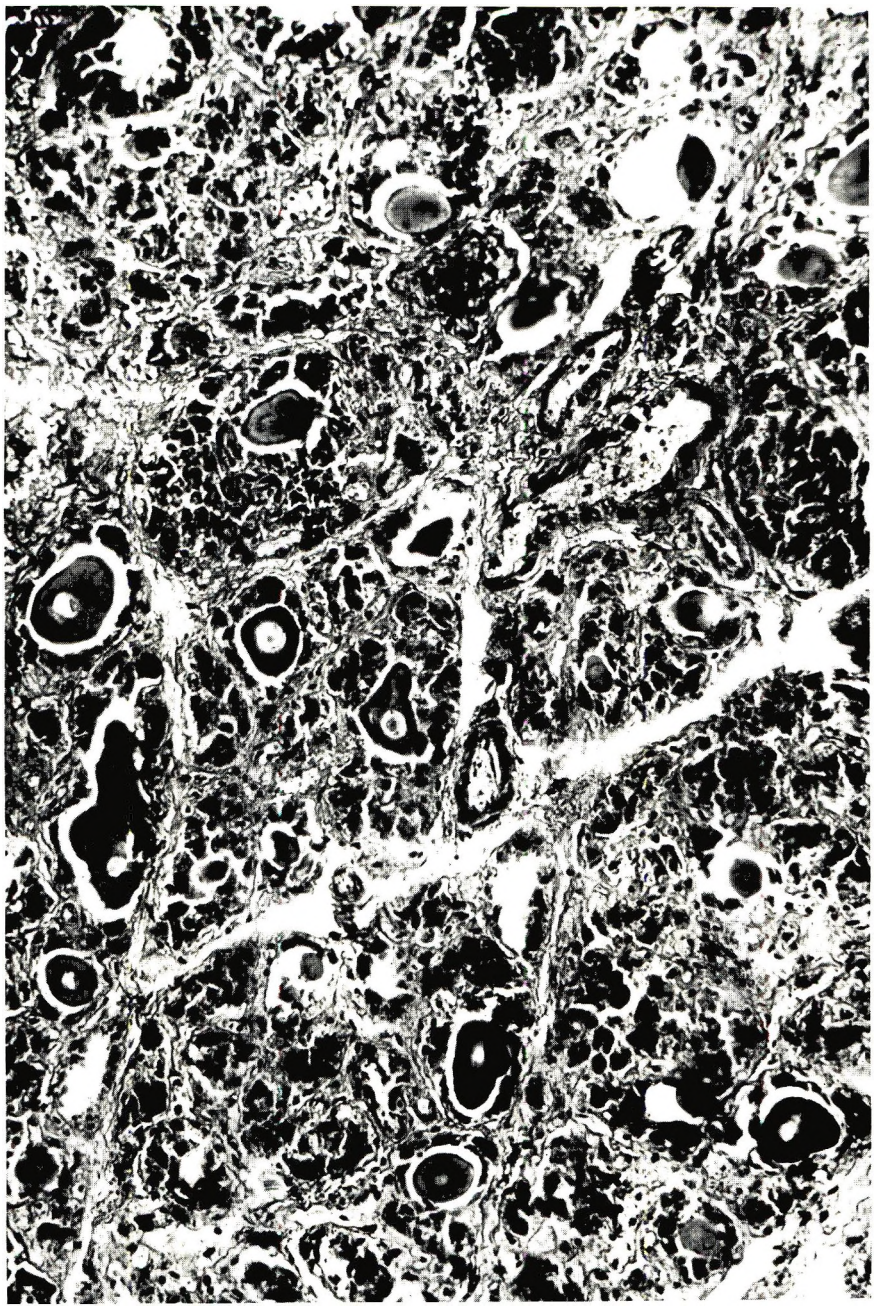

Fig. 1 Section of an enlarged thyroid from a rat fed oxidized soybean oil and killed *in extremis* at 890 days. Note psammoma bodies, absence of colloid, and low columnar epithelium. H & E. × 325.

TABLE 1

Thyroid weights and occurrence of pituitary tumors in male rats dying spontaneously after having been fed various fresh and oxidized fats from weaning [1,2]

Age at death	Thyroid weights															
	Butter		Lard		Beef fat		Chicken fat		Soybean oil		Corn oil		Cottonseed oil		Olive oil	
	Fresh	Oxidized	Fresh	Oxidized	Fresh	Oxidized	Fresh	Oxidized	Fresh	Oxidized	Fresh	Oxidized	Fresh	Oxidized	Fresh	Oxidized
days	*mg*		*mg*		*mg*		*mg*		*mg*		*mg*		*mg*		*mg*	
200–299											70			38	100	
300–349															50	
350–399												62	158		78	
400–449		46		36										68		
									40		68			52		
450–499			40						56	56				44		
			50	54							104		82		284	
500–549	54	52	44	38					56		40	46				
	40		42						72		38	60		80		
												44				
550–599	54	56									140		174	1100	68	
		42	38(T)								196	56	650	148		
	90			67	115				76				54	420	154	122
600–649		64	54(T)	36			290		240				610(T)		192	608
		44	152						448(T)		163(T)	50	186	307		223
			60		92									234	66	92

650–699			80						97(T)		72			132	800	
	88	40(T)	35					88	—(T)				142	170	278	418
	63	165	42					92		150			62		346(T)	
		140		58(T)				636(T)						320	460	246(T)
700–749	100(T)	80		52	52	40	160	94(T)					320		732(T)	76
	56(T)	154(T)	68					58			60		730	62	157	610
	50	53				76	330	190(T)			125		200	64	408	
	42	60	68(T)				480(T)	78(T)					559	58	132	228
		99					48	220		106						
750–799	88	59	82		96	56			112(T)			64		114(T)		
		98(T)	36			58				326(T)		59		292		34
	102	127						84			550					
	61	73(T)								553						126
800–849	251(T)	67	54	54						138	258					238
	165	108(T)	88		100(T)											
	84	54(T)	174(T)										68(T)			
	162		38	81						683(T)		340				
	72									50						
850–899	93	80	58(T)						380(T)			177				456
	69	100	137						60			164				
	88	70	62		42(T)		66			580		50				154
	50	859(T)	413(T)									102				
		1217(T)	325(T)				334									
			35													
900+	79		207	70	—				129	46	54	217(T)				124
	53		56	97					55	238						
				189(T)						794						132
										46(T)						
										160			64(T)			

[1] Rats dying at approximately the same age are indicated on the same level.
[2] The occurrence of pituitary tumors is indicated in parentheses (*T*).

TABLE 2

Average iodine content of thyroids from rats fed various fresh and mildly oxidized fats in a diet deficient in iodine for 95 weeks (control values are from rats fed commercial ration).

	No. of rats	Avg iodine content
		μg/specimen
Commercial ration [1]	12	15.7 ± 0.98 [2]
Cottonseed oil		
Fresh	6	2.9 ± 0.41
Oxidized	6	2.6 ± 0.52
Olive oil		
Fresh	6	3.5 ± 0.27
Oxidized	6	2.3 ± 0.27
Chicken fat		
Fresh	6	2.2 ± 0.27
Oxidized	6	2.4 ± 0.39
Beef fat		
Fresh	6	2.9 ± 0.20
Oxidized	6	2.4 ± 0.17

[1] Rockland Rat Diet, Teklad Inc., Monmouth, Illinois.
[2] SE.

ing 100 mg or more and 13 among the 68 rats with smaller thyroids (chi-square = 8.8; $P < 0.01$). Among the rats fed vegetable oils, although the overall incidence of tumors was the same as for those fed animal fats, there were only 14 pituitary tumors among the 59 rats with thyroids weighing more than 100 mg (chi-square for the difference between this and the corresponding group fed animal fats = 11.0; $P < 0.01$). One wonders whether the difference in tumor incidence between the animals fed fresh and oxidized vegetable oils (chi-square = 4.1; $P < 0.05$) is real.

Consideration has to be given to the question of the extent to which iodine deficiency was responsible for the large thyroids and for the occurrence of pituitary tumors and to what extent differences in iodine intake may have been responsible for differences between groups.

Although salt mixture USP XIII no. 2 contains practically no iodine, the diet mix without fat contained 16.5 μg/100 g. The iodine content of the fats was in μg/100 g: beef fat, 5; chicken fat, 3; butter, 10; lard, 10; cottonseed oil, 2; olive oil, 2; corn oil, 2; and soybean oil, 5. Therefore, the dietary levels ranged from 15.2 μg/100 g in the butter and lard diets to 13.2 μg in three of the vegetable oil diets. Food intakes ranged from 11 to 17 g/day, which meant that iodine intakes could have varied from 1.5 to 2.6 μg/day, with a great deal of overlapping among the groups. The iodine content of New York City tap water given the animals was not determined,[8] but Money et al. (9) have shown that tap water did not influence their results in short-term experiments with iodine-deficient rats. The 1.5 to 2.6 μg of iodine/day afforded our rats was approximately the amount Levine et al. (10) had found would prevent significant thyroid enlargement in their experiments. Furthermore, the growth rate of our rats was essentially normal for 18 months, and at least 50% of the animals were alive after 2 years. This suggests that the degree of iodine deficiency, although sufficient to produce large thyroids, was not great enough to overwhelm the animals.

The occurrence of many more thyroids weighing over 100 mg among the animals fed vegetable oils could have been due to differences in iodine intake, to increased iodine requirements among the rats fed vegetable oils, or to the occurrence of goitrogenic substances in the vegetable oils. However, the iodine content of those thyroids which we analyzed (table 2) was approximately the same in all groups; and there were more enlarged thyroids in the groups fed soybean oil than in those fed chicken and beef fats, although these fats had the same iodine content. Therefore, the differences in iodine intake were probably not the cause. The possibility that the vegetable oils increased the iodine requirements cannot be entirely discounted, but the fact that soybeans, at least, are known to contain goitrogenic substances (11) makes it appear more probable that the differences were due to the presence of such substances in the other vegetable oils. The fact that the incidence of large thyroids was the same in groups fed fresh and oxidized vegetable oils showed that the goitrogenic substances were not destroyed by mild heating and oxidation, un-

[8] We have been informed by the New York City Department of Water Supply that, unlike other minerals, records of the iodine content of city water are not being kept. The Albert Chaney Laboratory of Glendale, California, also informed us that data as to iodine determinations on tap water are probably not available. In any event, all rats drank approximately the same amounts of water at any time. Moreover, any trace amounts present in the water would probably be negligible in comparison with the total intake.

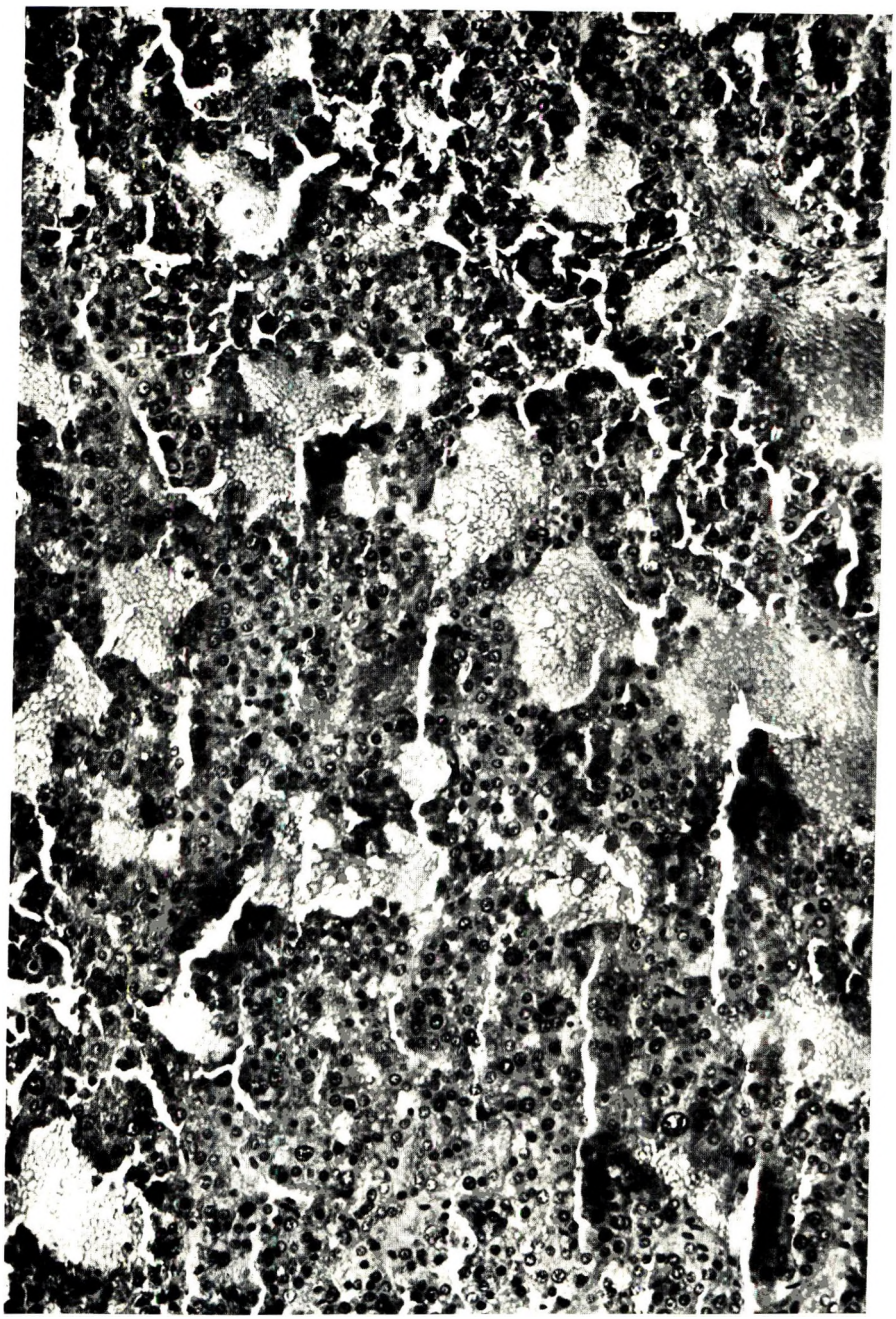

Fig. 2 Section of a hemorrhagic, chromophobe pituitary adenoma of a rat fed oxidized soybean oil. The animal was the same as that described in figure 1. H & E. × 325.

like the substances in vegetable oils which affected life span (2).

Pituitary tumors may occur spontaneously in a high percentage of old rats, depending upon the strain (7,8,12), and they are usually of the somatotropic type (12). Iodine deficiency has been shown to induce pituitary tumors (13) which se-

crete thyroid-stimulating hormone (TSH) (14). Inasmuch as hormonal assays could not be carried out, evaluation of the relative importance of these factors in the overall incidence of pituitary tumors in our study had to be made on indirect evidence. Examination of the body weight curves of rats with and without tumors did not show any significant differences in body weight attained, which suggests no significant increase in growth hormone. Furthermore, histologically, all thyroids, whether accompanied by a pituitary tumor or not, showed epithelial changes indicative of increased levels of TSH.

The apparently greater numbers of pituitary tumors associated with thyroids weighing over 100 mg in rats fed animal fats does not appear to be due to tumor-inducing substances in these fats because the overall incidence of tumors was approximately the same for the groups fed animal and vegetable fats. Therefore, the apparent difference in the incidence of tumors associated with large thyroids in the 2 sets of animals is probably a consequence of the occurrence in the rats fed vegetable oils of a number of large thyroids which are induced by goitrogenic substances and which are not necessarily associated with pituitary tumors.

ACKNOWLEDGMENTS

The general plan and execution of these experiments were carried out in cooperation with Drs. Helen Oldham and Mildred Adams of the Human Nutrition Research Division, U. S. Department of Agriculture; Mr. Lewis Pegus gave us most valuable technical assistance; and we are grateful to Dr. Jacob Furth of this department for his help in the evaluation of the pituitary tumors. Oxidation of the fats was carried out by Mr. N. D. Farel of the Archer-Daniels-Midland Company, Minneapolis, and iodine analyses were carried out in the Albert L. Chaney Chemical Laboratory, Glendale, California.

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Pyruvate Metabolism in Thiamine-deficient Calves [1,2]

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ABSTRACT Three to four hours after feeding, 2 thiamine-deficient and 2 control calves were injected intravenously with pyruvate-2-^{14}C. The specific activity of respired CO_2 reached maximal values 20 minutes after the injection of pyruvate-2-^{14}C in the control animals and after 35 and 60 minutes in the two deficient calves. The rate of CO_2 excretion was the same in control and deficient calves. The proportion of CO_2 arising from pyruvate was 40 and 30%, respectively, for one control and deficient calf. The amount of ^{14}C incorporated into liver glutamate, aspartate, alanine, serine, and glycine per millicurie of ^{14}C injected/kg body weight was higher in deficient than in control calves. The intramolecular distribution of ^{14}C in liver glutamate indicated that relatively more of the carbon from pyruvate was incorporated into the tricarboxylic acid cycle via CO_2 fixation in the deficient calves compared with their controls. These data indicate that thiamine deficiency affects the route of metabolism of pyruvate in the calf, apparently diminishing the fraction metabolized through acetyl-CoA.

The results of a limited number of studies have led to the generalization that metabolism of pyruvate is unaltered in thiamine-deficient animals. This conclusion was arrived at after it had been shown that thiamine-deficient rats and mice expired as much $^{14}CO_2$ from pyruvate-^{14}C or lactate-^{14}C as pair-fed controls (1, 2),[4] and had similar ^{14}C labeling patterns in liver glutamic acid after metabolizing pyruvate-2-^{14}C (3). This result was was not compatible with the observations that the conversion of pyruvate to acetyl-CoA and CO_2 requires thiamine pyrophosphate as a cofactor, and that the activity of pyruvate dehydrogenase is markedly depressed in animals fed thiamine-deficient diets (4–7). Furthermore, the routine observation of elevated pyruvate levels in blood and body fluids of thiamine-deficient animals has been used as an index of thiamine deficiency and has been considered the result of a depression in pyruvate oxidation.

The results of these experiments were complicated by 2 factors: first, the experiments were conducted when the animals exhibited terminal symptoms of thiamine deficiency and, hence, may not have been suffering from a single deficiency; and second, the animals were usually fasted 18 to 24 hours prior to injection of isotope. It has been shown unequivocally that reduced food intake decreases the oxidation of ^{14}C-pyruvate (1, 3).

The purpose of the present experiment was to evaluate the effects of a metabolic deficiency of thiamine on the extent of and alterations in the pathway of pyruvate metabolism in calves force-fed deficient diets in order to maintain the same relative food intakes as control calves.

MATERIALS AND METHODS

Two pairs of male Holstein calves were used in this study. The diet used to produce the thiamine deficiency, the procedures and criteria used to evaluate the deficiency and the data relating to states of the calves used have been reported (7). The deficient calves were force-fed portions of their diets when necessary in order to maintain intakes equivalent to those of the control calves. When the deficient member of a pair was diagnosed as "deficient" (7) that calf, and 2 days later, its respective control, was injected intravenously with sodium pyruvate-2-^{14}C via a jugular catheter 3 to 4 hours after feeding. The specific activities of $^{14}CO_2$, blood

Received for publication July 25, 1966.

[1] Supported in part by Public Health Service Research Grant no. AM-05745-02 from the National Institute of Arthritis and Metabolic Diseases; and by the National Institutes of Health Predoctoral Research Fellowship 1-F1-GM-17660-01A1.

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pyruvate and liver amino acids and the distribution of ^{14}C within liver glutamate were determined after the administration of pyruvate-2-^{14}C to pair-fed control and deficient calves.

Total CO_2 production was determined with an infrared CO_2 analyzer and $^{14}CO_2$ production was determined as described previously (8). In trials 3 and 4, 25 ml of jugular blood were collected from a jugular catheter at intervals of 5, 10 and 15 minutes during the first, second, and third one-hour periods, respectively, for determination of specific activity of blood pyruvate. After deproteinization of the blood samples, pyruvate was isolated as its phenylhydrazone and counted at "infinite thinness" (7, 11). Previous work showed that only pyruvate phenylhydrazone was formed in sample preparations (12).

The animals were killed 6 hours (trials 1 and 2) and 3 hours (trials 3 and 4) after injection of the isotope. The livers were removed, quickly frozen and stored frozen until analyzed. Liver protein was hydrolyzed, the amino acids were isolated and glutamate was degraded according to methods described previously (8).

The specific activities (SA) of all compounds oxidized and degraded are expressed as microcuries per gram atom of carbon (μCi/GAC). To compare data from different experiments, this unit has been modified further to adjust for variations in animal weights and injected doses by calculating the standardized specific activities (SSA) as follows:

$$SSA = \frac{\mu Ci/GAC}{\mu Ci \text{ injected/kg body wt}}.$$

The ratio of the specific activity of expired CO_2 to that of blood pyruvate (transfer quotient) was used in the calculation of the amount of CO_2 originating from pyruvate in order to correct for differences in pyruvate pool sizes in the control and deficient calves (9). Incorporation of ^{14}C into aspartate, alanine, glycine and serine and the ^{14}C distribution within glutamate were used to assess the rates of pyruvate carbon entry into the tricarboxylic acid cycle (3, 10).

RESULTS

Calf weights, amounts of isotope injected, rates of CO_2 excretion and percentage of injected doses excreted as CO_2 for all the trials are listed in table 1. The thiamine-deficient calves excreted considerably less of the injected ^{14}C as CO_2 than their respective controls within 3 hours after injection of the pyruvate-2-^{14}C. The deficiency did not appear to affect total CO_2 excretion, in agreement with data reported for the rat (13). These results and reported tissue α-ketoglutarate dehydrogenase activities in thiamine deficiency (4–6) indicate that the tricarboxylic acid cycle functions normally in thiamine-deficient animals at the time they exhibit deficiency symptoms.

The maximal standardized specific activities (fig. 1) of respired CO_2 occurred between 20 and 25 minutes after the isotope injection in the control animals and between 30 and 40 mintues in one and after more than 60 minutes following isotope injection in the other deficient calf.

Pyruvate pool sizes were estimated from plots of the specific activity of blood pyruvate against time and from blood levels of pyruvate and lactate determined at the beginning of the trial and total body water (14, 15). These pool-size estimates agreed remarkably well (table 2), indicating that lactate and pyruvate come into equilibrium rapidly and that they equilibrate rapidly throughout the total body water. The pyruvate pool in the deficient animal was over 3 times greater than in the control calf.

TABLE 1
Summary of data related to isotope experiments

Trial no.	Animal	Wt	Wt	Injected dose	CO_2/hr/kg$^{3/4}$	Dose excreted as $^{14}CO_2$ in 3 hours
		kg	*kg*$^{3/4}$	*μCi*	*moles*	*%*
1	Deficient	64.1	22.6	491	0.0445	29.2
2	Control	65.0	22.9	544	0.0465	46.9
3	Deficient	69.5	24.1	1336	0.0423	22.0
4	Control	60.0	21.6	1178	0.0378	36.3

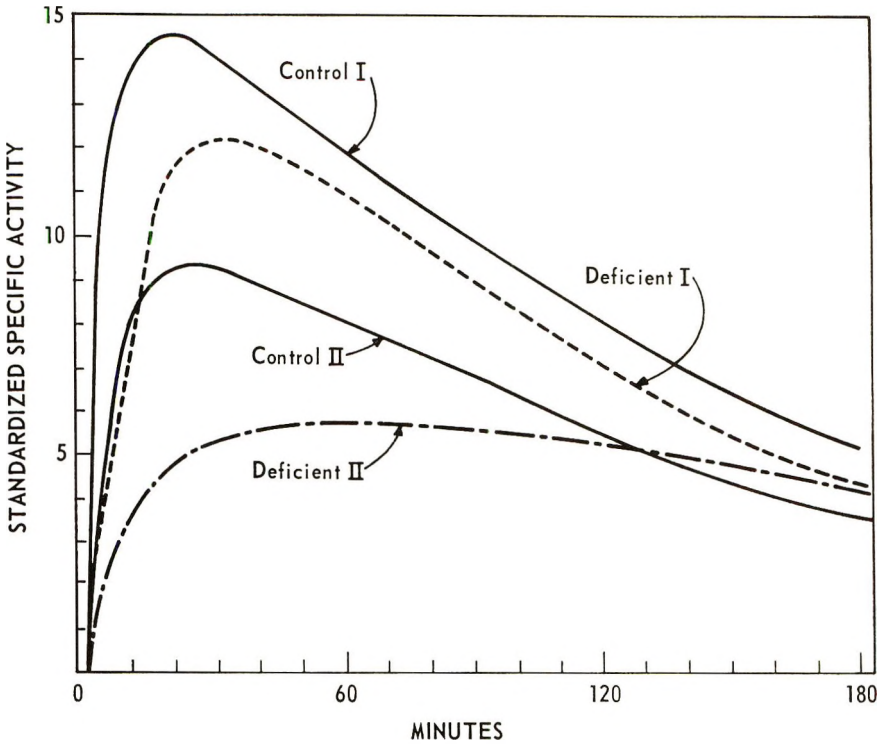

Fig. 1 The standardized specific activity of CO_2 from the two deficient control pairs of calves. (See table 3, footnote 1 for calculation of standardized specific activity.)

TABLE 2

Pyruvate pool calculated by isotope dilution and from blood pyruvate plus lactate concentrations

Animal	1 Lactate + pyruvate	2 Total body water	Pyruvate pool size [1]	
			Col. 1 × col. 2	Isotope dilution
	mg/liter	*liters*	*g*	*g*
Control	211	51.4	10.9	12.1
Deficient	891	44.4	39.6	44.4

[1] Rapid equilibration between lactate and pyruvate throughout total body water assumed. Values represent results of trials 3 and 4.

The standardized specific activities of liver glutamate, aspartate, alanine, serine and glycine (table 3) were higher in the two deficient calves than in their respective controls.

A summary of the results obtained by degrading glutamic acid is presented in table 4. The recovery of isotope was calculated by dividing the summated specific activities for positions 1 through 5 by the value obtained from combustion of a small aliquot of the same sample. The ratio of

TABLE 3

Standardized specific activity [1] of amino acids isolated from liver

Trial no.	Animal	Glutamate	Aspartate	Alanine	Serine	Glycine
1	Deficient	0.458	0.279	0.342	0.210	0.167
2	Control	0.422	0.171	0.300	0.190	0.148
3	Deficient	0.294	0.158	0.330	0.151	—
4	Control	0.274	0.149	0.243	0.105	0.064

[1] $\frac{\mu Ci/gram\ atom\ of\ carbon}{\mu Ci/kg\ body\ wt}$

TABLE 4

Distribution of ^{14}C in liver glutamic acid after intravenous injection of pyruvate 2-^{14}C

Trial no.	Animal	C-1	C-2	C-3	C-4	C-5	Recovery of isotope	C-2 + C-3 / C-5
		%	%	%	%	%	%	%
1	Deficient	19.6	17.3	26.8	7.0	29.2	101	151
2	Control	18.3	9.6	22.5	2.7	46.8	103	68
3	Deficient	15.8	12.7	22.8	2.6	46.3	98	77
4	Control	22.8	12.5	11.5	1.1	52.0	88	46

the activity in carbon-2 plus that in carbon-3 relative to that in carbon-5 in glutamate can be used to estimate the proportion of pyruvate entering the citric acid cycle via CO_2 fixation and the acetyl CoA pathways (3, 10). The ratio of $C_2 + C_3/C_5$ in glutamate from deficient calves was about twice that found in their controls indicating that a greater proportion of the pyruvate that entered the tricarboxylic acid cycle entered via the CO_2 pathway in the deficient calves.

DISCUSSION

The data presented show clearly that pyruvate oxidation is decreased in animals metabolically deficient in thiamine, and indicate that the proportion of pyruvate metabolized by the CO_2 fixation route is markedly increased in thiamine-deficient calves.

The lower recovery of label from pyruvate in CO_2 in the deficient as compared with control calves was due, in part, to the larger pyruvate pool in the deficient calf (table 2) with the accompanying isotope dilution and, in part, to decreased rates of pyruvate oxidation. When the fraction of expired CO_2 arising from pyruvate was calculated by relating the average specific activity of CO_2 to that of blood pyruvate (transfer quotient (9)) it was found that 30% of the CO_2 excreted came from pyruvate in the deficient calf (trial 3) and 40% in the control calf (trial 4) during the 3 hours of comparison. These results show that the deficiency depressed pyruvate oxidation since the total CO_2 production was the same in deficient and control animals (table 1), whereas the proportion of CO_2 carbon arising from pyruvate was smaller in the deficient animal.

The greater specific activities of amino acids in the deficient animals reflected a marked increase in pyruvate conversion into these amino acids, because the specific activity of the precursor pyruvate was reduced in deficient animals by the more than threefold increase in the pyruvate pool. In addition, in the cases of aspartic and glutamic acids it can be shown by considering the theoretical labeling patterns of the tricarboxylic acid cycle intermediates that preferential metabolism of pyruvate via CO_2 fixation increased the relative amount of ^{14}C in both of these amino acids. The theoretical labeling patterns outlined in table 5 show that the aspartate specific activity would be 3 times higher if pyruvate-2-^{14}C was metabolized via the CO_2-fixation pathway rather than via

TABLE 5

Theoretical labeling patterns of glutamic and aspartic acids based on position of label and pathway of metabolism of acetate and pyruvate [1]

	Isotope [2] pathway	Ac-1, Pyr-2, Ac-CoA	Ac-2, Ac-CoA	Pyr-2, CO_2-fix
		Aspartic acid		
COOH		50	50	50
CH_2		—	100	100
CNH_3		—	100	100
COOH		50	50	50
Specific activity		25	75	75
		Glutamic acid		
COOH		100	—	—
CH_2		—	100	—
CH_2		—	100	100
CNH_3		—	100	100
COOH		50	50	50
Specific activity		30	70	50

[1] Theoretical labeling patterns at equilibrium for 100 units of activity entering the cycle at each "turn" of the cycle.

[2] Abbreviations Ac-1, Pyr-2, Ac-2, Ac-CoA, CO_2-fix refer to acetate-1-^{14}C, pyruvate-2-^{14}C, acetate-2-^{14}C, and the pathways of pyruvate utilization involving conversion to acetyl-CoA and CO_2 fixation, respectively.

acetyl-CoA. Similarly, the specific activity of glutamic acid would be increased 60% if pyruvate-2-^{14}C was metabolized via CO_2 fixation. This indication of a relative shift in pathways of pyruvate metabolism was consistent with the elevated $C_2 + C_3/C_5$ ratios in liver glutamate (table 4) in these calves. Both observations indicated that the thiamine-deficient calves metabolized relatively more pyruvate via the CO_2-fixation pathway, involving pyruvate carboxylase, than via the pathway involving pyruvate dehydrogenase and acetyl-CoA.

The decrease in and delay of the maximal standardized specific activities of the respired CO_2 in the deficient animals (fig. 1) relative to their controls reflected differences in the distribution of label from pyruvate-2-^{14}C within the intermediates of the tricarboxylic acid cycle. The theoretical labeling patterns presented in table 5 show that the tricarboxylic acid cycle intermediates — α-ketoglutarate and oxalacetate — are labeled identically when acetate-1-^{14}C and pyruvate-2-^{14}C are metabolized via the acetyl-CoA pathway. Also, nearly the same labeling patterns result when acetate-2-^{14}C is metabolized via acetyl-CoA as when pyruvate-2-^{14}C is metabolized via the CO_2 fixation pathway. Respired air patterns have been shown to be very different when acetate-1-^{14}C is administered than when acetate-2-^{14}C is used (16). The maximal standardized specific activity (SSA) of CO_2 from acetate-1-^{14}C occurs at 6 minutes and is twice that of acetate-2-^{14}C, which occurs at 12 minutes. Hence, the decrease in and delay of the SSA of CO_2 from pyruvate in the deficient calves could be directly related to labeling patterns of tricarboxylic acid cycle intermediates. Therefore, the altered respired air patterns of the deficient calves were in accord with increased pyruvate-2-^{14}C metabolism via the CO_2-fixation pathway as was deduced from the $C_2 + C_3/C_5$ ratios in liver glutamic acid.

Several metabolic shifts could account for the apparent change in flow of carbon from pyruvate into the tricarboxylic acid cycle. One possibility would be that the activity of the CO_2-fixation pathway remained constant while the rate of conversion of pyruvate to acetate decreased; a second is that the activity of the CO_2-fixation pathway increased while the utilization of pyruvate via the acetyl-CoA pathway remained constant; and a third is that the oxidation of body fat increased markedly without an alteration in the route of pyruvate entrance into the tricarboxylic acid cycle.

The second alternative does not account for the increased levels of blood lactate and pyruvate (7) unless the rate of glucose breakdown increased sharply at the time of the deficiency. It has been shown previously that levels of blood glucose are not affected by thiamine deficiency (7). The third alternative implies that liver acetyl-CoA levels would be increased (17, 18) and pyruvate-2-^{14}C entering the tricarboxylic acid cycle via the acetate pathway would undergo a greater dilution than it would in the normal animal. Then decreased activity in carbon-5 relative to carbon-2 and carbon-3 in liver glutamate would occur without a shift in the relative proportions of pyruvate entering the tricarboxylic acid cycle via the CO_2 fixation and acetyl-CoA pathways. Accordingly, the standardized specific activity of glutamate would be expected to be lower in deficient than control animals since ^{14}C would be diluted with cold carbon from the fat. However, the glutamate standardized specific activities of the thiamine-deficient animals were greater than those of their controls.

The respired air patterns shown in figure 1 are of no value in assessing which of the 3 possibilities prevailed since they directly reflect citric acid cycle intermediate labeling patterns and therefore support equally all 3 possibilities. The second and third alternatives proposed as explanations for the decreased rate of pyruvate oxidation and shift in pathways of pyruvate metabolism in deficient calves have been refuted in the above discussion. Therefore, the first alternative appears most tenable and is supported by the observations of elevated blood pyruvate and lactate levels and decreased pyruvate dehydrogenase activities in thiamine-deficient calves (7).

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Metabolic Responses of White Rats to Balanced and Imbalanced Protein Fed with Different Carbohydrates in 15% and 5% Fat Diets [1,2,3]

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ABSTRACT This study was designed to investigate effects of different dietary carbohydrates (fed with 2 levels of fat) on liver lipid changes in male, weanling rats subjected to threonine deficiency; and to observe influences of the amino acid imbalance on responses of certain glycolytic enzyme systems to carbohydrate source. In rats fed low protein, threonine-deficient diets containing 15% of corn oil, moderate amounts of excess lipid accumulated in livers regardless of carbohydrate source (glucose, fructose, or an equal mixture of glucose and fructose). However, when similar diets containing only 5% of corn oil were fed, the effect of threonine deficiency on lipid content of liver depended on the type of dietary carbohydrate: with glucose, no increase in liver lipid; with fructose or sucrose, marked fatty infiltration. The degree of stimulation of the glucose 6-phosphatase and fructose-1,6-diphosphatase enzyme systems by a dietary source of fructose was influenced by both threonine deficiency and level of fat in the diet. Also, in contrast with previous observations with 20% protein diets, responses in fructose-1,6-diphosphatase activity were as great as those in glucose 6-phosphatase activity.

The liver lipid deposition commonly associated with an amino acid imbalance, such as threonine deficiency, is influenced by the type and amount of dietary carbohydrate [4] (1-3) and by the type and amount of dietary fat [5] (4). Previous studies conducted in this laboratory (5-7) have emphasized some interrelationships of responses to dietary fats and carbohydrates. The present study was designed to observe the influence of different dietary carbohydrates in 15% and 5% fat diets on the responses of white rats to balanced and imbalanced protein. At the same time, we were able to make some observations on the influence of the amino acid deficiency on the activity of certain glycolytic enzyme systems.

EXPERIMENTAL

In part 1, male weanling rats of the Sprague-Dawley strain [6] were separated into 6 groups of 6 rats each. In part 2, there were 6 groups of 8 rats each, and one group of 4 rats. All animals were given food and water ad libitum.

All rations for part 1 contained the following: (in per cent) vitamin-free casein, 9; salts W,[7] 4; corn oil,[8] 15; choline chloride,[9] 0.2; vitamin mixture,[10] 0.25; DL-methionine, 0.3; and DL-tryptophan, 0.1. Rations 1–3, but not 4–6, were supplemented with 0.36% of DL-threonine. The remainder of each ration was carbohydrate: glucose for groups 1 and 4, fructose for groups 2 and 5, and an equal mixture of glucose and fructose for groups 3 and 6. The animals were killed at 2 weeks.

Diets fed to groups 1 through 6 in part 2 were the same as corresponding diets in part 1 except that the percentage of corn

Received for publication July 15, 1966.
[1] Supported in part by Public Health Service Research Grant no. AM-04854-04 from the Institute of Arthritis and Metabolic Diseases.
[2] Published with the approval of the Director of the Arkansas Agricultural Experiment Station.
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[9] Added as a 20% solution.
[10] Vitamin mixture contained the following: (in mg/kg of ration) thiamine·HCl, 8.0; riboflavin, 6.0; pyridoxine, 4.0; Ca pantothenate, 40.0; niacin, 50.0; inositol, 200.0; folic acid, 4.0; vitamin B_{12}, 0.04; biotin, 0.2; vitamin A powder (20,000 units/g), 100.0; calciferol (850,000 units/g), 1.8; *dl-a*-tocopherol (250 units/g), 300.0; and menadione, 3.8.

oil was 5 instead of 15, and sucrose replaced glucose-fructose in rations 3 and 6. An additional control group (7, 4 rats) was fed a 25% casein diet containing 5% of corn oil and glucose. All diets were made to 100% with the component carbohydrate. One-half of the rats in groups 1–6 was killed at 2 weeks, and the remainder at 3 weeks, in order to include the period of maximal lipid response (8) as well as that of maximal enzyme adaptation (5). All rats of group 7 were killed at 3 weeks.

Livers of all animals were assayed for activities of glucose 6-phosphatase and fructose-1,6-diphosphatase enzyme systems, and for content of glycogen, nitrogen, total lipid, cholesterol, phospholipid and labile phosphorus from ADP and ATP; serum was assayed for cholesterol concentration. Methods used have been described previously (5, 6).

In part 2, the lipid content of the epididymal fat pads was also determined. Both pads were removed, washed in distilled water, blotted dry, and frozen. Later the pads were thawed, weighed and placed with 2 to 4 g of sand into extraction thimbles in weighed extraction beakers. This combination (beaker, thimble, sand, and pad) was immediately weighed, then dried to a constant weight at 95°. The pads were ground in the thimbles with a glass stirring rod before extraction. Total lipid and cholesterol were determined by the methods used for liver tissue.

RESULTS

Body weights, food intakes, and food efficiency ratios

Part 1 (15% fat diets). Weight gains of rats fed threonine-deficient diets were generally less than those of rats fed the corresponding threonine-supplemented diets, as shown by the average final body weights (table 1). The reduction of growth induced by threonine deficiency was most pronounced in rats fed the fructose-minus-threonine diet (fructose-plus-threonine vs. fructose-minus-threonine, $P < 0.01$).[11] Substitution of fructose for glucose in threonine-supplemented diets also resulted in somewhat smaller body weights at the end of 2 weeks ($P < 0.05$). Substitution of glucose:fructose (1:1) for glucose had no depressing effect on weight gains.

[11] Student's *t* test.

TABLE 1

Body weight, food intakes, and food efficiency ratios of rats fed balanced and imbalanced protein with different types of carbohydrates in 5 and 15% fat diets for 2 and 3 weeks

% fat in diets [1]	No. weeks fed diets	9% protein diets						25% protein
		Glucose		Fructose		Glucose and fructose or sucrose [2]		
		With Thr	Without Thr	With Thr	Without Thr	With Thr	Without Thr	Glucose diet
		Final body weight, g						
15	2(6) [3]	106±1 [4]	101±1	99±2	87±3	113±3	102±2	
5	2(4)	119±6	100±4	97±4	85±2	94±10	91±2	
5	3(4)	140±3	133±4	122±4	98±5	129±1	105±8	193±7
		Total food intakes, g						
15	2(6)	114±5	113±3	102±6	93±6	121±3	118±6	
5	2(8) [5]	143±3	141±7	106±6	95±2	110±6	108±6	155±11
5	3(4)	243±4	254±3	190±3	157±2	200±11	179±22	273±17
		Food efficiency ratios [6]						
15	0–2(6)	37±2	34±1	36±1	28±1	39±1	34±1	
5	0–2(8) [5]	36±1	32±1	35±1	26±1	33±4	26±1	53±1
5	0–3(4)	32±1	25±1	32±1	27±2	33±1	30±2	42±1

[1] Diets in part 1 contained 15% of fat and diets in part 2 contained 5%.
[2] Glucose and fructose (equal parts) were used in part 1, and sucrose in part 2.
[3] No. of rats/group.
[4] Average ± SE of mean.
[5] Only 4 rats in 25% protein group.
[6] Weight gain, g/100 g food intake.

Intakes of each of the rations, either with or without supplemental threonine, were comparable, except for slightly reduced intake of the 2 rations containing fructose. Variations in weight gain and food intake of groups fed threonine-supplemented rations were approximately parallel, so that food efficiency ratios (FER) of the 3 groups were essentially the same. On the other hand, efficiency of utilization of the threonine-deficient diets as compared with that of their respective supplemented control diets was dependent on the carbohydrate. For example, FER of the glucose-minus-threonine ration was not greatly different from that of the glucose-plus-threonine ration, whereas the FER of the fructose-minus-threonine ration was significantly less than that of the fructose-plus-threonine ration ($P < 0.01$).

Part 2 (5% fat diets). Effects of threonine deficiency and dietary fructose on average body weights at 2 weeks were similar to those noted with the higher fat diets, although differences among groups were greater (table 1). Again, final body weights were lowest in the groups subjected to the double stress of a diet deficient in threonine and providing fructose as the only carbohydrate. However, sucrose, unlike glucose:fructose (1:1) in part 1, appeared to have almost as great a depressing effect on weight gain as did fructose.

During the first 2 weeks, intake of each of the 4 diets containing fructose or sucrose was significantly lower ($P < 0.01$) than intake of the corresponding glucose diet; but during the third week, intake of only the 2 fructose diets was consistently less than that of the glucose diets ($P < 0.01$). The 3 threonine-supplemented rations fed in part 2, like those in part 1, were utilized with equal efficiency. Also, each threonine-deficient diet was utilized less efficiently than the corresponding supplemented diet. At 2 weeks, the severity of the depression was greatest in the fructose-minus-threonine group ($P < 0.01$), but significant also in the sucrose-minus-threonine group ($P < 0.02$). However, during the third week, the FER of the glucose-minus-threonine group was depressed as severely as that of the fructose-minus-threonine group (glucose-minus-threonine vs. glucose-plus-threonine, $P < 0.01$), whereas the FER of the fructose-minus-threonine group was unchanged, and that of the sucrose-minus-threonine group slightly improved, suggesting some adaptation to the latter diet.

Relative liver weights and gross composition of livers

Relative liver weights (g/100 g of body weight) and percentages of protein,[12] moisture, glycogen and lipid for all groups are given in table 2. Omission of the threonine supplement from 9% protein diets had practically no effect on relative liver size, but substitution of a fructose source for glucose resulted in substantial increases. In part 1 (15% fat), average relative liver weights of both groups fed fructose were 33% greater, and those of groups fed glucose:fructose (1:1) approximately 18% greater than the respective glucose control groups. All differences were significant at the 1% level. In part 2, (5% fat), relative liver sizes were more than 40% greater ($P < 0.01$) for the 2 groups fed fructose than for the corresponding groups fed glucose. Response to sucrose diets, as compared with the glucose diets, was 20% and 30% greater in the threonine-supplemented and threonine-deficient groups, respectively. Relative liver weights tended to decrease during the third week in groups fed threonine-deficient but not in groups fed threonine-supplemented diets.

The percentage of protein in the liver (table 2) was essentially the same for all groups fed 9% protein diets. In contrast with the lack of effect of threonine deficiency on percentage of protein in liver, the amount of protein in the diet had a striking effect — approximately 30% more protein ($P < 0.01$) in livers of rats fed 25% of protein than in livers of rats fed 9% of protein (glucose-plus-threonine). The greater proportion of protein was accompanied by a generally smaller proportion of glycogen in livers of the 25% protein group as compared with those of the 9% protein groups. For example, liver glycogen of the high protein group was approximately 30% less than

[12] Nitrogen x 6.25.

TABLE 2

Relative weights and gross composition of livers of rats fed balanced and imbalanced protein with different types of carbohydrates in 5 and 15% fat diets for 2 and 3 weeks

% fat in diets [1]	No. weeks fed diets	9% protein						25% protein
		Glucose		Fructose		Glucose and fructose or sucrose [2]		Glucose diet
		With Thr	Without Thr	With Thr	Without Thr	With Thr	Without Thr	
		Liver weights, g/100 g body wt						
15	2 (6) [3]	4.17±0.13 [4]	4.37±0.13	5.56±0.27	5.80±0.37	4.87±0.10	5.21±0.18	
5	2 (4)	4.49±0.07	4.20±0.42	6.40±0.32	6.52±0.14	5.40±0.32	6.06±0.27	
5	3 (4)	4.25±0.15	4.10±0.03	6.51±0.32	5.66±0.40	5.41±0.02	5.43±0.32	4.77±0.33
		Protein, [5] % wet wt						
15	2 (6)	13.9 ±0.4	13.2 ±0.6	12.8 ±0.9	13.1 ±0.6	13.1 ±0.4	13.2 ±0.7	
5	2+3 [6](8) [7]	13.5 ±0.6	13.2 ±0.2	13.7 ±0.3	13.0 ±0.4	12.7 ±0.2	11.9 ±0.3	17.3 ±0.4
		Lipid, % wet wt						
15	2 (6)	4.0 ±0.3	5.9 ±0.3	3.5 ±0.3	4.7 ±0.5	4.0 ±0.1	5.2 ±0.5	
5	2+3 (8)	4.2 ±0.2	4.8 ±0.3	4.2 ±0.3	7.2 ±0.6	4.9 ±0.1	9.9 ±1.2	4.0 ±0.3
		Moisture, % wet wt						
15	2 (6)	73.8 ±0.8	72.7 ±1.0	73.7 ±1.4	73.3 ±1.5	73.1 ±1.8	72.6 ±1.0	
5	2+3 (8)	71.7 ±0.2	69.8 ±2.1	72.0 ±0.4	67.0 ±1.3	71.4 ±0.5	68.1 ±0.9	71.4 ±0.1
		Glycogen, % wet wt						
15	2 (6)	6.7 ±0.3	7.4 ±0.7	8.6 ±0.3	8.1 ±0.6	8.3 ±0.8	8.8 ±0.6	
5	2+3 (8)	8.7 ±0.7	8.2 ±0.3	8.7 ±0.3	8.7 ±0.3	8.5 ±0.5	8.3 ±0.3	6.0 ±0.6
		Glycogen, mg/100 g body wt						
15	2 (6)	329±38	330±33	477±39	462±33	402±35	465±29	
5	2 (4)	414±65	335±38	561±58	542±37	450±75	487±14	
5	3 (4)	356±34	346±21	567±86	523±41	472±31	460±37	286±30

[1] Diets in part 1 contained 15% of fat and diets in part 2 contained 5%.
[2] Glucose and fructose (equal parts) were used in part 1, and sucrose in part 2.
[3] No. rats/group.
[4] Average ± SE of mean.
[5] Nitrogen × 6.25.
[6] Values for 2 and 3 weeks were the same, and hence they were combined.
[7] Only 4 rats in the 25% protein group.

that of the corresponding low protein group (glucose-plus-threonine).

The percentage of glycogen in the liver was essentially the same for all groups fed the 9% protein diets, except that the fructose-plus-threonine (15% fat) diet resulted in a slightly greater percentage than that of the comparable glucose diet ($P < 0.01$). On the other hand, the total amount of liver glycogen (mg/100 g body weight) available as a source of blood glucose was influenced both by the type of carbohydrate and by the adequacy of the protein (table 2). In part 1 (15% fat), the substitution of fructose for glucose resulted in about 40% increase in total glycogen regardless of the adequacy of the protein ($P < 0.05$), whereas substitution of glucose:fructose (1:1) resulted in an increase of this magnitude only if the diet was deficient in threonine ($P < 0.02$). In part 2 (5% fat), at 2 weeks, total glycogen was increased by the substitution of fructose or sucrose for glucose if the rations contained no threonine supplement, but by 3 weeks, the increases due to substitution of these fructose sources were significant with both threonine-supplemented and threonine-deficient rations.

Total liver lipid, expressed as percentage of liver, wet weight, is shown in table 2. Omission of threonine from 15% fat diets resulted in deposition of about 30% more lipid in the liver, regardless of the source of carbohydrate (glucose and glucose:fructose (1:1), $P < 0.01$). On the other hand, with rats fed 5% fat diets, omission of threonine from the glucose ration had no significant effect on lipid deposition, whereas omission from fructose or sucrose rations resulted in increases of approximately 80% and 100%, respectively ($P < 0.01$). These increases in liver lipid in groups fed fructose-minus-threonine and sucrose-minus-threonine diets were accompanied by significant decreases in moisture content of the liver tissues as compared with those from rats fed the corresponding threonine-supplemented diets ($P < 0.05$). Sucrose also enhanced lipid deposition in livers of rats fed threonine-supplemented diets (sucrose-plus-threonine vs. glucose-plus-threonine, $P < 0.01$), although fructose had no such effect. Lipid content of livers of rats fed 25% of protein was comparable to that of rats fed the glucose-plus-threonine diet.

Liver and fat pad lipids

Expressing liver lipids on the basis of liver nitrogen (table 3) indicates absolute amounts, independent of differences in proportions of glycogen and moisture in the tissue. On this basis, differences in total lipid among groups in part 1 were similar to those expressed per gram of liver tissue; whereas in part 2, differences between fructose-minus-threonine and sucrose-minus-threonine and the corresponding threonine supplemented groups were even greater.

Although the type of carbohydrate in 15% fat diets (part 1) did not influence the amount of total lipid deposited in response to threonine deficiency, it did affect the amount of cholesterol (table 3). For example, increased lipid resulting from omission of threonine from the glucose diet was accompanied by a proportional increase in cholesterol, but the same degree of lipid accumulation in the fructose-minus-threonine group was accompanied by no increase in cholesterol. Thus the amount of cholesterol in livers of the fructose-minus-threonine group was about 30% less than that in livers of the glucose-minus-threonine group ($P < 0.01$). Also, among threonine-supplemented groups, livers of rats fed fructose-plus-threonine contained less cholesterol than livers of rats fed glucose-plus-threonine ($P < 0.05$). Values for groups fed glucose:fructose (1:1) fell between those for the glucose and fructose groups. Phospholipid content of livers was variable, but tended to parallel total lipid.

Neither total lipid nor cholesterol content of livers of rats fed glucose in 5% fat diets (part 2) was significantly altered by omission of threonine. On the other hand, the accumulation of large amounts of liver lipid resulting from omission of threonine from fructose or sucrose diets was accompanied by relatively smaller increases in cholesterol. Thus the amount of cholesterol in livers of the fructose-minus-threonine group was no greater than that of the glucose-minus-threonine group; and the amount of cholesterol in livers of the sucrose-minus-threonine group was only

TABLE 3

Liver and fat pad lipids of rats fed balanced and imbalanced protein with different carbohydrates in 5 and 15% fat diets for 2 and 3 weeks

% fat in diets [1]	No. weeks fed diets	9% protein						25% protein
		Glucose		Fructose		Glucose and fructose or sucrose [2]		Glucose diet
		With Thr	Without Thr	With Thr	Without Thr	With Thr	Without Thr	
		Total liver lipid, mg/100 mg liver nitrogen						
15	2 (6) [3]	182 ± 10 [4]	241 ± 13	175 ± 22	228 ± 28	190 ± 2	246 ± 14	
5	2+3 [5] (8) [6]	200 ± 13	232 ± 13	185 ± 15	349 ± 27	241 ± 6	528 ± 77	144 ± 6
		Liver cholesterol, mg/100 mg liver nitrogen						
15	2 (6)	23 ± 1	28 ± 2	18 ± 2	19 ± 2	20 ± 1	23 ± 1	
5	2+3 (8)	19 ± 1	21 ± 1	16 ± 1	22 ± 1	19 ± 1	29 ± 1	14 ± 1
		Liver phospholipid, mg/100 mg liver nitrogen						
15	2 (6)	45 ± 5	50 ± 4	44 ± 9	50 ± 7	44 ± 4	51 ± 8	
5	2+3 (8)	57 ± 2	42 ± 4	33 ± 4	56 ± 5	42 ± 5	69 ± 7	25 ± 2
		Total fat pad lipid, mg/100 g body wt						
5	2+3 (8)	465 ± 34	509 ± 37	319 ± 13	339 ± 19	412 ± 44	378 ± 26	584 ± 41
		Fat pad cholesterol, mg/g lipid						
5	2+3 (8)	16 ± 2	18 ± 1	22 ± 1	24 ± 1	22 ± 2	19 ± 2	15 ± 1

[1] Diets in part 1 contained 15% of fat, and diets for part 2 contained 5%.
[2] Glucose and fructose (equal parts) were used in part 1 and sucrose in part 2.
[3] No. rats/group.
[4] Average ± SE of mean.
[5] Data for 2 and 3 weeks were essentially the same, and hence they were combined.
[6] The group fed 25% of protein consisted of 4 rats, maintained with the diet for 3 weeks.

38% greater than that of the glucose-minus-threonine group, whereas total lipid was 119% greater. As in part 1, livers of rats fed fructose-plus-threonine contained less cholesterol than livers of rats fed glucose-plus-threonine, but the difference was not highly significant. The effect of threonine deficiency on phospholipid content of the liver varied with the type of carbohydrate in the diet, namely, a 30% decrease with glucose ($P < 0.01$), and a 60 to 70% increase with either fructose or sucrose ($P < 0.01$).

The amount of lipid deposited in epididymal fat pads, in proportion to body weight (table 3), was not influenced by threonine deficiency. However, total fat pad lipid of groups fed fructose was approximately 30% less than that of corresponding groups fed glucose ($P < 0.01$). Sucrose exerted a significant depressant effect ($P < 0.02$) only when fed in a threonine-deficient diet. Rats fed the 25% protein diet had more fat pad lipid per 100 g of body weight than any of the groups fed 9% protein except the glucose-minus-threonine group. Concentration of cholesterol in fat pad lipid was greatest in groups having the least amount of total lipid, indicating that the difference in total lipid was probably due primarily to differences in triglyceride content.

Enzyme activities and labile phosphorus from ADP and ATP

Part 1 (15% fat diets). The general pattern of response of glucose 6-phosphatase to the various carbohydrates was the same with threonine supplementation and threonine deficiency, but the degree of stimulation by a source of fructose was much greater in the deficient groups (table 4). For example, units of activity per 100 g of body weight were only 30% greater in the fructose-plus-threonine group than in the glucose-plus-threonine group, but more than 80% greater in the fructose-minus-threonine group than in the glucose-minus-threonine group ($P < 0.01$). The difference in the 2 responses can be attributed primarily to a significant increase in specific activity in the threonine-deficient group ($P < 0.05$).

Increases in fructose-1,6-diphosphatase activity in response to substitution of a fructose source for glucose roughly paral-

TABLE 4

Enzyme activities and nitrogen in livers of rats fed balanced and imbalanced protein with different carbohydrates in 5 and 15% fat diets for 2 and 3 weeks

% fat in diets [1]	No. weeks fed diets	9% protein						25% protein
		Glucose		Fructose		Glucose and fructose or sucrose [2]		Glucose diet
		With Thr	Without Thr	With Thr	Without Thr	With Thr	Without Thr	
		Glucose 6-phosphatase activity, units [3]/100 g body wt						
15	2(6) [4]	116±5 [5]	99±11	151±19	181±12	141±14	152±13	
5	2(4)	120±8	115±6	174±19	184±18	158±16	156±15	
5	3(4)	108±7	103±7	187±2	171±3	141±6	162±6	99±2
		Glucose 6-phosphatase activity, units/100 mg liver nitrogen						
15	2(6)	123±7	108±12	139±14	159±13	142±17	136±8	
5	2(4)	125±6	115±6	124±13	131±7	140±6	131±6	
5	3(4)	128±14	120±9	135±8	147±9	129±5	159±11	75±3
		Fructose-1,6-diphosphatase activity, units/100 g body wt						
15	2(6)	48±1	49±5	70±10	84±8	58±6	73±8	
5	2(4)	45±2	49±7	78±8	93±14	64±11	67±7	
5	3(4)	58±4	49±2	89±4	85±10	77±2	81±3	64±2
		Fructose-1,6-diphosphatase activity, units/100 mg liver nitrogen						
15	2(6)	51±2	53±5	75±9	70±6	58±6	66±6	
5	2(4)	47±2	48±6	55±6	67±9	57±7	58±7	
5	3(4)	64±10	56±2	64±5	72±8	71±3	81±6	49±1
		Liver nitrogen, mg/100 g body wt						
15	2(6)	92±3	92±2	115±10	121±7	102±2	109±2	
5	2(4)	97±5	101±3	141±4	139±6	112±6	116±5	
5	3(4)	91±8	86±1	141±7	118±8	109±2	103±3	132±3

[1] Diets in part 1 contained 15% of fat, and diets in part 2 contained 5%.
[2] Glucose and fructose (equal parts) were used in part 1 and sucrose in part 2.
[3] One unit = activity catalyzing release of 1 μmole of inorganic phosphorus/minute, at 37.5°.
[4] No. rats/group.
[5] Average ± SE of mean.

leled increases in glucose 6-phosphatase activity. However, in the fructose-plus-threonine group, a significant increase in specific activity ($P < 0.01$) contributed to a slightly greater increase in total activity of fructose-1,6-diphosphatase than of glucose 6-phosphatase. Differences in total fructose-1,6-diphosphatase activity between groups fed fructose and corresponding groups fed glucose were significant at the 1% level.

Part 2 (5% fat). At 2 weeks, the general pattern of responses of the 2 enzyme systems was similar to that noted in part 1, namely, marked increases in total activity with substitution of fructose for glucose and lesser increases with substitution of sucrose (table 4). However, the magnitude of response of the enzyme systems to dietary fructose was more pronounced with 5% than with 15% fat diets supplemented with threonine. No consistent effect of fat level in threonine-deficient diets was observed (table 4). By 3 weeks, responses of both glucose 6-phosphatase and fructose-1,6-diphosphatase to sucrose in threonine-deficient diets were equal to the responses to fructose. When data from 2 and 3 weeks were combined, all differences between activities in groups fed fructose or sucrose and the corresponding groups fed glucose were significant at the 1% level.

A decrease with time in total liver nitrogen (table 4) was accompanied by variable increases in specific activities of the enzyme systems, with the net result that total activities at 3 weeks were not depressed below the 2-week level. In fact, in the group fed sucrose-minus-threonine, total fructose-1,6-diphosphatase activity was greater at three than at two weeks. The same type of homeostatic mechanism is illustrated by a comparison of enzyme

activities in the group fed 9% protein plus glucose with activities in the group fed 25% protein plus glucose. Although specific activities were considerably greater for the low protein group than for the high protein group, total nitrogen content was lower, with the net result that total activities of both enzyme systems were the same for the 2 groups.

Labile phosphorus values showed no significant effect of carbohydrate source or threonine supplementation. However, average values for all groups fed 9% of protein, with or without threonine supplementation, were more than double that of the group fed 25% of protein. Average for the 25% protein group, in micrograms/100 mg of liver nitrogen, was 125 ± 9 as compared with 344 ± 49 (2 and 3 weeks combined) for the glucose-plus-threonine group ($P < 0.01$).

DISCUSSION

Metabolic changes associated with amino acid imbalance have been attributed to low food consumption (3, 9). We found that intake of the threonine-deficient diet containing 5% fat and fructose was less than that of the corresponding supplemented diet, but acceptance of each of the other threonine-deficient diets was comparable to that of its supplemented control. In fact, intake of the 9% protein diets containing glucose, with or without supplemental threonine, was equal to that of the 25% protein diet at 2 weeks, and only slightly less at 3 weeks. Depression of intake by fructose was more pronunced in this study than in previous experiments with diets containing 20% of protein (5, 6).

Increases in total liver lipid (mg/100 g body weight) resulting from the omission of threonine from the diet was accompanied by either smaller or equal increases in cholesterol and phospholipid. This is in accord with observations of others (10), indicating that the major component of the excess lipid is probably triglycerides. On the other hand, the type of carbohydrate in the diet affected lipid composition to some extent. For example, dietary fructose, as compared with glucose, tended to reduce the proportion of cholesterol in total liver lipids, regardless of the adequacy of the dietary protein.

The lesser degree of fat accumulation in livers of the fructose-minus-threonine group in part 1 as compared with the fructose-minus-threonine group in part 2 was probably due to the difference in fat content of the 2 diets. Reduction in severity of fatty infiltration of liver in threonine-deficient rats with increasing levels of dietary fat has been reported previously (11). Yoshida and Harper (12) proposed that higher fat diets might lessen the severity of fatty livers by inhibiting fatty acid synthesis, which increased during the period of fat accumulation in livers of rats fed threonine-deficient, 5% corn oil diets. This could also account, in part, for difference in effects of glucose:fructose (1:1) in part 1 and sucrose in part 2. However, feeding sucrose in either threonine-deficient or threonine-supplemented diets resulted in higher levels of liver lipid than feeding either glucose or fructose in comparable diets. Weiner et al. (3) suggested that the greater accumulation of liver fat induced by substitution of sucrose for dextrin in low protein diets was due to lower intake of the sucrose diet. In our study, however, average intakes of the 2 sucrose diets was as great or greater than intakes of the corresponding fructose diets. Dietary sucrose appears to favor lipid accumulation to a greater extent than can be accounted for by its fructose content.

The fact that omission of threonine from 5% fat diets containing glucose had no effect on the amount of lipid in liver, whereas its omission from a comparable diet containing fructose resulted in severe fatty infiltration, could be related to differences in metabolism of the 2 sugars. Previous work in this laboratory indicated that pathways of glucose and fructose metabolism influenced accumulation of liver lipid induced by feeding hydrogenated coconut oil in 20% protein diets (6). Macdonald and Roberts (13) reported that adult rats fed 20% protein, low fat diets containing fructose converted more labeled fructose into liver lipids the longer they were fed the diet, but a similar adaptation did not take place with glucose. If the influence of fructose on lipid metabolism is mediated through stimulation of

lipogenesis, fructose might be expected to aggravate threonine-deficiency symptoms by further stimulation of the already rapid rate of fatty acid synthesis.

Evidence that the type of carbohydrate in the diet influences lipid metabolism in tissues other than the liver is provided by the fat-pad data. The smaller amount of fat stored in epididymal fat pads of rats fed fructose as compared with those fed glucose could be related to the removal from portal blood of the major part of absorbed fructose by the liver (14), with subsequent slower release of the carbohydrate as glucose to the general circulation and hence to adipose tissues, than is the case with absorbed glucose. Also, the initial load of carbohydrate to the liver after fructose ingestion might affect modes of disposal, including lipogenesis in that tissue.

One objective of this study was to observe the influence of amino acid imbalance on responses of enzymes to different carbohydrates. We found that not only did threonine deficiency affect activities of glucose 6-phosphatase and fructose-1,6-diphosphatase (table 4), but also that protein level appeared to influence relative responses of the 2 systems. With all low protein-fat combinations used, the degree of stimulation of total fructose-1,6-diphosphatase activity by a dietary source of fructose equaled or was greater than stimulation of glucose 6-phosphatase activity in the same rats. This observation contrasts with the consistently greater stimulation of glucose 6-phosphatase (fig. 1) observed with 20% protein diets (5–7). This is of particular interest in that the lack of parallelism between responses of fructose-1,6-diphosphatase and glucose 6-phosphatase to dietary fructose was interpreted as evidence for an alternate route, possibly involving enzymes of the hexose monophosphate shunt (HMS), for conversion of fructose to glucose 6-phosphate under certain dietary conditions. The parallelism in this study therefore suggests the possibility that synthesis of enzymes of the Embden-Myerhof pathway (including fructose-1,6-diphosphatase) has priority over synthesis of enzymes involved in an alternate route when the supply of protein is low. Such an interpretation appears to be plausible in view of the selective increase in activities of glucose 6-phosphatase and fructose-1,6-diphosphatase with decrease in total nitrogen in this study; and the reported sensitivity of glucose 6-

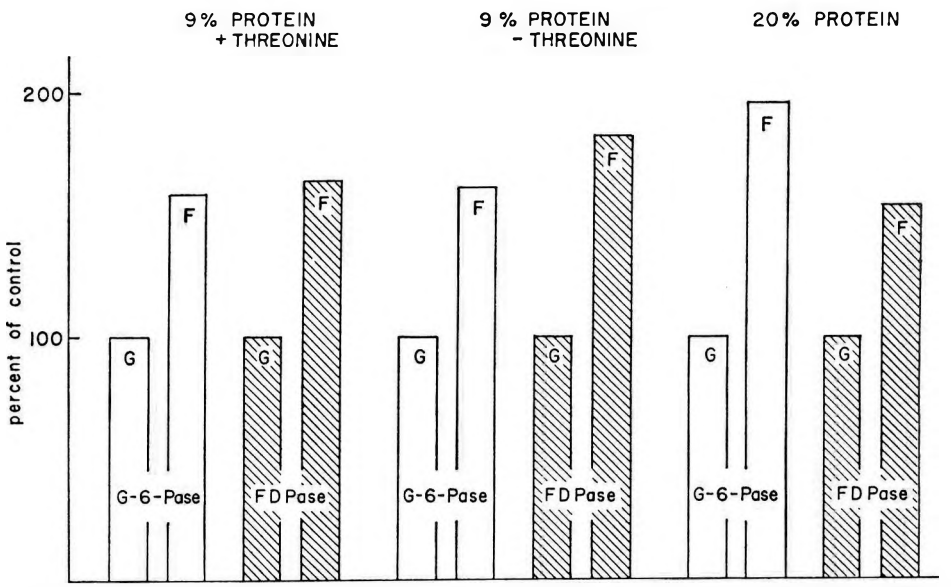

Fig. 1 Response of liver glucose 6-phosphatase (G-6-Pase) and fructose-1,6-diphosphatase (FDPase) enzyme systems to dietary fructose, expressed as percentage of glucose control, in diets containing different quantities and qualities of protein. Data for 20% protein groups are from J. Nutr., 87: 202, 1965. G = glucose, F = fructose; dietary fat = 5% corn oil.

phosphate dehydrogenase, an HMS enzyme, to level of dietary protein in refed rats (15).

Greater response to dietary fructose with the lower level of dietary fat (part 2 vs. part 1) could be expected on the basis of previous work with different fat levels (7). The occurrence of this effect in supplemented but not in deficient groups suggests that the amino acid imbalance modified the adaptation. However, increases in activities of both enzyme systems in response to fructose and to the mixture of glucose and fructose in rats fed threonine-deficient, 15% fat diets were much greater than increases in comparable supplemented groups (table 4). Perhaps maximal adaptation possible with the inadequate amount of protein available was attained with the fructose-minus-threonine, 15% fat diet. The greater response to threonine-deficient than to threonine-supplemented diets could be a reflection of gluconeogenesis from inefficiently utilized amino acids of the imbalanced protein.

Labile phosphorus from ADP and ATP is used as an indication of the ATP content of the liver (16). The much higher values from groups fed 9% protein diets as compared with that of the group fed 25% of protein were unexpected. This effect could possibly be related to the observation of Sidransky (17) that in livers of animals fed imbalanced protein diets, protein synthesis is considerably increased, despite the fact that net stores of nitrogen are being depleted.

The results of this study show that changes in lipid metabolism resulting from amino acid imbalance can be modified by the type of dietary carbohydrate, and that adaptation of glycolytic enzyme systems to different dietary carbohydrates is influenced by the protein content of the diet. These observations re-emphasize the difficulties of studying metabolic effects of single nutrients.

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Utilization by the Rat of 1,3-Butanediol as a Synthetic Source of Dietary Energy [1]

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ABSTRACT To test the ability of the rat to utilize 1,3-butanediol (BD) as a source of dietary energy, rats were fed high fat diets in which carbohydrate was replaced by BD. These tests indicated that following an adaptation period of at least one week, BD had a caloric value of approximately 6 kcal/g. In a 30-week long-term study, rats were fed 30% fat diets containing 20 and 30% added BD. Other diets included in the study for comparative purposes contained 10 to 60% fat and variations in protein level. The principal effect was an impairment in utilization of the diet when BD was fed at the 30% level. No impairment in utilization was observed when rats were fed diets containing 20% BD. At the end of this 30-week study, urine was collected and blood and liver were taken for various assays. The assays included urine and serum ketone bodies, liver glycogen and phosphohexose isomerase, and serum glucose. Increasing levels of fat resulted in an increase in both serum and urinary ketone bodies. In contrast, increasing dietary BD levels produced essentially no change in both parameters. When the results of the other assays were examined, a similar pattern was obtained. Fat produced a decrease in liver phosphohexose isomerase and in serum glucose, whereas liver glycogen tended to increase. Feeding BD, however, resulted in an increase in liver phosphohexose isomerase, no significant change in serum glucose, and an increase in liver glycogen. The results of these studies do not support the hypothesis that BD is oxidized through β-hydroxybutyric acid.

Synthetic sources of dietary calories, which do not require land for production, are of interest as potential food source and may aid in the study of the manner in which living organisms utilize a variety of structures for energy. An inexpensive, commercially available,[3] petroleum-derived compound, 1,3-butanediol (BD), is one of the first synthetic energy sources to be studied extensively.

About 1949 reports on BD began to appear in the literature. Fischer et al. (1) reported that BD had low toxicity as indicated by an oral LD_{50} of 29.42 cm^3/kg in rats. Further studies by Meyer (2) in 3 generations of rats indicated that relatively low levels of BD had no adverse effect on growth, fertility, and reproduction. Bornmann (3–5), in a series of papers, also concluded that BD was of low acute and chronic toxicity.

Specific nutritional use of polyhydric alcohols, including BD, was reported by Schussel (6, 7). The polyols were fed to rats at levels of 5 to 40% of dietary calories. Of the 7 polyols tested, BD was best tolerated. Low concentrations appeared to stimulate growth. As a result, the author concluded that the low levels of BD may have nutritional value and may enlarge the sources of our food supply.

The present series of studies constitute the animal-feeding phase of studies on BD. They were designed to determine the ability of the rat to utilize BD for energy and to elucidate the factors which affect utilization when the rat is fed this 4-carbon diol as a carbohydrate replacement in high-energy diets. In addition the influence of prolonged feeding of BD upon a number of metabolic products and intermediates was examined at the completion of the 30-week feeding study reported in this paper. This was done to obtain pre-

Received for publication May 9, 1966.

[1] Contribution no. 743 from the Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts. Supported by a contract with the Biomedical Laboratory, 6570th Aerospace Medical Research Laboratories, Aerospace Medical Division, Air Force Systems Command, Wright-Patterson Air Force Base, Ohio and by Public Health Service Research Grant no. AM-06837 from the Institute of Arthritis and Metabolic Diseases.

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liminary information concerning the manner in which BD was utilized by the rat.

EXPERIMENTAL

In addition to caloric bioassays the studies consisted of the following sequence of experiments: (a) 4-week ad libitum feeding of graded levels of BD; (b) pair-feeding of 2 levels of BD for 3 weeks; (c) intubation pair-feeding of a high level of BD for 4 weeks; and (d) a long-term 30-week feeding test. At the end of the 30-week experiment, samples were taken of urine, serum, and liver.

Commercial grade BD was used in all the experiments. The compound met specifications as follows: boiling point, 207.5°; specific gravity 20/20 C, 1.006; and a minimal purity of 99% by weight. While BD has unusual chemical stability, it is a hydroscopic compound; therefore, care was taken to prevent water absorption in storage and in handling. The gross energy content of BD was determined with a ballistic bomb calorimeter (8) and found to be 7.5 kcal/g. However, the available metabolic energy (ME) content of BD, estimated with a bioassay procedure developed in this laboratory,[4] was only 5 kcal/g. When animals adapted to the feeding of BD were used, the ME increased to 6 kcal/g.

All animals used in these studies were male rats of the Charles River C.D. strain. Initial average weights of the animal used in the 4-week ad libitum feeding, 3-week pair-feeding, 4-week pair-intubation, and the 30-week long-term feeding test were 47, 50, 87, and 145 g, respectively.

The first feeding experiment was a 4-week test in which 5 groups of 10 weanling rats each were fed ad libitum diets containing BD.[5] All diets contained 20% protein on a dry basis. One group was fed a 10% fat control diet, and 4 groups received 25% fat diets containing 0, 5, 10, and 20% BD on a dry basis. The ingredients of the basal 25% fat diet on a dry basis were as follows: (in per cent) casein, 22; sucrose, 14.4; dextrose, 28.9; lard, 15; corn oil, 10; salt mix W, 4 (9); liver powder, 1.0; vitamin mix, 1.2; and agar, 3.5. The diet was made into a semisolid agar-gel by mixing equal amounts of diet and hot water containing the dissolved agar and allowing them to set, according to the procedure of Miller and Allison (10). Test diets containing 5, 10, and 20% BD were prepared by adding BD at the expense of the carbohydrate sources. In the 10% fat control diet, the dextrose and the sucrose were increased, but, as in all diets, the 2-to-1 ratio of these carbohydrate sources was maintained.

In the second feeding test, 3 groups of 10 weanling rats each were pair-fed four of the diets from the previous experiment for 3 weeks. The diets pair-fed were the 10% fat control and the 25% fat diets containing 0, 5, and 20% BD. Amount of the diet fed was controlled by the group with the lowest food intake.

The third feeding experiment consisted of force-feeding equal volumes of isocaloric diets to 2 groups of 10 rats each for 4 weeks. One diet contained 30% fat, and the other contained 30% fat plus 20% BD. The animals were usually intubated 3 times daily with a syringe and a 16-gauge ball-point, 76-mm stainless steel needle. A control group was fed ad libitum a commercial laboratory animal feed and was intubated with the same volume of water that the other groups received in the liquid diet. Composition of the diets is shown in table 1. The diets were prepared by first mixing all the dry ingredients to form a stable dry mix. A 2-day supply of the liquid intubation diets was prepared by adding distilled water and corn oil or BD to the dry mix in a Waring Blendor. After preparation, the diets were stored in a refrigerator.

Fifteen groups of 10 rats each were used in the fourth feeding test which was a 30-week study. Protein, fat, and BD levels were the principal experimental variables. The principal components of the diets are shown in table 2. In addition all the diets contained 4% salt mix W (9), 1.2% vitamin mix (table 1), and 4% agar. Where supplied, carbohydrate was furnished by sucrose, dextrose, and dextrin in the ratio of 2:1:1, respectively. The fat source was 1 part corn oil to 2 parts lard. To facilitate the feeding of high levels of fat and BD, all diets were prepared in semisolid agar-

[4] Dymsza, H. A., and S. A. Miller 1963 Bioassay for caloric value and energy utilization of dietary components. Federation Proc., 22: 610 (abstract).

[5] The authors wish to express their appreciation to the Celanese Chemical Corporation for supplying the 1,3-butanediol used in these studies.

TABLE 1
Composition of diets used in isocaloric, pair-fed, intubation study

	30% fat (control)	30% fat + 20% 1,3-butanediol
Dry mix:	*g*	*g*
Lactalbumin	200	222
Sucrose	100	47
Dextrose	211	95
Dextrin	100	47
Cellulose	35	35
Salts W (9)	40	40
Vitamin mix [1]	14	14
Liquids: [2]		
Corn oil	300	300
1,3-Butanediol	0	200
Water	500	646
Total	1500	1646

[1] The vitamin mix supplied/kg of diet: (in milligrams) thiamine·HCl, 10; riboflavin, 10; pyridoxine, 10; Ca pantothenate, 30; niacin, 25; menadione, 4; inositol, 500; biotin, 0.5; choline, 1000; and α-tocopherol, 40 IU.
[2] Added to dry mix before use.

gel form by incorporating 1000 g of the dry diet with 750 ml of hot water containing the dissolved agar.

At the end of the 30-week feeding period the animals were placed in metabolism cages and fasted for 18 hours. Urine was collected in a container surrounded by ice, and the volume was measured. The urine was then kept frozen in sealed vials at −40° until analysis could be performed. After removal from the metabolism cages, the animals were decapitated, blood was collected, and livers were removed and weighed. The blood was kept refrigerated, centrifuged, and the serum was removed and frozen. An aliquot of liver was taken immediately upon dissection, weighed, and placed in KOH. The remainder was frozen.

Measurements were made of serum glucose (11) and ketone bodies (12). Liver determinations were made of glycogen (13) and phosphohexose isomerase (14). Urinary ketone bodies were also determined (12).

All data obtained were statistically analyzed by analysis of variance methods described by Snedecor (15).

RESULTS

The results of the feeding experiment in which 5, 10, and 20% BD diets were fed for a 4-week period are presented in table 3. In this test the highest body weight gain (175 g) was made by the group fed 5% BD, and the lowest body weight gain (145 g) was obtained with rats fed 20% BD. Animals fed 20% BD gained significantly less body weight ($P = 0.05$) than rats fed the 10% fat, 25% fat, and the 25% fat plus 5% BD diets. The two lowest weight gains which were obtained with 10% BD (159 g) and 20% BD (145 g) were not statistically different.

A similar pattern was observed for food intake (table 3). Rats fed 10 and 20% BD consumed significantly less ($P = 0.01$) food than animals fed any of the other diets. However, when compared with the 25% fat unsupplemented group, a high level of food, protein, and caloric efficiency was maintained at all 3 levels (5, 10, and 20%) of BD.

The results of the pair-feeding study are shown in table 4. Since rats in this test did not readily consume the 20% BD diet, total food consumption and body weight gain of all the animals were low. Nevertheless, compared with animals fed the unsupplemented 25% fat diet, animals pair-fed diets containing 5 and 20% BD had essentially similar body weight gains and food and protein efficiencies.

TABLE 2
Characteristics of diets used in 30-week long-term study [1]

	Diet														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Protein,[2] %	18	36	18	36	18	36	36	18	36	18	36	18	18	18	28
Fat,[3] %	10	10	30	30	30	30	30	30	30	50	50	60	60	60	60
1,3-Butanediol, %					20	20	20	30	30						
Carbohydrate,[4] %	62	42	42	22	22	2		12		22	2	12	11	6	

[1] Dry basis.
[2] Casein.
[3] 1:2 mixture of corn oil:lard.
[4] 2:1:1 mixture of sucrose:dextrose:dextrin.

TABLE 3

Effect of 4-week feeding of graded levels of 1,3-butanediol as a carbohydrate replacement in high fat diets [1]

Diet [2]		Body wt gain [3]	Nutrient intake [2]			Nutrient efficiency [2]		
Fat	1,3-Butanediol		Food [4]	Protein	Calories	Food [5]	Protein [6]	Calorie [7]
%	%	g	g	g	kcal			
10	—	165 ± 3 [8]	329	65.8	1349	50.1	2.51	12.22
25	—	168 ± 8	317	63.3	1552	52.8	2.64	10.77
25	5	175 ± 5	318	63.6	1590	55.0	2.24	11.00
25	10	159 ± 9	296	59.2	1510	53.2	2.66	10.43
25	20	145 ± 7	257	51.5	1363	56.0	2.80	10.57

[1] Ten rats/group.
[2] Dry basis.
[3] LSD at 5% = 19 g; at 1% = 25 g.
[4] LSD at 5% = 14 g; at 1% = 18 g.
[5] LSD at 5% = 1.4; at 1% = 1.8.
[6] No significant differences among groups.
[7] LSD at 5% = 0.3; at 1% = 0.4.
[8] Mean ± SE.

TABLE 4

Effect of 3-week paired-feeding of 25% fat diets with and without 1,3-butanediol [1]

Diet [2]		Body wt gain [3]	Nutrient intake [2]			Nutrient efficiency [2]		
Fat	1,3-Butanediol		Food	Protein	Calories	Food [4]	Protein [5]	Calorie [6]
%	%	g	g	g	kcal			
10	—	56 ± 4 [7]	112	22.4	459	50.0	2.50	12.20
25	—	74 ± 6	112	22.4	549	66.0	3.30	13.48
25	5	75 ± 4	112	22.4	560	67.0	3.35	13.39
25	20	73 ± 5	112	22.4	594	65.0	3.25	12.29

[1] Ten rats/group.
[2] Dry basis.
[3] LSD at 5% = 4 g; at 1% = 6 g.
[4] LSD at 5% = 3.80; at 1% = 5.00.
[5] LSD at 5% = 0.18; at 1% = 0.24.
[6] LSD at 5% = 0.73; at 1% = 1.00.
[7] Mean ± SE.

Table 5 shows the average weekly weight gains of animals in the third feeding experiment, in which rats were intubated with equal volumes of isonitrogenous and isocaloric liquid diets. The differences in weight gained by animals fed the 30% fat diets with and without 20% BD indicate that a one-week adaptation period was required for maximal utilization.

During the first week animals fed the BD-containing diet gained 8 g as compared with 16 g for rats fed the unsupplemented liquid diet. Second-week body weight gains were 24 and 22 g for animals fed BD and the unsupplemented diet, respectively, indicating the completion of the adaptation period. Thereafter, average second- and third-week body weight gains with the 20% BD diet were generally similar to those of animals not receiving BD in their diet. Eliminating the first-week adaptation period, the average total 2- to 4-week weight gains with the intubated diets are 57 g for both the BD and the unsupplemented groups. Since the diets were designed to be isocaloric, it appears that the estimated caloric value for BD of 6 kcal/g is correct in adapted animals.

No effect of protein level was noted in any of the parameters studied during the 30-week long-term study. This observation is similar to that of French et al. (16). For this reason and in an effort to simplify presentation of the data, the results of groups fed similar levels of fat and BD were combined.

At 4 weeks groups fed diets containing 30% BD consumed less food and gained significantly less body weight than rats fed any of the other diets (table 6). In addition food and calorie efficiency were also depressed in animals fed these diets.

At 30 weeks (table 7) weight gains of animals fed 20% BD were similar to those of animals fed the 10, 30, 50 and 60% fat diets. In contrast, weight gains of rats fed diets containing 30% BD were considerably lower than those of animals fed the other diets; these results are similar to those obtained at 4 weeks. Rats fed the 30% fat diet gained weight at a rate equal to rats fed the higher fat diets.

Similar variations were observed when nutrient efficiencies were considered. Food and calorie efficiency were reduced when 30% BD was added to the diet (table 7). However, utilization of diets containing 20% BD was not significantly impaired.

TABLE 5

Weight gains of rats pair-intubated with equal volumes of isocaloric and isonitrogenous liquid diets

Diet	Avg body wt gain/week				Total wt gain in 4 weeks	Total wt gain 2–4 weeks
	1 week	2 weeks	3 weeks	4 weeks		
	g	*g*	*g*	*g*	*g*	*g*
30% Fat	16±2 [1](9) [2]	22±2(9)	17±0.8(9)	18±2(9)	73	57
30% Fat+20% 1,3-butanediol	8±2 (10)	24±1(9)	17±0.8(9)	16±0.9(9)	65	57
Water intubated control [3]	27±3 (10)	29±3(9)	29±3(9)	27±4(9)	112	85

[1] Mean ± SE.
[2] Number of rats.
[3] Fed stock diet ad libitum.

TABLE 6

Summary of 4-week weight gain, nutrient intake, and efficiency in rats fed various levels of fat and 1,3-butanediol

Groups combined	Diet		No. rats	4-wk body wt gain [1]	Nutrient intake			Nutrient efficiency		
	Fat	1,3-Butanediol			Food	Protein	Calories	Food [2]	Protein [3]	Calorie [4]
	%	%		*g*	*g*	*g*	*kcal*			
1,2	10	0	20	167	445	120.4	1795.5	37.5	1.53	9.3
3,4	30	0	20	176	392	105.4	1991.0	45.0	1.87	8.85
5,6,7	30	20	29	150	343	102.2	1865.0	43.9	1.58	8.1
8,9	30	30	19	110	305	69.9	1737.0	31.0	1.62	6.3
10,11	50	0	20	160	337	991.7	2035.0	47.5	1.92	7.9
12–15	60	0	40	160	327	69.1	2151.0	48.8	2.44	7.4

[1] LSD at 5% level = 25 g; at 1% level = 35 g.
[2] LSD at 5% level = 6.86; at 1% level = 9.53.
[3] LSD, no significant differences.
[4] LSD at 5% level = 1.4%.

TABLE 7

Summary of 30-week weight gain and nutrient efficiency in rats fed various levels of fat and 1,3-butanediol

Groups combined	Diet		No. rats	Body wt gain [1]	Efficiency	
	Fat	1,3-Butanediol			Food [2]	Calorie [3]
	%	%		*g*		
1,2	10	0	19	447	13.4	3.32
3,4	30	0	18	514	16.5	3.30
5,6,7	30	20	25	459	16.3	3.00
8,9	30	30	17	368	14.0	2.45
10,11	50	0	16	483	17.4	2.88
12–15	60	0	34	518	18.9	2.87

[1] LSD at the 5% level = 75 g; at the 1% level = 104 g.
[2] LSD at the 5% level = 2.5; at the 1% level = 3.5.
[3] LSD at the 5% level = 0.34; at the 1% level = 0.47.

Food efficiency with these diets was 16.3 as compared with 16.5 for the unsupplemented 30% fat diets. Similarly, average caloric efficiency was 3.0 with the 20% BD diets as compared with 3.3 for unsupplemented 30% fat diets and 2.9 with the 50 and 60% fat diets.

Liver and kidney weights were recorded at the termination of the 30-week experiment. There were no significant differences among any of the values when compared as a percentage of body weight. Mortality was not excessive in any of the groups and did not appear to be related to the type of diet.

Dietary fat and BD appeared to have different effects on the metabolic parameters studied at the end of the 30-week feeding period. Changes in serum ketone bodies with variations in dietary fat were as expected (table 8 and fig. 1A). Serum ketone bodies generally increased with increasing dietary fat up to 50%. When dietary fat was increased to 60%, the level decreased markedly, falling to 50% of the levels of the animals fed the 50% fat diet. However, when BD was added to the diet, serum ketone bodies did not change significantly.

In contrast, the pattern of change in urinary ketone bodies was different (table 8 and fig. 1B). In this case little if any change occurred at dietary fat levels up to 30%. At 50% dietary fat urinary ketone bodies began to increase, reaching a maximum at 60% dietary fat. No such effect was observed when BD was added to the diet. In general, urinary ketone bodies remained essentially constant with increasing dietary levels of BD.

Urine volumes (table 8) also tended to increase with increasing levels of dietary fat. At 60% dietary fat, urine volume was more than twice that at 10% fat. However, increasing the level of BD to 30% tended to decrease urine volume.

Changes in those parameters associated with carbohydrate metabolism also tended to fit a pattern (fig. 2). Liver phosphohexose isomerase (PHI) generally decreased with increasing fat in the diet (fig. 2A). The exception to this pattern was higher activity at 50% dietary fat. In contrast, when BD was added to the

TABLE 8

Ketone bodies [1] in serum and urine at 30 weeks

	10% fat (19) [2]	30% fat (18)	50% fat (16)	60% fat (34)	30% fat + 20% BD (25)	30% fat + 30% BD (17)
Serum ketone bodies, mg/100 ml	8.37 ± 1.11 [3]	9.60 ± 0.70	12.33 ± 1.36	5.97 ± 0.45	8.48 ± 0.67	6.84 ± 0.75
Urine volume, ml/18 hr	6.85 ± 1.25	8.20 ± 2.37	11.40 ± 2.35	17.31 ± 1.31	8.17 ± 1.09	5.55 ± 0.78
Urine ketone bodies, μg/ml	74.00 ± 12.28	48.40 ± 11.77	70.70 ± 11.13	130.23 ± 18.05	63.90 ± 18.33	63.00 ± 9.18
Total urine ketone bodies, mg/18 hr	0.39 ± 0.05	0.33 ± 0.03	0.81 ± 0.04	2.25 ± 0.21	0.35 ± 0.04	0.34 ± 0.06
Urine ketone bodies, mg/kg/18 hr	0.63 ± 0.08	0.53 ± 0.11	1.11 ± 0.15	3.39 ± 0.29	0.59 ± 0.06	0.63 ± 0.10

[1] As acetone.
[2] Numbers in parentheses refer to number of rats/group.
[3] SE.

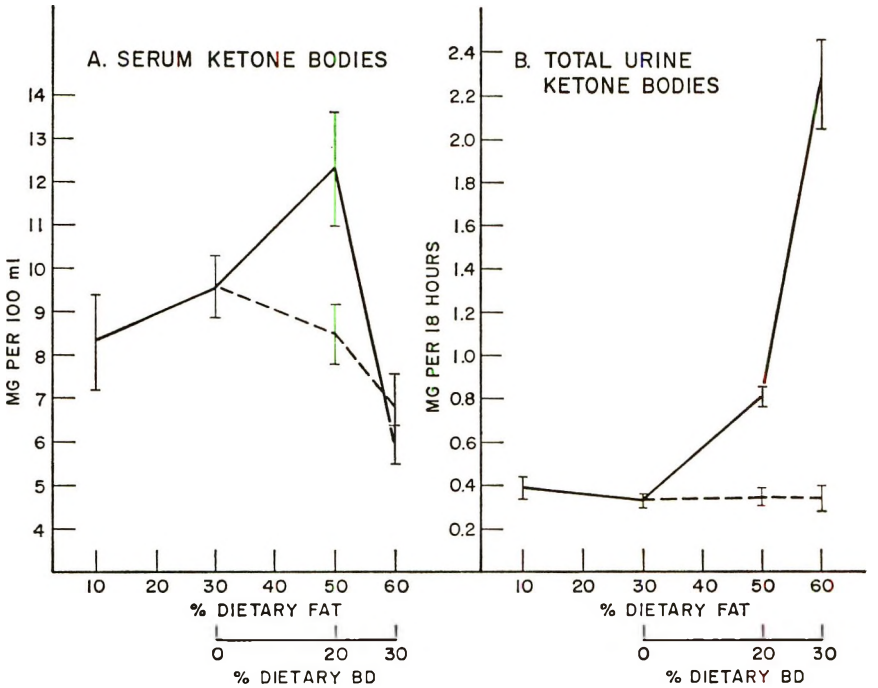

Fig. 1 Changes in serum and urine ketone bodies with increasing dietary levels of fat (solid line) and 1,3-butanediol (BD, dashed line). The vertical lines represent the standard error of the mean. The number of animals at each point is shown in table 8.

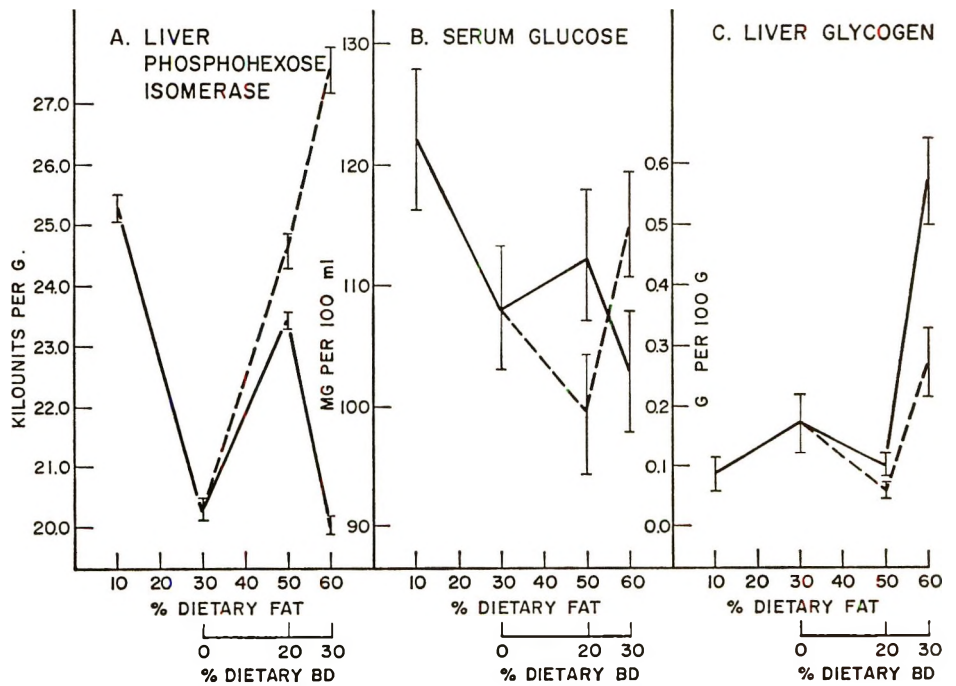

Fig. 2 Changes in liver phosphohexose isomerase, glycogen, and serum glucose with increasing dietary levels of fat (solid line) and 1,3-butanediol (BD, dashed line). The vertical lines represent the standard error of the mean. The number of animals at each point is shown in table 8.

30% fat diet, PHI activity increased with increasing dietary concentration.

Changes in serum glucose with increasing levels of dietary fat were very small although the pattern in these changes was similar to those observed for PHI including the anomalous increase at 50% (fig. 2B). The addition of BD to the diet had no clear effect.

As dietary fat levels were increased to 60% there appeared to be an increase in liver glycogen with an anomalous decrease appearing at 50% dietary fat (fig. 2C). Similarly, when the level of BD was increased to 30%, liver glycogen also increased significantly.

DISCUSSION

The initial attempt in our laboratory to feed high levels of BD to weanling rats (exp. 1) indicated that the poor performance in weight gain, but not in nutrient efficiency, was caused by reduction in food intake. Further study of this problem (exp. 2) showed that the response of animals pair-fed 5 and 20% BD for 3 weeks was similar to that of unsupplemented control rats in weight gain and in food and protein conversion efficiency. However, in all tests, BD apparently was not utilized as well during the first week as in the succeeding weeks of the test.

An explanation may be found in the isocaloric force-feeding study (exp. 3) which demonstrated the need for an adaptation period of at least one week for maximal utilization of BD. Further confirmation of this phenomenon was found in the first 4 weeks of the long-term study (exp. 4). Animals fed 20% BD in the diet demonstrated a reduction in growth and in protein conversion parameters in the first week, which was overcome in succeeding weeks. This response was not as clearly marked in animals fed a 30% BD diet.

In the case of the diets containing 30% BD it must be concluded that, from a consideration of all utilization and growth parameters measured, there was a significant impairment in utilization of the diet. Since Hess and Kopf (17) have reported that BD retards the absorption of drugs, it is possible that absorption is one of the factors responsible for the lower utilization of diets containing high levels of BD.

Although utilization of diets containing 30% BD was impaired, no such effect was noted when 20% dietary levels of BD were used. In this case, following the adaptation period, the rats were able to utilize these diets as well as equivalent diets containing carbohydrate. These results are in agreement with the conclusions of Bornmann (5) concerning the low order of toxicity of BD and the report of Schussel (7) that levels of BD up to 20% did not cause a growth depression in the rat.

The superior weight gains of rats fed the 30% fat diets appear to confirm the reports of Schaer et al. (18) that 30% may be the optimal level of dietary fat for the rat. However, in that experiment there appeared to be as many obese rats in groups fed 30% fat as in groups fed 60% fat. This observation is in contrast with the studies of Mickelsen et al. (19) in which a diet containing 63% fat was used to produce obesity. Furthermore, unlike the observations of Yoshida et al. (20), the caloric consumption of rats in our experiment generally increased as the level of fat in the diet was raised from 10 to 30, 50 and 60%.

In terms of the metabolic parameters examined in this study, our results do not appear to confirm the report of Gessner et al. (21) who proposed that BD was completely oxidized through β-hydroxybutyric acid (BHBA) to carbon dioxide and water. This conclusion was based upon the observation that when a single relatively small dose of the compound was administered to rabbits, no metabolite of it could be found excreted in the urine or feces. If this hypothesis was correct, feeding large amounts of BD under conditions which inhibit the oxidation of BHBA should result in ketosis. This then should result in a marked excretion of BHBA and result in ketonuria. That conditions for ketonuria existed in our study is indicated by the increased ketonuria and ketonemia observed with increasing levels of fat in the diet, an observation reported by others (22). Furthermore, the diuresis observed in animals fed the 60% fat diet is also

consistent with ketosis in the rat (23). In contrast, when the 30% fat diet was supplemented with large amounts of BD, no such changes could be observed. In this case, moreover, there was a tendency for both urinary and serum ketone levels to remain constant with increasing dietary levels of BD.

The observation of increased urine volume at 60% dietary fat might also explain the marked drop in serum ketone levels and increase in urinary ketone level when the level of dietary fat is increased from 50 to 60%. Under these conditions the clearance of ketone bodies from the blood may be increased, resulting in a "washing" of ketone bodies with the urine.

Dietary fat and BD were different also in their effects on carbohydrate metabolism. In general, increasing levels of dietary fat produced changes in the parameters studied in this investigation similar to the patterns reported by other investigators (24, 25). On the other hand, increasing dietary levels of BD produced a different pattern in these parameters. Liver PHI increased as the level of BD in the diet increased, whereas in contrast, PHI decreased when fat was fed. Although changes in serum glucose were small, fat and BD again tended to produce opposite responses. The exception to this pattern was liver glycogen. When either fat or BD was fed, liver glycogen tended to increase.

Some comment should be made concerning the apparently anomalous behavior of the groups fed 50% fat. In assays for PHI, glucose, and glycogen these animals consistently demonstrated a response opposite to that expected. The reasons for this behavior are unknown but probably represent an artifact in the experiment.

It is apparent, therefore, that the feeding of BD at levels of up to 20% of the diet results in no gross deleterious effect in terms of nutrient utilization and weight gain. In addition, in contrast with fat, at dietary levels of up to 30%, BD does not cause any increase in urinary and serum ketone body levels. These results support the original viewpoint of Schussel (6) that synthetic compounds such as BD can be used to broaden the base of nutrition.

ACKNOWLEDGMENT

The authors wish to express their appreciation to Mrs. Anne Browning for her technical support.

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Effect of Levels of Nitrogen Intake on Tryptophan Metabolism and Requirement for Pregnancy of the Rat [1,2]

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ABSTRACT Investigations were performed to determine the effect of levels of dietary nitrogen on the requirement of tryptophan for pregnancy and on the conversion of tryptophan to N [1]-methylnicotinamide and to hepatic pyridine nucleotide. Niacin-free diets varying in tryptophan content from 0.03 to 0.13% and in nitrogen content from 0.3 to 3% were fed to pregnant rats for 3 weeks and to nonpregnant rats for 8 weeks. One series of diets contained crystalline amino acids as the only source of nitrogen. Another series contained casein as the source of tryptophan and either tryptophan-free proteins or equinitrogenous amounts of diammonium citrate, glycine and glutamic acid as sources of additional nitrogen. With both series of diets, augmentation of dietary nitrogen induced a lowering of N [1]-methylnicotinamide excretion. The extent of the lowering of the hepatic pyridine nucleotide levels resulting from increased dietary nitrogen depended on the magnitude of the increase and the duration of the feeding period. Changes from the 1% to the 2% level of dietary nitrogen had an adverse effect on the condition and viability of fetuses of rats fed the 0.09 and 0.07% tryptophan diets but appeared to have no such effect at the 0.13 and 0.11% levels of tryptophan intake.

Results reported previously from this laboratory (1) indicated that for rats fed during gestation a niacin-free diet containing 3% of protein nitrogen, the minimal tryptophan intake required for pregnancy to proceed with normal growth and viability of the fetuses was more than 0.093% and possibly less than 0.136% of the diet. According to Pike (2) an intake of over 0.2% of tryptophan is needed by the pregnant rat fed niacin-free, 14.7% casein hydrolysate diets for the normal maternal weight gain and protection of the fetuses from congenital cataract. Changes in the intake of essential and nonessential amino acids and nonspecific nitrogenous substances affect the metabolism and the requirements for individual essential amino acids. Although the tryptophan requirements for the growth of young animals at different levels of protein intake have been studied by several investigators (3,4) relatively little has been reported on the effect of levels of nitrogen intake on the tryptophan requirement for pregnancy.

The present paper reports the results of a study of the effect of levels of intake of other amino acids and nonspecific nitrogenous substances on the tryptophan requirement for pregnancy, and on the levels of excretion of N[1]-methylnicotinamide (MNA) and the concentration of hepatic pyridine nucleotide of the pregnant and nonpregnant rats.

EXPERIMENTAL

Rats. Four- to six-month-old rats of the Osborne-Mendel strain were used for these investigations. The females weighed 250 to 300 g and the males, 400 to 500 g. In most cases 8 rats were used for each experiment.

Diets. The pre-experimental or control diet consisted of: (in per cent) casein, 21.2; salt mixture (5), 2.5; corn oil, 8.0; complete vitamin mixture,[3] 1.0; niacin-

Received for publication July 18, 1966.

[1] Supported in part by Public Health Service Research Grant no. HD-01216 from the National Institutes of Health.

[2] Preliminary results of this investigation were presented at the 49th annual meeting of the Federation of American Societies for Experimental Biology, Atlantic City, New Jersey, 1965.

[3] The vitamin mixture contained per gram: (in IU) vitamin A, 950; vitamin D, 100; (in milligrams) α-tocopherol, 5.0; ascorbic acid, 45.0; inositol, 5.0; choline chloride, 75.0; menadione, 2.25; p-aminobenzoic acid, 5.0; niacin, 4.5; riboflavin, 1.0; pyridoxine·HCl, 1.0; thiamine·HCl, 1.0; Ca pantothenate, 3.0; (in micrograms) biotin, 20; folic acid, 90; and vitamin B_{12}, 1.35.

free vitamin mixture,[4] 1.5; and glucose monohydrate,[5] 65.8. The experimental diets varied in their tryptophan content from 0.03 to 0.13% and in their total nitrogen content from 0.3 to 3%. In one series of diets, casein was used as the source of tryptophan, and the total nitrogen content of the diets was increased by the addition of either tryptophan-free proteins (gelatin and casein hydrolysate), or of isonitrogenous amounts of diammonium citrate, glycine and glutamic acid. In the other series of diets, crystalline amino acids were used as the only source of nitrogenous substances. The amino acid mixtures were prepared according to the FAO pattern (6) for the essential amino acids, with the exception of tryptophan, and according to the milk protein pattern (7) for the nonessential amino acids, and were incorporated into the diets at an essential-to-nonessential amino acids ratio of 0.8. The total nitrogen levels of the diets were altered either by increasing the amount of the amino acid mixtures, or by supplementing either glycine, glutamic acid, diammonium citrate or threonine. In addition to the nitrogenous substances all diets contained 2.5% of a niacin-free vitamin mixture[6]; 2.5% of salt mixture (5); 8.0% of corn oil and glucose monohydrate to 100%.

Procedure. All the rats were fed the pre-experimental or control diet for 2 to 3 weeks. Twenty-four-hour urine samples were collected for 3 to 5 days toward the end of that period for the determination of the animals' pre-experimental MNA values. For the pregnancy studies, virgin rats were mated and fed the experimental diets from the first day of gestation. Twenty-four-hour samples for MNA determination were collected on days 2, 7, 14, 19 and 20 of pregnancy. Because of the repeatedly observed large individual variations in the MNA excretion even between littermates, the MNA excretions for all the experimental animals were expressed as percentages of their pre-experimental MNA excretion values. On the twenty-first day of pregnancy the animals were decapitated, their livers were analyzed for pyridine nucleotide and observations were made on the condition, number, weight and viability of the fetuses. For studies of the effect of longer feeding periods, nonpregnant rats were fed the experimental diets for 8 or 9 weeks. Samples for MNA determinations were collected at weekly intervals and the livers were analyzed for pyridine nucleotide. Analytical methods described previously (1) were used for MNA and pyridine nucleotide determinations.

RESULTS

The trend of the changes in the levels of MNA excretion which occurred as the result of pregnancy and of feeding the niacin-free, low tryptophan amino acid-mixture diets are illustrated in figure 1. In animals fed the pre-experimental, or control diet (A) during the gestation period there was a steady increase in MNA excretion levels, which was especially pronounced during the third week of pregnancy. The feeding of niacin-free, low tryptophan diets to nonpregnant rats (NP-C-2) resulted in a rapid decrease in their MNA excretion during the first week, followed by a more gradual decline in MNA values during the second and third weeks of the experiment. In pregnant rats, the feeding of niacin-free, low tryptophan diets induced decreases in the MNA output during the first week of pregnancy, which were followed by increases in MNA excretion as pregnancy proceeded. The extent of the drop in MNA values resulting from the feeding of the experimental diets, as well as that of the increase induced by pregnancy, was influenced by both the tryptophan and the nitrogen content of the diets. In animals fed diets containing 0.13 and 0.11% of tryptophan and 1% of nitrogen (B-1, C-1) the initial decreases in MNA excretion were followed by increases of a magnitude which allowed the MNA values to approach their pre-experimental levels by the third week of pregnancy and to exceed them by the end of the period of gestation. The feeding of diets of lower tryptophan (D-1) and higher nitrogen content (B-2, C-2, D-2) resulted in more drastic lowering of MNA excretion which was followed by considerably smaller increases resulting from pregnancy. At the 2% ni-

[4] Similar to vitamin mixture in footnote 3, but free of niacin.
[5] Cerelose, Corn Products Company, Argo, Illinois.
[6] See footnote 4.

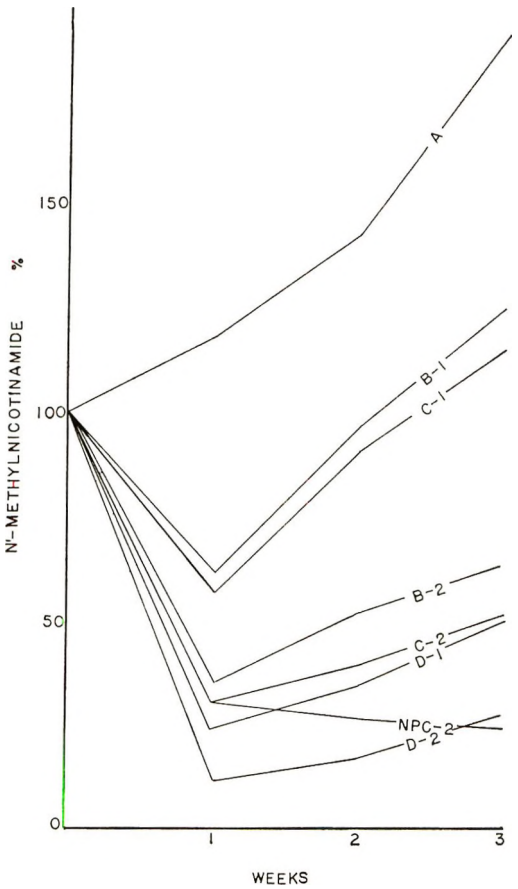

Fig. 1 Effect of pregnancy and of levels of tryptophan and nitrogen intake of rats fed the control and the amino acid-mixture diets on N^1-methylnicotinamide excretion, expressed as the percentage of the pre-experimental N^1-methylnicotinamide excretion values. Notations are for diets: A, control, containing 0.23% tryptophan, 3% N, 0.007% niacin; B-1, 0.13% tryptophan, 1% N; B-2, 0.13% tryptophan, 2% N; C-1, 0.11% tryptophan, 1% N; C-2, 0.11% tryptophan, 2% N; D-1, 0.09% tryptophan, 1% N; D-2, 0.09% tryptophan, 2% N; and NP-C-2, 0.11% tryptophan, 2% N, fed to nonpregnant rats.

trogen, 0.07 and 0.05% levels of tryptophan intake, pregnancy induced very small or no increases in MNA excretion. At the end of the 3-week experimental periods there were no significant changes in the weights of the nonpregnant rats.

The mean values of the MNA excretion and the hepatic pyridine nucleotide concentration at the end of the experimental periods of rats that were fed the crystalline amino acid diets are presented in table 1. Comparison of the values for the MNA excretion of rats fed diets of equal tryptophan and varying nitrogen content indicates that the amount of output of MNA was influenced by the levels of dietary nitrogen. With the diets containing 0.13, 0.11 and 0.09% of tryptophan, at the end of the period of pregnancy, the MNA excretion expressed as the percentage of the pre-experimental values was significantly lower ($P < 0.01$) for the animals fed diets containing 2% of nitrogen rather than 1% of nitrogen. At the 0.07% tryptophan level the difference between the MNA excretion of the groups of rats fed diets containing 1 and 2% of nitrogen was of low significance ($P < 0.05$), and at the 0.05% tryptophan level, no difference could be detected between the MNA excretion of the 2 groups of rats. At such low levels of tryptophan intake the concentration of MNA in the urine of the rats reached values which were on the borderline or below the limits required for the sensitivity of the analytical method used. At equal levels of tryptophan intake the animals' hepatic PN concentration appeared to be lower at the 2% than at the 1% level of dietary nitrogen. However, these differences at the end of the 3-week experimental periods were of either low or of no significance. Changes in levels of dietary nitrogen from 1 to 2% did not affect the animals' food intake.

At the 0.07% level of tryptophan intake, increases in nitrogen intake from 1 to 2% produced by supplementing the diets with isonitrogenous amounts of either diammonium citrate, glycine or glutamic acid resulted in decreases ($P < 0.05$) in MNA excretion which were of the same degree of magnitude as those produced by the addition of a complete amino acid mixture. At the 0.09% level of tryptophan in diets containing 2% of nitrogen, substitution of half of the amino acid mixture with an isonitrogenous amount of glycine induced no changes in the level of MNA excretion, whereas an increase in dietary nitrogen from 2 to 3% by the addition to the diet of 5.4% of glycine resulted in a lowering of MNA excretion. The hepatic pyridine nucleotide concentrations of the pregnant rats in these experiments were not affected

TABLE 1

N[1]-methylnicotinamide (MNA) excretion, expressed as percentages of pre-experimental values and hepatic pyridine nucleotide (PN) concentration of rats fed amino acid-mixture diets

Diets					Daily food intake	MNA	PN
Trypto-phan	N	EAA [1]	NAA [2]	Source of additional N			
%	%	%	%		*g/100 g body wt*	%	*μg/g*
				3-week experiment, females			
0.13	1.0	3.88	4.21		5.9 ± 0.2 [3]	134.0 ± 15.1	751 ± 31
0.13	2.0	7.89	8.41		6.1 ± 0.3	62.6 ± 9.0 [4]	703 ± 17
0.11	1.0	3.90	4.21		6.1 ± 0.5	117.2 ± 4.2	724 ± 29
0.11	2.0	7.91	8.41		6.0 ± 0.4	34.3 ± 5.5 [4]	673 ± 22
0.09	1.0	3.92	4.21		5.6 ± 0.3	45.9 ± 10.1	708 ± 16
0.09	2.0	7.93	8.41		5.9 ± 0.3	19.3 ± 3.8 [4]	668 ± 11
0.09	2.0	3.92	4.21	Glycine, 5.40%	5.3 ± 0.6	18.9 ± 1.9 [4]	661 ± 6
0.09	3.0	7.93	8.41	Glycine, 5.40%	5.6 ± 0.3	11.3 ± 3.1 [4]	634 ± 26 [5]
0.07	1.0	3.94	4.21		6.1 ± 0.4	18.6 ± 3.7	641 ± 26
0.07	2.0	7.95	8.41		5.3 ± 0.2	9.6 ± 2.2 [5]	628 ± 23
0.07	2.0	3.94	4.21	Diammonium citrate, 8.10%	6.7 ± 0.1	9.5 ± 2.3	664 ± 26
0.07	2.0	3.94	4.21	Glycine, 5.40%	6.0 ± 0.5	11.5 ± 1.7	660 ± 30
0.07	2.0	3.94	4.21	Glutamic acid, 10.50%	6.6 ± 0.5	12.0 ± 3.3	658 ± 26
0.05	1.0	3.96	4.21		6.1 ± 0.4	3.4 ± 0.3	621 ± 27
0.05	2.0	7.97	8.41		5.9 ± 0.4	3.7 ± 0.8	597 ± 29
				8-week experiment, males			
0.03	0.3	1.30	1.40				486 ± 25
0.03	0.3	1.26	1.40	Threonine, 0.40%			509 ± 10
0.03	2.0	8.00	8.41				326 ± 21 [6]
0.03	2.0	7.96	8.41	Threonine, 0.40%			330 ± 17 [6]

[1] Mixture of essential amino acids.
[2] Mixture of nonessential amino acids.
[3] Mean ± SE.
[4] Significance of difference from the corresponding values at the 1% level of nitrogen intake, $P < 0.01$.
[5] Significance of difference from the corresponding values at the 1% level of nitrogen intake, $P < 0.05$.
[6] Significance of difference from the corresponding values at the 0.3% level of nitrogen intake, $P < 0.01$.

by increases in nitrogen intake from 1 to 2%, but were reduced at the 5% probability level by increases from 1 to 3% nitrogen.

In animals fed diets containing 0.03% of tryptophan, at the end of the 8-week experimental periods the concentration of the liver pyridine nucleotide was very significantly smaller ($P < 0.01$) at the high level (2%) than at the low level (0.3%) of nitrogen intake. At this dietary tryptophan content the concentration of MNA in the samples was below the limits of sensitivity required for the analytical method used. Supplementation of these diets by 0.4% of threonine produced no changes in the animals' hepatic pyridine nucleotide concentrations.

In table 2 are presented the mean values for the MNA excretion and the hepatic pyridine nucleotide concentration of rats fed the casein diets for periods of 1, 3 and 8 weeks. With diets containing 7.7% of casein, increases from the 1% to the 3% level of nitrogen intake which were produced by the addition of either tryptophan-free proteins or of an isonitrogenous mixture of glycine, glutamic acid and diammonium citrate, resulted in a marked and prompt lowering of the animals' MNA excretion. These decreases could be observed within 24 hours after the ingestion of the experimental diets and were significant at the end of a one-week feeding period, as well as at the end of 3-week and 8-week periods, in both pregnant and nonpregnant

TABLE 2

N^1-methylnicotinamide (MNA) excretion and hepatic pyridine nucleotide (PN) concentration of rats fed diets containing 7.7% of casein and nitrogenous supplements for periods of 1, 3, or 8 weeks

Diets			Female rats					Male rats		
			1 week	3 weeks		8 weeks		3 weeks	8 weeks	
N	Supplements		MNA [1]	MNA	PN	MNA	PN	MNA	MNA	PN
%		%	%	%	μg/g	%	μg/g	%	%	μg/g
1	None		40 ± 3 [2]	49 ± 3	851 ± 35	30 ± 2	890 ± 32	37 ± 5	38 ± 5	1022 ± 24
1	Threonine	0.4	38 ± 3	44 ± 4	843 ± 30	—	—	—	—	—
3	Casein hydrolysate	4.0	11 ± 1 [3]	12 ± 1 [3]	768 ± 22 [4]	9 ± 1 [3]	700 ± 39 [3]	11 ± 2 [3]	7 ± 2 [3]	830 ± 13 [3]
	Gelatin	9.3								
3	Diammonium citrate	5.4	12 ± 2 [3]	13 ± 2 [3]	710 ± 33 [5]	10 ± 0 [3]	667 ± 12 [3]	9 ± 1 [3]	9 ± 2 [3]	698 ± 4 [3]
	Glycine	3.6								
	Glutamic acid	7.0								
2	Diammonium citrate	8.1						9 ± 1 [3]	10 ± 1 [3]	851 ± 52 [5]
2	Glycine	5.4						9 ± 1 [3]	8 ± 2 [3]	684 ± 21 [3]
2	Glutamic acid	10.5						10 ± 1 [3]	9 ± 1 [3]	874 ± 43 [5]

[1] Expressed as percentage of pre-experimental values $\frac{\text{MNA final}}{\text{MNA initial}} \times 100\%$.

[2] Mean ± SE.

[3] Significance of difference from the corresponding values at the 1% level of nitrogen intake, $P < 0.01$.

[4] Significance of difference from the corresponding values at the 1% level of nitrogen intake, $P < 0.05$.

[5] Significance of difference from the corresponding values at the 1% level of nitrogen intake, $P < 0.02$.

rats. Increases in dietary nitrogen from 1 to 3% resulted also in a reduction of the animals' hepatic pyridine nucleotide concentration. At the end of the period of pregnancy, the differences in the pyridine nucleotide concentration at the 2 levels of nitrogen intake were significant at the 2% probability level when the extra 2% nitrogen was supplied by a mixture of diammonium citrate, glycine and glutamic acid, and only at the 5% probability level when the casein hydrolysate and gelatin were used as the source of additional nitrogen. At the end of the 8-week feeding periods these differences for both the female and the male rats were significant at the 1% probability level. The decrease in pyridine nucleotide concentration was more marked when the extra nitrogen was supplied by an isonitrogenous mixture of glutamic acid, glycine and diammonium citrate than when the extra nitrogen was supplied by a mixture of gelatin and casein hydrolysate. Augmentation of dietary nitrogen from 1 to 2% by the addition of either diammonium citrate, glycine or glutamic acid resulted in decreases in the animals' MNA excretion which were of the same degree of magnitude for all those substances. It also produced, after 8-week feeding periods, some lowering in the rats' hepatic pyridine nucleotide concentration. The difference between the pyridine nucleotide values at the 1 and 2% levels of nitrogen intake were highly significant ($P < 0.01$) when the extra nitrogen was supplied by glycine, and of lower significance ($P < 0.02$) when the diammonium citrate or glutamic acid were the source of extra nitrogen.

There was no loss in the weight of the rats that were fed diets containing 7.7% of casein as the only source of nitrogen. The weight of the animals decreased on the average to 95% of their original values after 8 weeks of feeding diets which were brought to a 2 or 3% level of nitrogen content by the supplementation with either tryptophan-free proteins or isonitrogenous amounts of glycine, glutamic acid and diammonium citrate.

In tables 3 and 4 are summarized data on the condition, viability, size and weight of the fetuses that were removed at autopsy on the twenty-first day of gestation of the experimental rats. The criteria for evaluation of the condition of the fetuses were: their build, presence or absence of deformities, color, normal development of legs and ability to move around. The viability of the fetuses is expressed as the percentage of pregnancies in which either all, two-thirds, one-third or none of the fetuses in a litter survived a period of 24 hours. In the case of animals fed the amino acid-mixture diets containing 0.13 and 0.11% of tryptophan, 100% of the fetuses were in good condition both at the 1 and the 2% levels of nitrogen intake. With the diets containing 0.09% of tryptophan, augmentation in dietary nitrogen from 1 to 2 and 3% had an adverse effect on the condition of the fetuses which was especially severe when glycine was used as the source of supplementary nitrogen. In 50% of the pregnant animals fed diets containing 0.09% of tryptophan and 3% of nitrogen with 1% of the nitrogen supplied by glycine, the fetuses were either dead or dying. In 17% there was resorption, which had evidently taken place rather early in the gestation period: the uteri contained either only pinpoint implantation sites or fetuses and placenta, the combined weight of which amounted to only 1.5 to 3.0 g. Gross examination of the living fetuses in this group revealed that they were small, thin, with apparently little body fat and poorly developed musculature.

With diets containing 0.07% of tryptophan, augmentation in nitrogen intake from the 1% to the 2% level produced by supplementing the diets with either complete amino acid mixtures, glycine, diammonium citrate or glutamic acid, resulted in an increase in the percentage of pregnancies with dead or resorbed fetuses, and in an impairment in the condition and viability of the young. Among the abnormalities observed from gross examination of the fetuses of rats fed diets containing 0.07% of tryptophan and 2% of nitrogen, subcutaneous edema was common and in a few cases there were also subcutaneous and internal hemorrhages. Many of the fetuses in this group and in the group fed diets containing 0.09% of tryptophan and 3% of nitrogen had some depression of the cranial surface which appeared to be a result of delayed development of the

TABLE 3

Condition and viability of fetuses of rats fed the amino acid-mixture diets

Diets		Fetuses		Condition					Viability [1]			
									Alive for 24 hours in litter			
Trypto-phan	N	No./litter	Wt of each	Good	Poor	Dying	Dead	Resorbed	All	2/3	1/3	None
%	%		g	%	%	%	%	%	%	%	%	%
0.13	1	10.3 ± 0.9 [2]	4.8 ± 0.2	100	—	—	—	—	85	15	—	—
0.13	2	10.4 ± 0.4	4.6 ± 0.1	100	—	—	—	—	70	30	—	—
0.11	1	9.8 ± 1.2	4.5 ± 0.3	100	—	—	—	—	100	—	—	—
0.11	2	12.3 ± 0.6	4.7 ± 0.1	100	—	—	—	—	90	10	—	—
0.09	1	11.0 ± 1.0	4.4 ± 0.1	100	—	—	—	—	85	15	—	—
0.09	2	12.1 ± 0.7	4.6 ± 0.2	70	30	—	—	—	80	20	—	—
0.09	2 [3]	8.5 ± 0.9	4.2 ± 0.2	10	61	29	—	—	—	60	40	—
0.09	3 [3]	9.8 ± 0.8	3.6 ± 0.3 [4]	—	33	23	27	17	—	50	50	—
0.07	1	9.7 ± 0.8	4.3 ± 0.2	40	40	—	—	20	50	25	25	—
0.07	2	11.7 ± 0.7	4.1 ± 0.1	27	27	—	—	45	—	50	50	—
0.07	2 [5]	11.6 ± 0.8	3.4 ± 0.3	14	14	19	24	30	—	50	50	—
0.07	2 [3]	7.6 ± 1.3	4.0 ± 0.5	—	30	17	40	14	—	—	70	30
0.07	2 [6]	9.7 ± 0.6	4.2 ± 0.3	—	35	15	35	15	—	50	50	—
0.05	1	No living fetuses		—	—	—	33	67	—	—	—	—
0.05	2	No living fetuses		—	—	—	78	22	—	—	—	—

[1] Viability of the fetuses is expressed as the percentage of pregnancies in which either all, two-thirds, one-third or none of the fetuses in a litter survived a period of 24 hours.

[2] Mean ± SE.

[3] One per cent of nitrogen supplied by glycine.

[4] Significance of difference from the corresponding values at the 1% level of nitrogen intake, $P < 0.05$.

[5] One per cent of nitrogen supplied by diammonium citrate.

[6] One per cent of nitrogen supplied by glutamic acid.

TABLE 4

Condition of fetuses of rats fed diets containing 7.7% of casein and nitrogenous supplements

Diets			Daily food intake	Condition of fetuses						
N	Supplements	%	g/100 g body wt	No. in litter	Wt of each	Good	Poor	Dying	Dead	Resorbed
%					g	%	%	%	%	%
1	None		5.4 ± 0.2 [1]	11.0 ± 0.5	4.9 ± 0.1	100	—	—	—	—
1	Threonine	0.4	6.0 ± 0.3	11.1 ± 0.3	5.0 ± 0.2	100	—	—	—	—
3	Casein hydrolysate Gelatin	4.0 9.3	6.3 ± 0.2	8.6 ± 0.7	4.7 ± 0.3	52	24	12	12	—
3	Diammonium citrate Glycine Glutamic acid	5.4 3.6 7.0	5.1 ± 0.2	11.0 ± 0.2	3.8 ± 0.2 [2]	35	35	—	—	30

[1] Mean ± SE.
[2] Significance of difference from the corresponding values at the 1% level of nitrogen intake, $P < 0.05$.

cerebrum and possibly other parts of the brain.

At the 0.05% level of tryptophan intake there were no living fetuses on the twenty-first day of gestation with diets containing either 1 or 2% of nitrogen.

With the casein diet (table 4), at the 0.09% tryptophan, 1% nitrogen level, the fetuses were in good condition. Supplementation of the diet with 0.4% of threonine had no adverse effect on the condition, number and size of the fetuses. Increases in the dietary nitrogen from 1 to 3% resulted in an impairment of the condition of the fetuses and in case of the diammonium citrate, glycine, glutamic acid diet, in a decrease in the average weight of the fetuses.

DISCUSSION

The data presented in these experiments indicate that for rats fed niacin-free, low tryptophan diets, changes in the levels of nitrogen intake affect the tryptophan metabolism as judged by such criteria as the MNA excretion and the hepatic pyridine nucleotide concentration, and that these changes also appear to have an effect on the tryptophan requirement for pregnancy. Increases in the nitrogen intake, which were produced by the addition to the diets of either tryptophan-free proteins, amino acid mixtures, or isonitrogenous amounts of glycine, glutamic acid and diammonium citrate resulted in prompt and marked decreases in the animals' MNA excretion. These decreases were significant after feeding periods of one, three and eight weeks. It has been observed previously in experiments with niacin-free diets (1) that at constant levels of dietary nitrogen, lowering of tryptophan intake resulted in decreases of the MNA excretion of rats before any significant changes occurred in their hepatic pyridine nucleotide concentration, and that the magnitude of the resulting decreases in the MNA values was greater than that of the decreases of the pyridine nucleotide levels. A similar phenomenon was observed in the present experiments. With diets having tryptophan as the most limiting amino acid, augmentations of the severity of amino acid imbalance, created either by decreasing the percentage of dietary tryp-

tophan or by increasing the percentage of dietary nitrogen, were reflected earlier and to a greater extent in the animals' MNA excretion than in their liver pyridine nucleotide concentration. The extent of the changes in the pyridine nucleotide concentration was observed to depend on the magnitude of the increases in dietary nitrogen levels and on the length of the feeding periods. After feeding periods of 3 weeks, increases in dietary nitrogen from 1 to 2% did not produce any significant changes in the pyridine nucleotide values, but increases from 1 to 3% resulted in changes significant at low probability levels. After feeding periods of 8 weeks, augmentation in nitrogen levels from 1 to 3% and from 0.3 to 2% resulted in highly significant decreases in the hepatic pyridine nucleotide concentrations.

The decreases in MNA excretion and pyridine nucleotide concentration observed in these experiments could be due to interference of the nitrogenous substances either with the absorption of tryptophan from the gastrointestinal tract or with conversion of tryptophan to MNA and pyridine nucleotide, possibly by channeling tryptophan metabolism into some other pathway. Decreases in MNA excretion which resulted from the addition of 4% of DL-methionine to niacin-free diets have been reported [7] to be accompanied by concomitant marked increases in urinary indoleacetic acid.

Among the nitrogenous substances used to produce amino acid imbalances in this study, glycine, when used alone or in combination with diammonium citrate and glutamic acid appeared to be the most effective in depressing the concentration of pyridine nucleotide in the rats' liver. Gelatin in combination with casein hydrolysate induced somewhat smaller changes. Both gelatin and glycine have been reported (8), to produce no decrease in the rats' hepatic pyridine nucleotide but to depress the synthesis of pyridine nucleotide in response to supplement with tryptophan.

An amino acid imbalance created by supplementing low tryptophan niacin-free diets with 0.36% of threonine has been reported (9) to affect the growth, but not the pyridine nucleotide concentration of young rats. In the present studies supplementation with 0.4% of threonine had no effect on either the weight, the MNA excretion or the hepatic pyridine nucleotide concentration of adult rats.

Amino acid imbalances have been observed (10) to affect the animals' growth by restricting the appetite and reducing their food intake. Under the conditions of these experiments the adverse effect of increased levels of dietary nitrogen on the development of the fetuses and on the excretion of MNA could not be attributed to decreased food intake.

A 0.13 and a 0.11% level of tryptophan intake in diets containing either 1 or 2% of nitrogen, as well as a 0.09% level of tryptophan intake in diets containing 1% of nitrogen were sufficient to meet the rats' requirements for pregnancy to proceed with normal development and viability of the fetuses. At the 0.09 and 0.07% levels of tryptophan intake, increases in dietary nitrogen from 1 to 2% and especially to 3% resulted in an impairment in the development of the fetuses. Supplementation of the diets with glycine had a more adverse effect on the condition and viability of the fetuses than did the other supplementations used in these experiments. It appears that there is a relationship between the effect of nitrogenous substances on tryptophan metabolism and on the requirement of tryptophan for pregnancy. These observations suggest the conclusion that the amino acid imbalances created by increasing the nitrogen content of the niacin-free, low tryptophan diets produced a decrease in the utilization of tryptophan both for the formation of MNA and pyridine nucleotide and for satisfying the needs of pregnancy.

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Effect of Vitamin E Deficiency on Collagen Metabolism in the Rat's Skin [1,2]

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ABSTRACT The effects of avitaminosis E on collagen metabolism in the skin of rats were studied. Hides from littermate male rats raised with vitamin E-sufficient or vitamin E-deficient diets were fractionated into neutral salt-soluble, acid-soluble and insoluble collagen. The rate of gel formation at 37°, pH 7.4, of both crude and purified neutral-salt and acid-soluble collagen solutions from the skin of these animals was measured together with the stability of those gels to 4° temperatures for various time periods. It was found that the animals raised with the vitamin E-deficient diets had a greater percentage of soluble collagen in their skins. The neutral salt-soluble collagen solutions from the deficient group did not form gels as readily as did those from their controls nor were these gels as stable to 4° temperature. These results suggest a defect in the formation of intermolecular and intramolecular crosslinkages in the collagen from the vitamin E-deficient group.

Steinburg (1) suggested that changes in collagen metabolism noted in rheumatic diseases may be the result of derangements in vitamin E metabolism, although no experimental evidence for this statement was presented. Hauch (2) demonstrated that topical application of vitamin E was useful in healing pressure sores which were not amenable to conventional therapy. Bartolomucci (3) reported that the addition of vitamin E to the diet had no effect on the rate of healing of experimental fractures in rats, but when vitamin E was deleted from the diet there was a significant retardation of fracture healing.

Previous work [5] from this laboratory has shown that avitaminosis E results in a decrease in the mechanical strength of the aorta of the rat similar to that noted in lathyrism (4), a condition in which a change in collagen metabolism is known to result. The present study was undertaken to determine the effects of vitamin E deficiency on collagen metabolism in rat skin.

EXPERIMENTAL PROCEDURE

Tissue fractionation. Weanling, male, littermate rats of the Wistar strain were fed the experimental diets described in table 1 for 16 weeks. The animals were decapitated under ether anesthesia, and the hides were removed promptly and stored at −20°. Although no storage period exceeded 2 weeks, all hides compared in this experiment were stored for equal lengths of time to eliminate any variable due to storage.

The hides were freed of hair, fat and connective tissue. A section of the dorsal side measuring approximately 10 by 15 cm was removed and minced with dissecting scissors. The minced skins were serially extracted by shaking at 4° with 5 changes of 5 volumes (w:v) of 1.0 M sodium chloride solution and 5 changes of 5 volumes (w:v) of 5 N acetic acid. The insoluble residue was mixed with 4 volumes of water and converted to gelatin by steam hydrolysis at 122° for 24 hours.

The neutral-salt and acid-soluble fractions from each extraction were freed of solids by centrifugation (16,000 × *g* at 0° for 30 minutes) and pooled. These pooled supernatants were filtered through a 1.3-cm pad of fine cheese cloth backed with Whatman no. 42 filter paper. Aliquot portions of the clear filtrate were taken for hydroxyproline analysis by the method of Neuman and Logan (5) as modified by Mar-

Received for publication May 25, 1966.

[1] Published by the permission of the Director, Tennessee Agricultural Experiment Station.

[2] Preliminary report read at the Annual Meeting, Federation of American Societies for Experimental Biology, 1964, Chicago.

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TABLE 1
Composition of experimental diets

	g/100 g of diet
Casein, vitamin-free [1]	15.00
DL-Methionine [1]	0.35
Sucrose	30.83
Cornstarch	31.82
Stripped lard [2]	6.00
Non-nutritive bulk [3]	10.00
Cod liver oil	2.00
Vitamin mixture [4]	1.00
Salt mixture [5]	3.00

[1] Nutritional Biochemicals Corporation, Cleveland.
[2] Distillation Products Industries, Rochester, New York.
[3] Alphacel, Nutritional Biochemicals Corporation.
[4] Vitamin mixture: (in g/400 g) niacin, 8.000; pyridoxine·HCl, 0.200; thiamine·HCl, 0.200; riboflavin, 0.002; Ca pantothenate, 0.400; folic acid, 0.200; biotin, 0.002; menadione, 0.010; vitamin B_{12} (0.1% in mannitol), 1.800; choline·Cl, 40.000; inositol, 40.000; *p*-aminobenzoic acid, 3,000; triturated in sucrose to make 400 g. *dl*-α-Tocopheryl acetate was added to the vitamin E-sufficient diet at a level of 28 mg/100 g of diet.
[5] Hubbell et al. (26).

tin and Axelrod (6). The collagen content was calculated by multiplying the hydroxyproline content by 7.46 as suggested by Fry et al. (4). Portions of these clear, crude solutions were further purified by the method used by Martin et al. (7) and lyophilized from acetic acid.

A second series of extractions, in which the minced skins were fractionated into 0.14 M, and 0.45 M and 1.0 M sodium chloride-soluble collagen was conducted using the same general procedure. The minced skins were serially extracted with 0.14 M, and 1.0 M salt solutions until the supernatant at each concentration of sodium chloride was free of amino nitrogen as determined by a negative ninhydrin test. The supernatants of each extraction were analyzed for hydroxyproline as described above. The total amount of sodium chloride-soluble collagen was assumed to be equal to the summation of these fractions. The proportion of the total sodium chloride-soluble collagen which the 0.14, 0.45 and 1.0 M sodium chloride-soluble collagens represented was then calculated as a percentage of this total.

Procedure for measuring gelatinization of collagen extracts. The crude, neutral salt-soluble and acid-soluble solutions were assayed for hydroxyproline (5) and diluted with 0.1 M phosphate buffer, pH 7.4, so that the constant concentration of hydroxyproline was nearly the same for all solutions used in this experiment.

In all cases, sufficient buffer was used so that the final pH was 7.1 to 7.2. Five-milliliter portions were pipetted into a series of three matched Klett tubes for each animal for each time period. The extracts were allowed to equilibrate at room temperature and then were incubated at 37°. It has been reported that the rate of gelatinization is directly related to increasing optical density (8); therefore, at specified times a tube was withdrawn and the optical density measured with a Klett-Summerson photo-electric colorimeter using a no. 42 filter.

The instrument had been set at zero using distilled water. The zero-time reading for each solution was then measured and served as a base line for subsequent measurements made on collagen solutions from that animal. The 3 values obtained for each time for each animal were averaged and that average figure was considered to be the measurement of opacity for that specific time and animal. For statistical comparison, the average value for each time was compared with that of the animal's littermate for the same time by the method of paired comparisons. In practice, there was little variation in reading between the 3 tubes for any animal and any time. Because of small errors in dilution, all opacity values were corrected by placing them on a per milligram of hydroxyproline basis for comparison.

Procedure for measuring the dissolution of collagen gels. The purified neutral salt and acid-soluble collagen fractions were dissolved in the phosphate buffer, centrifuged at 21,000 × *g* at 0° for 30 minutes and filtered by vacuum through Whatman no. 42 filter paper. This treatment produced a clear solution which was analyzed for hydroxyproline and nitrogen. Three 16 × 150 mm Pyrex test tubes were used for each determination. Gelation was carried out by the procedure described above with an incubation time of one hour, although the rate of gelation was not measured. After incubation one of the tubes from each experimental set was centrifuged in an International clinical centrifuge for 10 minutes and the amount of nitrogen in the supernatant fluid was measured by the micro-Kjeldahl method (9). The remaining gels were cooled

promptly and stored at 4°. After 24 and 48 hours, duplicate samples were centrifuged at 6,000 × *g* at 4° for 30 minutes and the nitrogen content of the supernatant fluid was determined.

The optical methods which are used for determining the rate of gelation and solubilization of collagen gels were first proved by measuring the rate of disappearance of soluble protein (8). Using this concept, the percentage reversibility was determined by the following formula:

$$\frac{\text{mg N/ml}_{(4\text{ n. hr})} - \text{mg N/ml}_{(37\text{ 1 hr})}}{\text{mg N/ml}_{(37\text{ 0 hr})} - \text{mg N/ml}_{(37\text{ 1 hr})}} \times 100 = \%\text{ dissolved}$$

where *n* hours is either 24 or 48 hours and the mg N/ml is the nitrogen in the supernatant fluid.

Other measurements. Since inanition conceivably could cause changes in collagen metabolism, the growth of the animals was recorded regularly during the experimental period. Vitamin E deficiency was assessed by the erythrocyte hemolysis technique of Friedman et al. (10).

A failure to store vitamin A has been reported in vitamin E-deficient rats (11). Vitamin A deficiency has been reported to effect the elaboration of ground substance (12) which, in turn, is essential to collagen formation. For this reason the liver vitamin A levels of rats raised with the same experimental diets were determined by the method of Embree et al. (13).

Littermate pairs of animals were used in all experiments. Statistical analysis of the data was made by the paired-comparison method (14).

RESULTS

Growth, erythrocyte hemolysis, and vitamin A storage. The vitamin E-deficient animals exhibited 95 to 100% erythrocyte hemolysis, whereas the vitamin E-sufficient animals exhibited only zero to 5% red blood cell hemolysis. These observations were interpreted as indicative of vitamin E deficiency in the groups fed the vitamin E-deficient diets. No significant difference in the growth rates occurred between the different dietary groups of either series of animals. The control group gained 360 ± 10 g[6] and the vitamin-deficient group gained 341 ± 3 g.

The analysis of the livers from vitamin E-deficient animals showed that they contained 164 ± 50 IU [7] of vitamin A/g of dry tissue. Since this value is within the published range (11) for animals fed adequate diets, it is unlikely that the observations of this experiment were due to induced vitamin A deficiency.

Fractionation of skin collagen. Table 2 presents data from the first fractionation study. The skin of the vitamin E-deficient animals contained amounts of total soluble collagen that were statistically greater than those present in the skin of their vitamin E-sufficient littermates. The soluble collagen prepared from skin of the vitamin E-deficient animals contained a significantly higher proportion of the acid-soluble fraction than that of the vitamin E-sufficient group. There was less, although not significantly less, neutral salt-soluble collagen per 100 g of collagen isolated from the skins of the vitamin E-deficient animals. Although the differences between the 3 pairs of littermates were small, these differences were statistically significant ($P < 0.05$). It is inconceivable that these differences could be the result of undissolved material present in the filtrate, since this would have to result from preferential contamination of the filtrates from the vitamin E-deficient group. Attention is called to there being no difference in the total amount of collagen produced per 100 g of wet skin by either group. All of the figures reported in table 2 are somewhat higher than the figures commonly reported in the literature. There may be several reasons for this. In the case of the values for total collagen, the higher value may be the result of the total being obtained by summation of the concentration of 3 collagen fractions, which would introduce some experimental error. Some dehydration may also have occurred during storage, or the higher values for the soluble fractions may have resulted from analytical error. However, this error appears to apply uniformly and apparently does not influence the validity of the comparison between the groups.

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[6] Weight gains and vitamin A levels are expressed as average ± SEM.

[7] See footnote 6.

TABLE 2

Total collagen and relative distribution of soluble collagen fractions from the skin of vitamin E-sufficient and vitamin E-deficient animals

Diet group [1]	Neutral salt-soluble	Acid-soluble	Total soluble [2]	Soluble: insoluble ratio	Total collagen
	g/100 g collagen	*g/100 g collagen*	*g/100 g collagen*		*g/100 g wet skin*
Vitamin E-sufficient	10.5	32.0	42.0	0.74	36.8
Vitamin E-deficient	7.4	37.3	44.6	0.81	37.2
Difference	3.1	−5.7	−2.6	−0.07	0.4
P value	< 0.10	< 0.05	< 0.05	< 0.01	< 0.90

[1] Three pairs of littermates in each group.
[2] Data are averages of 3 separate determinations; therefore, the total soluble does not always equal the summation of the acid plus neutral salt-soluble.

TABLE 3

Proportional distribution of sodium chloride-soluble collagen fractions in vitamin E-deficient and vitamin E-sufficient animals

Diet group [1]	Fraction			0.45 M : 1.0 M ratio
	0.14 M	0.45 M	1.0 M	
	g/100 g neutral salt-soluble collagen			
Vitamin E-sufficient	3.1	33.9	62.6	0.54
Vitamin E-deficient	3.1	27.8	68.9	0.40
Average difference	0.0	6.1	−6.2	0.14
P value	—	< 0.10	< 0.05	< 0.05

[1] All values are the average for 5 rats.

Table 3 presents the data from the more extensive fractionation of the sodium chloride-soluble collagen fraction. Significantly greater amounts of the more mature, 1.0 M salt-soluble collagen were present in skins from the vitamin E-deficient animals, whereas there were no significant differences between the groups in the quantity of 0.14 M and 0.45 M salt-soluble collagen present.

Rate of gel formation in neutral salt-soluble and acid-soluble collagen. When heated to 37° the neutral salt-soluble collagen solutions from the vitamin E-deficient group formed gels at a rate which was significantly slower than that of solutions which came from vitamin E-sufficient animals (fig. 1). The lag-time for gelation of the collagen solutions from both groups of animals was about 3 minutes. After this lag period, the gelation curve for the collagen solutions from the control animals rose abruptly and reached a plateau after 20 minutes of incubation. In contrast, the curve for the vitamin E-deficient group rose in a somewhat more gradual manner and did not exhibit as sharp an inflexion point as in the curve from the control group.

Although there were small variations in the amounts of hydroxyproline in the solutions used in the gelation studies, the hydroxyproline content of the solutions from the vitamin E-deficient animals in no case was less than that of the controls. These data suggest that the growth phase of the fibrils in the collagen solutions from the vitamin E-deficient group was somewhat impeded. In both cases, however, gelation was essentially complete after 30 minutes of incubation.

The curves in figure 1 appear to indicate that there is also a difference in the amount of gelation. This may be the case, although there was sufficient variation in individual values that no statistical importance can be placed on the differences between the means at 60 minutes of incubation. When the same experiment was performed using acid-soluble collagen solutions, no vitamin E-dependent differences in the rate of gel formation could be found (fig. 2).

Dissolution of collagen gels at 4°. The vitamin E-deficient group exhibited a highly significant increase in the rate of dissolution of the thermal gels during the first 24 hours of incubation at 4° (table

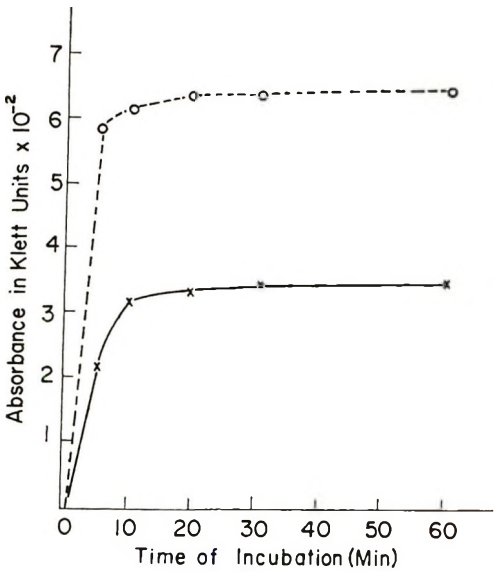

Fig. 1 Rate of gel formation in neutral salt-soluble collagen solutions. The abscissa is the time of incubation in minutes and the ordinate is the absorbance in Klett units $\times 10^{-2}$ per mg of hydroxyproline. The points are the averages of 3 pairs of littermates. The vitamin E-sufficient group is indicated as O-----O, and the vitamin E-deficient group, as ×——×. The differences in the rates of gel formation are significant ($P < 0.05$) for all times up to 30 minutes. The differences at 60 minutes are not significant ($P < 0.10$).

4). This group still showed a significantly greater gel dissolution at 48 hours. The greatest rate of breakdown in gels from the vitamin E-deficient group occurred during the first 24 hours. During this period the gels from the vitamin E-sufficient animals remained rather stable.

To determine whether contaminants of the crude collagen from the vitamin E-deficient group were preventing the formation of stable gels, the experiment was repeated using purified neutral salt-soluble collagen solutions. After 24 hours of incubation at 4° a significant increase in the breakdown of the gels formed from purified collagen solutions derived from vitamin E-deficient animals was observed (table 5). After 48 hours of treatment at 4° no significant difference was observed between the groups. The greatest rate of breakdown of the gels in the vitamin E-deficient group again occurred during the first 24 hours with only a slight increase during the 24- to 48-hour interval. The gels formed from purified collagen solutions from the vitamin E-sufficient group exhibited a greatly increased rate of break-

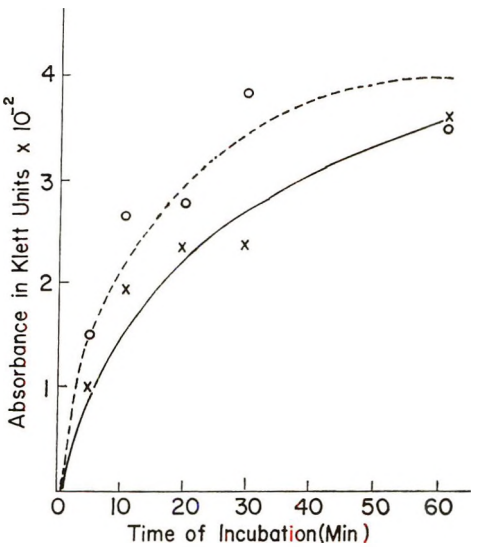

Fig. 2 Rate of gel formation in acid-soluble collagen solutions. The abscissa is the time of incubation in minutes; the ordinate is the absorbance in Klett units $\times 10^{-2}$ per mg of hydroxyproline. The points are the averages of 3 pairs of littermates. The vitamin E-sufficient group is indicated as O-----O, and the vitamin E-deficient group, as ×——×. There are no significant differences in the rates of gel formation.

TABLE 4

Stability of gels formed at 37° from crude, neutral salt-soluble collagen prepared from the hides of vitamin E-sufficient and vitamin E-deficient rats to storage at 4°

Time at 4°	Vitamin E-sufficient	Vitamin E-deficient	Difference	*t* value [1]	P value
hours	*% redissolved*	*% redissolved*	*%*		
24 [2]	11.5	26.2	14.7	5.846	< 0.01
48 [3]	32.7	39.4	6.7	8.291	< 0.02

[1] Student's *t* test.
[2] Four pairs of animals.
[3] Three pairs of animals.

TABLE 5

Stability of gels formed at 37° from purified neutral salt-soluble collagen gels prepared from the hides of vitamin E-sufficient and vitamin E-deficient rats to storage at 4°

Time at 4°	Vitamin E-sufficient [1]	Vitamin E-deficient [1]	Difference	*t* value [2]	P value
hours	*% redissolved*	*% redissolved*	*%*		
24	24.5	33.4	8.9	3.447	< 0.02
48	33.7	37.5	3.8	0.777	> 0.60

[1] Four animals/group.
[2] Student's *t* test.

TABLE 6

Stability of gels formed at 37° from purified acid-soluble collagen prepared from the hides of vitamin E-sufficient and vitamin E-deficient rats to storage at 4°

Time at 4°	Vitamin E-sufficient [1]	Vitamin E-deficient [1]	Difference
hours	*% redissolved*	*% redissolved*	*%*
24	2.1	4.2	2.1
72	9.1	14.9	5.8

[1] Four animals/group.
[2] These differences are not significant by the method of paired comparisons.

down during the first 24 hours when compared with that of gels produced from the crude solutions. Hausmann (15) also noted that gels from purified collagen solutions tended to break down more readily than those formed from crude solutions. Since purification did not negate the results found with crude solutions, the defect in the gel stability of the vitamin E-deficient group appeared to be an expression of a change in the molecule.

Table 6 presents data gathered using purified acid-soluble collagen. No difference in the percentage of gel breakdown could be detected.

DISCUSSION

The evidence found in this study strongly suggests that the metabolism of collagen in the skin of vitamin E-deficient rats was not normal. The studies on gel formation and the stability of the gels formed in soluble collagen solutions give perhaps the best indication that the defect is a change or decrease in intra- and intermolecular crosslinking. Gross (10) reported that there was a decrease in the solubility of collagen gels formed at 37° which was directly related to the length of incubation time. A direct relationship was suggested between the number and nature of the intermolecular crosslinkages formed between the tropocollagen units with the increased incubation time. It was found that if the gels were placed in the cold, that a certain fraction would always return to solution. Wood and Keech (8) found that these thermal gels contained fibrils with the characteristic 640 Å band-spacing and appeared similar to mature collagen fibrils from calfskin. The formation and the stability of these gels to cold has been found to be related to the degree of intramolecular crosslinkage.

Wood (17, 18) found the neutral salt-soluble collagen was heterogeneous and could be separated into three distinct fractions, A, B, and C. These fractions were found to vary with respect to their β- and less mature α-subunit composition. The A fraction had an α-:β- ratio of 4:1, whereas the C fraction had an α-:β- ratio of 2:1. The B fraction contained no β-component. The C fraction, which was found to contain the greatest relative amounts of the more mature β-subunits, was the fraction responsible for nucleation and subsequent fibril growth. Any factor which would interfere with the formation of the C fractions, which apparently are formed by intramolecular crosslinkage, would impede the formation of fibrils. It appears that the slower rate of gelation observed in the collagen solutions from the vitamin E-deficient group was an expression of a lack of this nucleating fraction or a lack of the more mature β-subunit.

The observation that the gelled solutions from the vitamin E-deficient group dissolved more readily may also be indicative of a failure to form B subunits. Wood found that the nucleating fraction remained insoluble, whereas the less mature fractions redissolved readily at 4°.

The inability of the collagen from vitamin E-deficient animals to form normal

intermolecular crosslinks may have resulted from the lack of some extrinsic factor necessary for the formation of normal crosslinks or from the presence of a factor which inhibited the formation of normal collagen fibrils. Alternatively, some intrinsic defect in the collagen molecule from the vitamin E-deficient animals may have been responsible for the observed failure to form crosslinkages. The latter explanation appears to be more tenable since the purified collagen solutions showed the same failure to form stable collagen gels that was noted using crude solutions. The rather rapid rate of the breakdown of the collagen gels from the vitamin E-deficient group during the first 24 hours indicates that the bonds formed by that collagen were quite unstable.

The acid-soluble fraction does not appear to be abnormal either in its gelation rate or its stability to cooling. This would indicate that vitamin E-deficiency affects primarily the newly synthesized, metabolically more active, neural salt-soluble collagen.

The exact nature of the defect in fibril maturation is not known or evident from these data. It is interesting to note that the defects noted in gel stability are almost identical to those reported in lathyrism (15). The authors suggest that quite possibly a common mechanism is affected in both lathyrism and vitamin E-deficiency, producing nearly similar results.

The data from the skin fractionation studies lend further support to the idea that avitaminosis E causes some changes in crosslinkage of the collagen molecule. As the collagen in the rat's skin ages, it forms more intermolecular crosslinkages or becomes more polymerized with a concomitant decrease in the amount of soluble collagen present (19–21). It is generally accepted that the most immature collagen is soluble in either water or neutral salt solutions. As this salt-soluble collagen matures, it may go either to a fraction which is soluble in dilute organic acids, or to an insoluble stage, which is the more mature. Recently evidence has been presented to show that the acid-soluble fraction is not a precursor of mature collagen (22), but rather another metabolic product.

The increase in soluble collagen noted in the vitamin E-deficient animals may be interpreted to be either the result of a blockage in the maturation of the collagen fibril, or of an increase in the rate of breakdown of previously formed, mature collagen. While the data presented do not make clear which modification occurs in avitaminosis E, the authors wish to suggest that a blockage in maturation is responsible for the observed changes. The origin of insoluble collagen may be considered to be the neutral salt-soluble collagen. If a blockage in the formation of insoluble collagen from neutral salt-soluble collagen occurred, a shift to the formation of acid-soluble collagen could be visualized. If, as has been suggested (22), the acid-soluble collagen fraction is not a precursor of insoluble collagen and is relatively inert metabolically, then an accumulation of this fraction could occur.

The mechanism by which vitamin E-deficiency affects collagen metabolism as observed in the fractionation studies is not known. Avitaminosis E has been reported to cause changes in the metabolism of mucopolysaccharides which appear to have some function in collagen metabolism (23, 24). Mathews has recently presented good evidence that mucopolysaccharides are involved in the crosslinkage of the collagen molecule (25). It is possible that avitaminosis E induced changes in mucopolysaccharide metabolism which are responsible for the changes in collagen metabolism. Alternatively, avitaminosis E may cause changes in fibroblast function which are reflected in changes both in the metabolism of mucopolysaccharides and of collagen.

ACKNOWLEDGMENTS

The authors wish to gratefully acknowledge the technical assistance of Rachel Sewell and Jayne Tigert.

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Effect of Ascorbic Acid on Certain Blood Fat Metabolism Factors in Animals and Man [1]

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ABSTRACT The possible relationship between certain fat metabolism disturbances and alterations in ascorbic acid metabolism was studied, including the influence of long-term, heavy-dose ascorbic acid therapy on blood cholesterol, lipoprotein lipase (LPL), and triglycerides in animals and man. One hundred and eighty rabbits were divided into 3 groups: (a) control; (b) fed cholesterol, 100 mg/kg body weight/day; and (c) cholesterol, 100 mg/kg body weight/day, plus ascorbic acid, 150 mg/kg body weight/day for 8 months. The total cholesterol was decreased from 1234 ± 8.8 (SD) in the cholesterol group to 308 ± 4.0 mg/100 ml in animals receiving ascorbic acid. The triglycerides were decreased from 195 ± 9.5 mg/100 ml, average, to 89 ± 1.4 mg/100 ml. The activity of LPL, 0.189 in the cholesterol group, increased almost to the normal level of 0.45 ± 0.02 unit. Histopathologic examination showed pronounced atheromatous-like lesions in the vascular system of the cholesterol group, and mild incipient pathologic alterations in the cholesterol-ascorbic acid group. Six hundred rats were also divided into 3 groups, observed for 8 months and given cholesterol, 100 mg/kg body weight/day, and ascorbic acid, 150 mg/kg body weight/day. The total cholesterol was reduced from 545 ± 4.14 to 170 ± 5.5 mg/100 ml; triglycerides from 142 ± 2.1 to 77 ± 3.2 mg/100 ml; and the LPL activity was increased from 0.184 ± 0.01 to 0.342 ± 0.01 unit. One hundred and twenty-two patients were given ascorbic acid, 2.0 to 3.0 g/day, for 4 to 30 months. In the cardiac patients, showing very low LPL activity, and high blood levels of triglycerides, the response to ascorbic acid therapy was markedly favorable with respect to these 2 factors.

For a number of years, it was believed that hypercholesterolemia was a chief causal factor in atheromatous alterations in blood vessels. Although the possible injurious influence of high plasma cholesterol levels on the vascular system is not excluded completely, more and more attention is given by nutrition scientists to other fat metabolism factors, such as triglycerides and lipoprotein lipase, as possible biological agents responsible for inducing atherosclerosis. By now it is generally accepted that the triglycerides enter the blood during the absorption of fatty meals in the form of lipid particles (chylomicra), which cause the plasma to become turbid. The work of Robinson and French (1), Robinson (2, 3) and others have demonstrated that the clearing reaction of turbid blood is due to the hydrolysis of triglycerides of the chylomicra by lipoprotein lipase (LPL), an enzyme also known as the clearing factor. The free fatty acids released as a result of the LPL action, combine with plasma albumin, forming a soluble molecule. With the disappearance of the chylomicra, the triglycerides are broken down, resulting in "clearing" of the plasma. Barritt (4), Albrink and Man (5), and others reported elevated fasting triglyceride levels in ischemic heart diseases and atherosclerosis. Pilgeram (6) reported a deficiency in LPL in atherosclerosis. It was demonstrated that there is wide distribution of LPL in the body tissues — in the rat heart, spleen, kidney, aortic wall and other organs. Since LPL appears in the blood almost immediately after heparin injection, it was postulated that LPL is located in the capillary wall, a fact which was proved by Robinson (2) experimentally on the capillary bed.

Localization of LPL in the capillary wall suggests that there may be some connection of this enzyme activity with ascorbic

Received for publication July 8, 1966.

[1] Aided by Public Health Service Research Grant no. HE-08505-03 from the National Heart Institute, and by a grant from the American Heart Association, Florida Division, and its Canaveral Area Heart Association.

acid, which exerts certain controlling and regulating influences on capillary functioning. These biological premises served us as a departing point in our present investigation.

Considerable work has been reported on the possible role of ascorbic acid in atherosclerosis and hypercholesterolemia. Aschoff (7) was the first to postulate in his analysis of the nature of atherosclerosis that, as its earliest morphologic lesion, it is a disturbance of the intercellular ground substance of the arterial intima. Willis (8) produced lesions in guinea pig arteries by maintaining the animals with a diet deficient in ascorbic acid. Intraperitoneal injections of ascorbic acid prevented or greatly decreased the lesions of experimental cholesterol atherosclerosis. Ascorbic acid appears to be essential for the maintenance of the physiological integrity of arterial ground substance. According to Yavorsky et al. (9), autopsy showed latent alterations of the ground substance due to ascorbic acid deficiency. Becker et al. (10) reported that the lipid accumulation in the arteries appears to be related to the increased rate of incorporation of ^{14}C-acetate into cholesterol which takes place in ascorbic acid deficiency. Willis and Fishman (11) investigated the arterial ascorbic acid content in cases of sudden death, routine hospital autopsy, and cases treated in the hospital with ascorbic acid prior to death. They concluded that ". . . a gross and often complete deficiency of ascorbic acid frequently exists in the arteries of apparently well-nourished hospital autopsy subjects." Using scorbutic guinea pigs, Willis (12) observed that early lesions of atherosclerosis are quickly resorbed with the use of ascorbic acid therapy, but more advanced lesions are considerably more resistant to reversal. Mueller and Cardon (13) reported a temporary increase in blood fatty acid levels in guinea pigs deprived of ascorbic acid. Booker et al. (14) noted that cholesterol, administered daily for 10 days, decreased plasma and cell ascorbic acid in rabbits, whereas in guinea pigs, plasma ascorbic acid increased as cell ascorbic acid decreased. When cholesterol was administered simultaneously with ascorbic acid, the blood plasma levels of ascorbic acid were less affected in both rabbits and guinea pigs. According to Mendoza (15), intravenous injections of ascorbic acid produced a temporary increase in blood serum cholesterol in 1 to 2 hours which was followed by a decrease. Sedov (16) conducted clinical investigations on the effect of large daily doses of ascorbic acid on blood cholesterol. One-half gram of ascorbic acid given to patients with hypercholesterolemia caused an abrupt decrease in serum cholesterol. Daily intravenous administration of 0.5 to 1.0 g of ascorbic acid for 10 to 30 days resulted in a significant decrease up to 30% in blood cholesterol in 92 of 106 atherosclerotic patients so treated. Similar results were obtained by Fedorova (17). Gandzha et al. (18) treated 96 patients affected with atherosclerosis of various degrees of gravity. Diet, low in fat and cholesterol, iodine therapy, heparin, adenosine triphosphate and ascorbic acid were tested on the patients. Neither the diet itself, nor iodine, heparin or adenosine triphosphate had any significant influence upon blood cholesterol levels. However, ascorbic acid, 0.5 g administered 3 times a day orally, decreased cholesterol 35 to 40%, with an increase of the total albumin and an increase in the albumin-globulin ratio. Several days after ascorbic acid therapy was stopped, the blood cholesterol levels increased again. Ostwald (19) stated that the need for vitamin C as an oxidation-reduction factor increases with age. Myasnikov (20) reported that ascorbic acid prevented the increase of blood cholesterol levels. Cholesterol-fed animals without ascorbic acid increased blood cholesterol levels 238%, and with ascorbic acid only 116%. Clinical administration of ascorbic acid, 1.0 g a day, decreased blood cholesterol levels in patients having persistent hypercholesterolemia. Bavina (21) observed that treating cholesterol-fed rabbits with 0.2 g ascorbic acid orally prevented the development of hypercholesterolemia in the animals.

As a continuation of our investigation on the effect of iodine value and carbon number of fatty acids on blood cholesterol in rabbits (22, 23), it was decided, 5 years ago, to initiate a study on the possible effect of ascorbic acid and related factors

on blood fat metabolism factors: cholesterol, lipoprotein lipase and triglycerides, both in animals (rabbits, rats, and guinea pigs) and humans.

Although the blood levels of cholesterol under the influence of ascorbic acid therapy were fully recorded in our experiment, our chief interest was directed to lipoprotein lipase and its close relationship to blood triglycerides.

EXPERIMENTAL

In the present study on the effect of long-range, heavy-dose ascorbic acid therapy on blood fat metabolism factors, 12 series consisting of 15 rabbits each, and 5 series of 120 rats each were conducted.

MATERIALS AND TECHNIQUES

Animals. New Zealand male rabbits, weighing 1500 to 1600 g and albino male rats, bred in our laboratory, with an average body weight 104 to 106 g, or 70 to 74 days of age, were used in our experiments.

Feeding. Laboratory ration [2] was used for all groups of animals, control or treated. A number of investigators studying experimentally induced cholestorolemia have administered cholesterol in an oil vehicle. The data supplied by Swell et al. (24), Wolf and Brignon (25) and others (26) emphasized that the intestinal absorption of cholesterol and its blood levels depended on the type of oil used as the vehicle. To avoid any possible influence of an oil vehicle on our results, we fed cholesterol to all animals in a water suspension, by pipette. Also some investigators have started their experiments by administering large doses of cholesterol, up to 1.0 g/day, from the first day of their tests. Such a method we feel was injurious to the health of experimental animals. Accordingly we started cholesterol feeding with a small dose of 15 mg/kg body weight /day, gradually increasing the daily dose to 100 mg/kg body weight. Ascorbic acid, 150 mg/kg body weight/day was administered orally, in water solution, by pipette.

Blood testing. In all our tests, blood specimens were taken after 16 hours' fasting. This means that the average blood levels of cholesterol were about 15% higher than in a normal nonfasting condition. Yet this procedure gave us an assurance of the exactness of the values obtained in the more than 2000 blood tests taken by us. Each experimental series continued for 7 to 8 months. Blood samples were taken once a month. In all series, the animals were divided into 3 groups: rabbits, 5 control, 5 fed cholesterol alone, and 5 given both cholesterol and ascorbic acid; rats: 40 control animals, 40 given cholesterol alone, and 40 administered both cholesterol and ascorbic acid.

The following blood factors were estimated in each sample taken either from a rabbit or rat: total cholesterol, free and esterified cholesterol, lipoprotein lipase, triglycerides and serotonin. We included the estimation of serotonin in view of certain evidence that it might contribute to the formation of arterial lesions (24). In 4 series of rabbit tests, the metabolite of serotonin, urine 5-HIAA, was also estimated. No rat urine 5-HIAA was checked.

The estimation techniques were as follows: serum cholesterol, modified method of Lieberman-Burchard (27); lipoprotein lipase, Grossman (28) and McDaniel and Grossman (29); triglycerides, the micromethod of Van Handel and Zilversmit (30); 5-HT and 5-HIAA, 5-HT in blood and tissue (31), urine 5-HIAA (32).

Histopathology. After the series of tests was completed, the animals, rabbits or rats, were killed, and the specimens of aortas, ascending and descending, heart, pulmonary artery, kidney and liver were removed and investigated histopathologically.

RESULTS

It was our practice to increase gradually the daily doses of cholesterol, beginning with 15 mg/kg body weight. As a result of such administration, all rabbits and rats remained in good condition during the 8 months of experimentation, gaining weight slowly, and never losing their appetite, in contrast with investigations when animals were given large doses of cholesterol at once, inducing hypercholesterolemia within 30 to 60 days.

Analysis of our observations as summarized in tables 1, 2, and 3, which covers 180 rabbits and 600 rats, indicates the

[2] Purina Rabbit Chow Checkers, Ralston Purina Company, St. Louis.

TABLE 1

Effect of long-range, heavy-dose ascorbic acid therapy on cholesterol-fed rabbits (series 6)

Month and year	10/64	11/64	12/64	1/65	2/65	3/65	4/65	5/65
Group A, control [1]								
Cholesterol, mg/100 ml	86.2 ± 4.1 [2]	90.2 ± 2.2	96.3 ± 3.3	90.0 ± 3.2	88.2 ± 2.3	92.0 ± 2.2	90.0 ± 3.0	93.0 ± 3.2
Lipoprotein lipase, units	0.578 ± 0.02	0.583 ± 0.04	0.572 ± 0.01	0.540 ± 0.01	0.570 ± 0.03	0.580 ± 0.04	0.570 ± 0.02	0.573 ± 1.04
Triglycerides, mg/100 ml	27.5 ± 1.5	25.4 ± 1.2	23.4 ± 2.1	22.2 ± 1.1	24.0 ± 2.2	23.3 ± 2.0	24.5 ± 2.1	22.3 ± 1.8
Serotonin, units/ml	1.26 ± 0.3	1.35 ± 0.5	1.4 ± 0.5	1.5 ± 0.6	1.8 ± 0.3	1.5 ± 0.4	1.7 ± 0.3	1.6 ± 0.3
Group B, cholesterol [3]								
Cholesterol, mg/100 ml	105 ± 2.1	182 ± 5.5	270.5 ± 3.2	333 ± 5.6	484 ± 6.0	880 ± 9.2	1160 ± 8.5	1390 ± 9.5
Lipoprotein lipase, units	0.538 ± 0.05	0.521 ± 0.02	0.495 ± 0.02	0.432 ± 0.05	0.298 ± 0.06	0.266 ± 0.06	0.203 ± 0.01	0.198 ± 0.04
Triglycerides, mg/100 ml	29 ± 1.5	38 ± 2.3	48 ± 2.4	79 ± 5.2	114 ± 2.3	144 ± 3.3	188 ± 2.1	201 ± 5.2
Serotonin, units/ml	1.3 ± 0.21	1.4 ± 0.2	1.3 ± 0.05	1.6 ± 0.65	1.7 ± 0.3	1.8 ± 0.3	2.07 ± 0.03	2.3 ± 0.03
Group C, cholesterol and ascorbic acid [4]								
Cholesterol, mg/100 ml	90.0 ± 5.2	138 ± 2.2	142 ± 5.5	155 ± 1.5	224 ± 4.2	235 ± 4.4	260 ± 3.0	280 ± 3.0
Lipoprotein lipase, units	0.565 ± 0.02	0.542 ± 0.02	0.531 ± 0.02	0.52 ± 0.03	0.502 ± 0.03	0.499 ± 0.02	0.451 ± 0.03	0.444 ± 0.03
Triglycerides, mg/100 ml	27 ± 2.1	28 ± 2.0	30 ± 2.5	40 ± 2.2	65 ± 2.1	76 ± 3.3	89 ± 2.2	90 ± 1.3
Serotonin, units/ml	1.25 ± 0.05	1.28 ± 0.12	1.38 ± 0.01	1.4 ± 0.02	1.48 ± 0.05	1.62 ± 0.22	2.01 ± 0.05	2.3 ± 0.2

[1] Five rabbits.
[2] Average for 5 animals ± SD.
[3] Five rabbits, cholesterol, 100 mg/kg body weight/day.
[4] Five rabbits, cholesterol, 100 mg/kg body weight/day and ascorbic acid, 150 mg/kg body weight/day.

TABLE 2

Effect of long-range, heavy-dose ascorbic acid therapy on cholesterol-fed rabbits (average for 12 series of tests)

Months	1	2	3	4	5	6	7	8
Group A, control [1]								
Cholesterol, mg/100 ml	84.0 ± 2.2 [2]	79.0 ± 2.0	85.3 ± 1.2	85.0 ± 4.4	82.2 ± 2.1	88.2 ± 3.3	83.4 ± 1.1	88.5 ± 2.2
Lipoprotein lipase, units	0.581 ± 0.01	0.582 ± 0.02	0.577 ± 0.01	0.565 ± 0.03	0.566 ± 0.04	0.566 ± 0.04	0.555 ± 0.01	0.576 ± 0.04
Triglycerides, mg/100 ml	28 ± 2.1	27.2 ± 1.2	23.4 ± 2.2	25.3 ± 1.2	25.2 ± 1.1	27.3 ± 3.2	23.2 ± 1.2	26.2 ± 1.2
Serotonin, units/ml	1.3 ± 0.1	1.2 ± 0.2	1.5 ± 0.4	1.3 ± 0.2	1.5 ± 0.3	1.4 ± 0.3	1.5 ± 0.2	1.4 ± 0.2
Group B, cholesterol [3]								
Cholesterol, mg/100 ml	103 ± 3.2	176 ± 2.3	255 ± 4.3	320 ± 4.4	465 ± 2.2	810 ± 5.4	1020 ± 6.5	1234 ± 8.8
Lipoprotein lipase, units	0.56 ± 0.03	0.524 ± 0.03	0.48 ± 0.03	0.41 ± 0.03	0.27 ± 0.02	0.25 ± 0.03	0.21 ± 0.04	0.189 ± 0.03
Triglycerides, mg/100 ml	25 ± 2.0	31 ± 3.2	51 ± 4.2	80.5 ± 1.5	105 ± 5.5	175 ± 5.5	176 ± 7.6	195 ± 9.5
Serotonin, units/ml	1.2 ± 0.6	1.2 ± 0.3	1.3 ± 0.4	1.7 ± 0.2	1.4 ± 0.7	1.6 ± 0.5	2.1 ± 0.1	2.0 ± 0.3
Group C, cholesterol and ascorbic acid [4]								
Cholesterol, mg/100 ml	88 ± 2.2	142 ± 2.3	151 ± 4.2	161 ± 5.2	210 ± 4.4	240 ± 3.4	270 ± 5.5	308 ± 4.6
Lipoprotein lipase, units	0.56 ± 0.03	0.57 ± 0.01	0.521 ± 0.01	0.52 ± 0.02	0.49 ± 0.03	0.47 ± 0.02	0.46 ± 0.02	0.45 ± 0.02
Triglycerides, mg/100 ml	26 ± 1.9	30 ± 1.0	31 ± 2.2	41.2 ± 1.3	59.9 ± 1.1	70.5 ± 1.1	90.2 ± 2.2	89 ± 1.4
Serotonin, units/ml	1.2 ± 0.1	1.3 ± 0.1	1.34 ± 0.02	1.5 ± 0.03	1.7 ± 0.01	1.8 ± 0.2	1.8 ± 0.1	2.1 ± 0.2

[1] Sixty rabbits.
[2] Average for 12 series ± SD.
[3] Sixty rabbits, cholesterol, 100 mg/kg body weight/day.
[4] Sixty rabbits, cholesterol, 100 mg/kg body weight/day and ascorbic acid, 150 mg/kg body weight/day.

TABLE 3

Effect of long-range, heavy-dose ascorbic acid therapy on cholesterol-fed rats (average for 5 series of tests, 120 rats each)

	Initial	2 months	4 months	6 months	8 months
		Group A, control [1]			
Cholesterol, mg/100 ml	42 ±2.2 [2]	40.2 ±1.2	41 ±2.2	48.5 ±2.3	54.5 ±3.3
Lipoprotein lipase, units	0.37 ±0.02	0.36 ±0.01	0.372±0.02	0.369±0.01	0.37 ±0.02
Triglycerides, mg/100 ml	28 ±1.2	27 ±1.1	28 ±1.4	29.2 ±1.1	30.1 ±1.2
Serotonin, units/ml	0.18 ±0.02	0.17 ±0.01	0.16 ±0.02	0.17 ±0.03	0.17 ±0.04
		Group B, cholesterol [3]			
Cholesterol, mg/100 ml	51 ±1.3	82 ±3.4	140 ±5.4	386 ±3.3	545 ±4.4
Lipoprotein lipase, units	0.35 ±0.03	0.33 ±0.03	0.32 ±0.01	0.228±0.03	0.164±0.01
Triglycerides, mg/100 ml	25 ±1.4	31.2 ±1.1	77 ±1.2	118 ±3.3	142 ±2.1
Serotonin, units/ml	0.16 ±0.03	0.16 ±0.02	0.24 ±0.01	0.34 ±0.01	0.38 ±0.01
		Group C, cholesterol and ascorbic acid [4]			
Cholesterol, mg/100 ml	53.2 ±1.3	66.2 ±3.3	70.5 ±2.2	148 ±3.5	170 ±5.5
Lipoprotein lipase, units	0.346±0.04	0.352±0.02	0.355±0.01	0.331±0.002	0.340±0.01
Triglycerides, mg/100 ml	26 ±1.2	28.2 ±2.1	54.2 ±1.3	68 ±3.3	77 ±3.2
Serotonin, units/ml	0.15 ±0.04	0.16 ±0.02	0.22 ±0.01	0.23 ±0.02	0.24 ±0.01

[1] Two hundred rats.
[2] Average for 5 series ± SD.
[3] Two hundred rats, cholesterol, 100 mg/kg body weight/day.
[4] Two hundred rats, cholesterol, 100 mg/kg body weight/day and ascorbic acid, 150 mg/kg body weight/day.

uniformity of the reaction pattern to ascorbic acid therapy for the cholesterol-fed rabbits and rats. In all series of tests the effect of ascorbic acid feeding was the same, with expected natural individual deviations of no scientific significance.

Table 1 shows the values for series 6 with rabbits. In the group of cholesterol-fed rabbits, the blood content of cholesterol increased gradually from the normal level of 105 mg up to 1390 mg after 8 months. Yet there was no significant change in the LPL activity for the first 3 months. The activity remained stationary between 0.538 and 0.495. Only when the actual hypercholesterolemia developed, did a steady decrease in LPL activity occur, reaching a level below 0.2 units at 8 months. The blood content of triglycerides increased slowly, almost paralleling the total cholesterol increase.

The effect of ascorbic acid therapy was insignificant on the LPL activity during the first 3 months, and only thereafter did its effectiveness become well-pronounced. At the eighth month, the cholesterol-fed rabbits had an LPL activity of 0.198 unit, whereas in the group given ascorbic acid it was 0.444 unit, only slightly lower than in the control rabbits.

The ascorbic acid therapy decreased the total cholesterol level from 1390 to 280 mg/100 ml and the triglyceride content from 201 to 90 mg/100 ml average. There was no significant influence of ascorbic acid on blood serotonin level. In both groups, cholesterol alone and cholesterol with ascorbic acid, there was a definite increase in blood serotonin: from 1.3 to 2.3 and from 1.25 to 2.3 units, respectively.

Table 2 summarizes the results of all 12 series of tests. The values presented indicate that the reaction patterns to ascorbic acid therapy for cholesterol-fed rabbits was similar, if not identical, to those of table 1. Here again the activity of LPL in the cholesterol group remained stationary for the first 3 months. When, however, the total cholesterol reached the level of 320 mg/100 ml, a gradual decrease in LPL became evident, reaching an activity of 0.189 units at the end of 8 months. With ascorbic acid administered, the LPL activity remained at much higher levels, with 0.45 units at the end of the experiments. The average values for 12 series show that the total cholesterol level of 1234 mg/100 ml for the cholesterol groups was reduced by ascorbic acid administration to 308 mg/100 ml, and of the triglyceride content from 195 to 89 mg/100 ml. The summarized values of all 12 series with rabbits indicate the same phenomenon as described in series 6. Ascorbic acid exerted no significant influence on the LPL activity during the first 3 months. Its effect was well-pronounced after hypercholesterolemia developed.

HISTOPATHOLOGY OF RABBITS

The specimens of blood vessels, heart and other organs were taken after the experiments of each series had been completed, 8 months after the tests were started. Since there were certain variations and individual deviations in rabbit pathology in all 3 groups, the present brief summary of pathologic changes presents their average picture.

The following stains were used: Giemsa, PAS, Weigert's, Spicer's, hematoxylin-eosin, and Sudan III.

Control group. No treatment was given. The animals were maintained with laboratory ration. Generally speaking, there were very few pathologic deviations in the vascular system of control rabbits. In several rabbits, luminal thickening of the pulmonary arteries was observed. Sudanophilic development in the aorta intima, as described by Bragdon (33) was detected in only five out of sixty control rabbits examined.

Cholesterol-fed groups. Ascending aorta. The whole lumen of the ascending aorta was considerably narrowed by closely adherent masses, composed of large foam cells, negative with PAS, and metachromatic reactions. The intima was poorly discernible and partially destroyed as was the media. The elastic membrane was fragmented along the whole wall. Around its fragments there were numerous metachromatic and PAS-positive substances containing lipids. There were pronounced fibrotic changes, an advanced intimal fibro-elastic type with lamellar fragmentation and mucopolysaccharide accumulation. *Descending aorta (thoracic).* The intima was considerably swollen with the elastic membrane fragmented; there was lipid deposition, and the masses of foam cells with endothelial cells were partially destroyed and swollen. *Descending aorta (abdominal).* There was pillow-like thickening of the artery wall, about 1.5-fold. The endothelial cells were swollen. Moderate mucopolysaccharide accumulation was noted, and the elastic membrane was partially destroyed and replaced in such places by intensive metachromatic substance. *Pulmonary artery.* The pulmonary arteries showed severe luminal narrowing by masses of subintimal lipid histiocytes. A concentration of large foam cells projected into the artery lumen. The intima was swollen, and the elastic membrane was partially destroyed. *Coronary arteries.* The coronary arteries were considerably involved. There was marked luminal narrowing due to a homogeneous deposition of lipid material in the subendothelial coat. Intramyocardial branches were affected with only small changes in the larger epicardial coronary arteries. The pericardium showed fibrosis and inflammatory infiltration. *Liver.* Lipid deposition was observed in the liver lobules within the hepatic polygonal cells. Many hepatic cells were swollen and vacuolated. *Kidney.* A moderate lipid deposition was noted.

Comment: The rabbits fed cholesterol for 8 months, 100 mg/kg body weight/day, showed pronounced atheromatous-like pathologic changes in the vascular system.

Cholesterol and ascorbic acid-fed groups. Ascending aorta. The whole aorta was slightly and irregularly swollen, with

small nuclei of foam cells detectable. The aortic lumen was not narrowed. The endothelial cells were mostly intact. The elastic membrane showed no fragmentation except in the upper part of the media. There was no mucopolysaccharide accumulation. *Descending aorta (thoracic).* No luminal narrowing was noted. The intima showed no fragmentation of elastic membrane. Several nuclei of foam cells were visible. *Descending aorta (abdominal).* There was slight thickening of the arterial wall, about 1- to 1.25-fold. The endothelial layer was moderately swollen in several places, and a slight deposition of lipids was noted. *Pulmonary arteries.* There was a slight narrowing of arterial lumen. Small nuclei of subintimal lipid histiocytes were noted and a few small-to-moderate concentrations of foam cells. *Coronary arteries.* A very slight luminal narrowing with a few lipid deposition nuclei in the subendothelial was seen. Intramyocardial branches were not affected, nor were the epicardial branches. The pericardium showed slight fibrosis. *Liver.* There was a moderate deposition of lipids in the hepatic cells. *Kidney.* No abnormalities were observed.

Comment: The rabbits fed cholesterol, 100 mg/kg body weight/day and ascorbic acid 150 mg/kg body weight/day for 8 months, showed fewer pathologic changes of the vascular system than the rabbits fed cholesterol alone. The pathologic changes observed in this group of animals may be termed as belonging to the initial or incipient phase of atheromatous-like condition.

Table 3 summarizes the results of our experiments with rats. Five series of tests were conducted, each composed of 3 groups of 40 rats. There is considerable similarity of the reaction pattern of cholesterol-fed rats given ascorbic acid to the reaction pattern observed in rabbit tests. The LPL activity remained almost stationary for the first 3 months both in the cholesterol-fed and cholesterol-ascorbic acid groups. The LPL activity was 0.32 to 0.35 units and 0.346 to 0.355 units, respectively. When, however, the total cholesterol level increased to 140 mg/100 ml in the cholesterol-fed group, the gradual decline in the LPL activity was well-pronounced, reaching 0.184 unit at the eighth month, whereas in the group receiving ascorbic acid, the value was almost normal: 0.342 units at the end of the experiment. The total cholesterol level of the cholesterol-fed group was 545 mg/100 ml at the eighth month. In the group given ascorbic acid it was reduced to 170 mg/100 ml, and with the triglycerides, levels decreased from 142 to 77 mg/100 ml. All animals appeared to be in good general condition, having gained normal weight and showing good appetite. Although the blood total cholesterol levels in the ascorbic acid-treated group were considerably higher than in the control, nontreated group (170 against 54.5 mg/100 ml, respectively), the activity of LPL was only slightly deviated in these 2 groups: control, 0.37 units, against 0.342 units in the ascorbic acid group.

The results of the present study on rabbits and rats show a uniformity of the reaction pattern of cholesterol-fed animals given ascorbic acid therapy. In not a single test was there any significant or even moderate deviation from the general rule. In all tests conducted for less than 4 months of cholesterol-feeding (100 mg/kg body weight/day), the effect of ascorbic acid was only slightly, if at all, pronounced. When, however, hypercholesterolemia developed, the influence of ascorbic acid was definitely evidenced. The blood fat metabolism factors investigated, total cholesterol, triglycerides, and LPL, were controlled or normalized to a considerable degree. The factor responding most strongly to ascorbic acid administration was LPL, which remained at an almost normal level in the ascorbic acid-fed group. This appears to indicate that ascorbic acid enhances the activity of LPL.

CLINICAL [3]

Although there was uniformity of the blood fat metabolism factors in the response of cholesterol-fed rabbits and rats to ascorbic acid therapy, quite different results were observed with humans. Here we observed great variety in the reaction pattern with this therapy.

During 4 years, 122 patients were under our observation for periods of from 4 to 30

[3] The clinical trials were conducted in cooperation with the Polk County Board of Health and Hospital, Florida.

months. They were given ascorbic acid daily, from 1.5 to 3.0 g. Blood tests were made every 3 to 4 weeks.

In addition, 55 placebo-treated patients served as controls. These patients in their deviations of fat metabolism factors, when observed, corresponded to the ascorbic acid-treated group. The results of our clinical observations are summarized in table 4. They represent 6 groups of patients, each with corresponding placebo-treated patients as controls. Group 1-A, 30 patients of a younger age, 18 to 35, and with the fat metabolism within normal limits apparently did not benefit from the ascorbic acid therapy, 2.0 g daily for 4 months. Group 2-A, 18 older patients, 45 to 70 years, with a mild deviation or almost normal fat metabolism did not respond significantly to this therapy, applied for 6 months, 2.0 g/day. Group 3-A, 24 patients, 47 to 72, responded moderately for 10 months to ascorbic acid administration, 2.0 g daily. As an average, the total cholesterol decreased from 272 to 221 mg/100 ml; the LPL activity increased from 0.30 to 0.39 unit; and triglycerides were reduced from 122.5 to 77.2 mg/100 ml. Group 4-A, with pronounced fat metabolism disturbance, covering 25 patients, age 53 to 73 and given 2.0 g ascorbic acid daily for 12 months, showed a more pronounced beneficial effect of this therapy. The total cholesterol level was reduced from 324 to 241 mg/100 ml, average; the LPL activity increased from 0.21 to 0.35 unit, whereas triglycerides decreased from 174.3 to 102.3 mg/100 ml, average. Group 5-A, 7 patients, 67 to 77 years of age, with similar fat metabolism deviations and given 2.0 g of ascorbic acid daily for 12 months, did not benefit from this therapy. Total cholesterol was reduced only slightly from 338 to 320 mg/100 ml, average. There was no significant increase in the LPL activity, 0.24 and 0.29 unit; triglycerides were lowered about 7%, from 156.3 to 146.1 mg/100 ml, average. Group 6-A, a group of 18 cardiac patients, 58 to 72 years of age, gave the most markedly beneficial response. The patients were given ascorbic acid, 2.0 to 3.0 g, for 15 to 24 months. The total cholesterol decreased from 320 to 245 mg/100 ml, average, the LPL activity was almost tripled, from 0.12 to 0.36 unit, and triglycerides were reduced from 209.4 to 102.3 mg/100 ml.

This group of cardiac patients, ten with one coronary occlusion and eight with two occlusions, were given ascorbic acid therapy from 15 to 60 days after the last attack, at which time their blood fat factors were estimated. In all cases but one, their LPL activity was very low, ranging from 0.05 to 0.18 unit. In one case it was 0.21. In six cases, 0.5 to 0.7 unit; in five, 0.07 to 0.10 unit; and in six, 0.10 to 0.14 unit. Triglycerides were considerably elevated in 14 cases, above the level of 250 mg/100 ml, while in 4 cases the range was be-

TABLE 4

Effect of ascorbic acid therapy on fat metabolism factors in man (177 cases)

Group	No. of patients	Age range	Ascorbic acid	Duration of therapy	Lipoprotein [1]					
					Cholesterol		Lipase		Triglycerides	
					Prior	After	Prior	After	Prior	After
		years	*g/day*	*months*	*mg/100 ml*		*units*		*mg/100 ml*	
1-A	30	18–35	2.0	5–6	204	200	0.41	0.42	67.2	65.6
1-B	10	17–36	— [2]	6	198	202	0.43	0.41	68.2	67.2
2-A	18	45–70	2.0	8	246	238	0.372	0.38	93.0	88.5
2-B	10	44–68	—	8	236	244	0.38	0.36	88.2	90.5
3-A	24	47–72	2.0	10	272	221	0.30	0.39	122.5	77.2
3-B	11	48–70	—	10	266	271	0.29	0.31	124.2	125.5
4-A	25	53–73	2.0	12	324	241	0.21	0.35	174.3	102.3
4-B	11	50–75	—	12	332	319	0.23	0.26	168.4	155.2
5-A	7	67–77	2.0	12	338	320	0.24	0.29	156.3	146.1
5-B	5	69–78	—	12	345	333	0.21	0.23	164.3	159.5
6-A	18	58–72	2.0–3.0	15–24	320	245	0.12	0.36	209.4	102.3
6-B	8	62–75	—	16	342	323	0.15	0.14	165.3	156.5

[1] Average values.
[2] Placebo-treated patients.

tween 100 to 225 mg/100 ml. The total cholesterol level showed great variety, from 250 up to 400 mg/100 ml. In 16 cases, there was an impressive uniformity in the LPL response to ascorbic acid therapy, which was started with 3.0 g/day, and reduced in 4 months to 2.0 g/day. As in all our experimental and clinical observations reported previously, the response appeared slowly in these cases as well. For the first 2 months of therapy, there was only a slight increase in the LPL activity. But by the third month of therapy, a gradual steady increase in the clearing factor activity was in evidence, bringing it to the level of 0.39 to 0.40 unit or more. The triglycerides followed the same pattern. The total cholesterol response to ascorbic acid treatment was not uniform. Only in the cases with original total cholesterol levels above 280 mg/100 ml was there a definite reduction in it.

The following 2 cases are described as illustrating the effects of long-range ascorbic acid administration on cardiac patients having greatly disturbed fat metabolism.

Case no. 9-28. Male, white, lawyer, 55 years of age; overweight. He had had two coronary occlusions within 2 years and a mild stroke, occurring shortly before the second attack. The treatment was started 20 days after the second attack while he was still hospitalized. He was receiving a restricted animal fat diet, and was given a daily dose of ascorbic acid for the first 4 months, 3 g. The dose was later decreased to 2 g/day. The patient was observed for about 2 years. The condensed report as shown in table 5 covers the first year of treatment.

In this case, there was a striking increase in the activity of the LPL which was close to zero for two to three months after the cardiac attack: from 0.07 to 0.34 unit. At the end of the second year of treatment, the LPL reached an almost normal level of activity of 0.41 unit, with the total cholesterol below 250 mg/100 ml and triglycerides between 75 to 90 mg/100 ml.

The following case was referred to us by Dr. William Hodges, cardiologist of the General Hospital in Lakeland, Florida.

Case no. 13-21. Female, white, age 64, with a history of diabetic background and diabetes of 30 years' duration. She had had gallbladder surgery and 2 coronary attacks in 1960 and 1964. Her general condition was poor. After the blood testing showed an unusually high level of triglycerides and a low activity of LPL, she was given ascorbic acid, 3 g/day, for 5 months. Table 6 summarizes the blood testing.

For the first 2 months, there was no significant change in her blood fat factors. The triglycerides remained at a level of 1000 to 710 mg/100 ml. But by the fifth month of therapy, there was a sharp improvement in the condition of the patient, with the triglycerides decreasing to 250

TABLE 5

Effect of ascorbic acid therapy on a cardiac patient

Month and year	12/63	2/64	4/64	6/64	8/64	10/64	12/64
Total cholesterol, mg/100 ml	355	330	310	290	265	270	258
Lipoprotein lipase, units	0.07	0.08	0.12	0.16	0.22	0.28	0.34
Triglycerides, mg/100 ml	245	240	220	205	186	178	145
Blood sugar, mg/100 cm^3	115	112	123	105	103	99	110
Blood pressure, mm Hg	155/78	143/78	148/82	154/79	165/88	152/78	145/77

TABLE 6

Effect of ascorbic acid therapy on a patient with triglyceridemia

Month and year	10/65	11/65	12/65	1/66	2/66
Total cholesterol, mg/100 ml	334	316	280	310	279
Lipoprotein lipase, units	0.122	0.140	0.227	0.227	0.371
Triglycerides, mg/100 ml	1050	1000	710	700	250
Serotonin, units/ml	0.28	0.28	0.14	0.16	0.18
Blood pressure, mm Hg	140/68	140/68	154/74	138/60	130/66

mg/100 ml and the activity of LPL increasing from the original 0.122 unit to 0.371 unit. The total blood cholesterol decreased only slightly (about 17%).

DISCUSSION

The observations presented in this paper indicate that long-range, heavy-dose ascorbic acid therapy tends to normalize considerably, but not completely, the disturbances in certain blood fat metabolism factors, such as total cholesterol, triglycerides and LPL activity. In experimental animals, rabbits and rats, the reaction pattern of these factors with ascorbic acid therapy was very uniform. In man, there were certain individual deviations in the response to this therapy.

The intrinsic mechanism of this influence of ascorbic acid is complex, and is apparently associated with alteration in the metabolism of this vitamin.

Other observations in the current investigation, which will be presented in a second paper, point to a marked decrease of ascorbic acid in hepatic cells in serious disturbances of fat metabolism, and to an increase of glutamic-pyruvic and glutamic-oxalacetic transaminases, and some involvement of the pituitary-adrenal axis.

The appearance of ascorbone, or reduced ascorbic acid, in increased amounts in the serum of animals and man with altered fat metabolism might also give us a proper interpretation of the salutary effect of this vitamin under such conditions.

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Effect of Some Dietary Factors and Drugs on Cholesterol Concentration in the Egg and Plasma of the Hen [1,2]

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ABSTRACT Studies were undertaken to determine the effect of dietary lipids and drugs on plasma and egg yolk cholesterol in the laying hen. Egg cholesterol concentrations were increased significantly when hens were fed diets containing either safflower oil, hydrogenated safflower oil, or coconut oil at a level of 30%, but only slightly when corn oil or animal fat was fed. Safflower oil at levels up to 15%, however, did not increase egg cholesterol. Egg cholesterol concentrations did not parallel blood levels when the different types of fat were fed except in cases where hypercholesterolemia was produced by feeding diets containing cholesterol. Increases in plasma and egg cholesterol due to feeding diets containing cholesterol (1%) were enhanced by dietary inclusion of either safflower oil (29%), lecithin (5%), or Tween 80 (5%), which increased the absorption of cholesterol. β-Sitosterol (1%) retarded the increase in plasma and egg cholesterol concentrations caused by dietary cholesterol. Feeding high or low vitamin A diets or niacin supplementation (0.1%) did not result in significant changes of the egg cholesterol content or counteract the effect of safflower oil. Administration of D-thyroxine by injection or as a dietary supplement resulted in decreased blood and increased egg cholesterol content.

Cholesterol metabolism in the laying hen has been studied by determining the effect of diet on the levels of blood and egg cholesterol. Previous work in this laboratory indicated that the cholesterol content of egg yolk increased as the unsaturation of the egg yolk lipids increased when hens were fed diets containing either safflower oil or linseed oil at a level of 30% (2). Various dietary fats included in the hen's diet up to a level of 30% have been reported to have little influence on the egg cholesterol content (3–9), and an increase has been reported by Combs and Helbacka (9) upon feeding corn oil at a level of 10%. Different dietary fats have been reported to have no effect on serum or plasma cholesterol levels (3–5, 10–13). Decreases upon feeding either 20% sunflower oil (14) or soybean oil at levels of 20% (14) and 12% (6), and increases with 10% tallow (15) and 5 to 10% animal fat (16) have been reported.

Cholesterol added to the hen's diet has been reported to increase egg cholesterol levels (8, 17–22). There are also reports of no increase in the egg cholesterol content when either cholesterol (23) or dried egg yolk (24) was fed. There is disagreement on whether feeding cholesterol to hens results in a significant increase in the concentration of blood cholesterol (8, 19–22, 25). Stamler et al. (26) showed that the greater resistance of hens to dietary-induced hypercholesterolemia, compared with cocks, was abolished by oviduct ligation. Cholesterol fed to hens in conjunction with dietary fats increased both blood and egg cholesterol levels (8, 21, 22). It is possible that surface-active agents, such as lecithin and polyoxyethylene sorbitan monooleate (Tween 80),[3] can influence cholesterol absorption in the laying hen. Tween 80 fed to rats in diets containing cholesterol enhanced the absorption of the latter (27). β-Sitosterol and other plant sterols, which are believed to interfere with cholesterol absorption, reduce hypercholesterolemia in man and ex-

Received for publication July 15, 1966.

[1] Part 2 of a series entitled "The Effect of Dietary Fat and Other Factors on Egg Yolk Cholesterol." A preliminary report of this investigation has been presented (1).

[2] This work was supported in part by grants from the National Institutes of Health, U. S. Public Health Service (training grant number 5 T1 ES 17), the Ohio Poultry Research Fund, and the Ohio Agricultural Research and Development Center.

[3] Atlas Powder Company, Wilmington, Delaware.

perimental animals, including cockerels (28,29). Even in the absence of dietary cholesterol, ingested β-sitosterol was found to decrease cholesterol concentrations in the serum, aortas, and livers of aged hens (30).

The use of ethyl *p*-chlorophenoxyisobutyrate (CPIB), nicotinic acid, and D-thyroxine as hypocholesterolemic agents has been reviewed (31). Nicotinic acid ingestion has been reported to decrease blood cholesterol levels in chicks fed diets with (32) and without (33) added cholesterol. Both L-thyroxine and the D-analogue, which is one-fifth as active, were found to stimulate liver cholesterol synthesis, release and peripheral turnover in the thyroidectomized chick, similar to that found in the euthyroid animal (34). It has been suggested that some of the dietary effects upon blood cholesterol levels may be mediated through the thyroid gland (35).

Conflicting reports have been given concerning the effect of vitamin A on cholesterol levels of humans and experimental animals. Vitamin A administered to aged, atherosclerotic hens had an anti-atherosclerotic effect, but only a slight change in serum cholesterol concentrations was observed (36). Large amounts of vitamin A fed to cockerels reduce the hypercholesterolemia resulting from cholesterol feeding (37–39). This response may be mediated through some effect on cholesterol absorption. Vitamin A deficiency has been reported to block cholesterol biosynthesis in rat liver (40,41), although this is in dispute. Vitamin A-deficient chickens probably do not have this metabolic block (42).

The present communication reports studies on the effect on egg and blood cholesterol levels of a variety of dietary lipids, other dietary factors, and drugs, which may affect cholesterol metabolism.

TABLE 1

Percentage composition of basal diets

	Low fat control	High fat basal	Low fat, low vitamin A basal	High fat, low vitamin A basal
Ground yellow corn	68.6	—	—	—
Ground wheat	—	—	70.5	—
Ground oat hulls	—	4.0	—	4.0
Soybean meal (44%)	10.9	37.5	11.5	37.5
Wheat middlings	2.0	—	2.0	—
Meat and bone scrap	2.5	3.0	2.5	3.0
Menhaden fish meal	2.5	3.0	2.5	3.0
Dried whey product	2.5	3.0	2.5	3.0
Alfalfa meal	2.5	3.0	—	—
Low fluorine rock phosphate	2.0	3.0	2.5	3.0
Feeding limestone	5.0	5.0	5.0	5.0
Iodized salt	0.5	0.5	0.5	0.5
Fat	—	30.0	—	30.0
Calcium silicate absorbant [1]	—	7.0	—	7.0
Premix	1.0	1.0	1.0	1.0
Premix for basal rations:				
Vitamin A, 20,000 IU/g	1.1	1.7	—	—
Vitamin D_3, 3,000 ICU/g	3.5	5.3	3.5	5.3
Riboflavin, 44mg/g	0.8	1.2	0.8	1.2
Ca pantothenate, 70 mg/g	0.6	0.8	0.6	0.8
Vitamin B_{12}, 53 μg/g	1.3	2.0	1.3	2.0
DL-Methionine	5.5	8.3	5.5	8.3
Butylated hydroxytoluene, 250 mg/g	5.5	11.0	5.5	11.0
Chlortetracycline supplement, 22 μg/g	5.5	8.3	5.5	8.3
Manganese sulfate	2.6	4.0	2.6	4.0
Choline chloride, 250 mg/g	—	44.0	—	44.0
Sucrose	73.6	13.5	74.7	15.2

[1] Micro-Cel E from Johns-Manville Corporation, Cleveland.

EXPERIMENTAL

White Leghorn hens, previously given a low fat control diet (table 1), were fed the diets and treated with the drugs listed in table 2. The low fat control diet contained 16% crude protein and 2110 kcal of productive energy per kilogram. It is a standard practical diet known to support excellent reproductive performance. The high fat diets contained 30% fat at the expense of starch from ground yellow corn. The protein, mineral and vitamin content of the high fat diet was increased in proportion to the total increase in caloric density of the diet. Hence the ratio of all critical nutrients to energy content of the diet was approximately the same in both diets. With the supplementary methionine added, both diets provided the minimal amino acid requirements of the laying hen. Plasma and egg yolk cholesterol concentrations were compared before and after treatment. Egg yolks were extracted and cholesterol determined by a modification of the Zlatkis method (2), which eliminated the interference of fatty acids. Blood samples from the hens' brachial veins were collected in heparinized tubes. One-half milliliter of plasma was saponified for each cholesterol determination. Plasma cholesterol values for hens that had ceased egg production are not included in the averages unless otherwise noted.

In experiment 1, the treatment period was 3 weeks. Two or three eggs from each

TABLE 2

Drug and dietary treatments

Designation	Treatment
LF	Low fat, 16% protein control
7.5 SO	7.5% safflower oil,[1] 3/4 low fat basal, 1/4 high fat basal
15 SO	15% safflower oil, 1/2 low fat basal, 1/2 high fat basal
30 SO	30% safflower oil (iodine value 150) high fat basal
HSO	30% hydrogenated safflower oil [2] (iodine value 75) high fat basal
CCO	30% coconut oil,[2] high fat basal
AF	30% animal fat,[3] high fat basal
CO	30% corn oil,[4] high fat basal
MO	30% menhaden oil,[5] high fat basal
C	1% cholesterol, low fat basal
C/SO	1% cholesterol, 29% safflower oil, high fat basal
L	5% lecithin, low fat basal
C/L	1% cholesterol, 5% lecithin, low fat basal
T	5% Tween 80 (polyoxyethylene sorbitan monooleate),[6] low fat basal
C/T	1% cholesterol, 5% Tween 80, low fat basal
S	1% β-sitosterol,[6] low fat basal
C/S	1% cholesterol, 1% β-sitosterol, low fat basal
S/SO	1% β-sitosterol, 29% safflower oil, high fat basal
C/S/SO	1% cholesterol, 29% safflower oil, 1% β-sitosterol, high fat basal
S/L	1% β-sitosterol, 5% lecithin, low fat basal
A−	16% protein, low vitamin A basal
A+	Low vitamin A basal, vitamin A(9091 IU/kg)
SO/A−	High fat, low vitamin A basal, 30% safflower oil
SO/A+	High fat, low Vitamin A basal, 30% safflower oil, vitamin A (9091 IU/kg)
N	Low fat basal, niacin (0.1%)
N/SO	Niacin (0.1%), 30% safflower oil, high fat basal
D-t	Low fat basal, D-thyroxine [7] (20, 40, 80 μg/100 g body wt) injected
NaD-t	Low fat basal, sodium D-thyroxine hydrate [8] (0.8, 1.6 mg/100 g diet)
D-t/SO	D-thyroxine (20 μg/100 g body wt), 30% safflower oil, high fat basal
CPIB	Ethyl *p*-chlorophenoxyisobutyrate [9] (0.1%, 0.2%, 0.4%, 0.6%), low fat basal

[1] Pacific Vegetable Oil Corporation, Richmond, California.
[2] Procter and Gamble. Cincinnati.
[3] Swift and Company. Columbus, Ohio.
[4] Capital City Products Company, Columbus, Ohio.
[5] Marine Products Company, Port Monmouth, New Jersey.
[6] Nutritional Biochemicals Corporation, Cleveland.
[7] Sigma Chemical Company, St. Louis.
[8] (8.36% moisture content), Baxter Laboratories,Inc., Morton Grove, Illinois.
[9] Ayerst Laboratories, Rouses Point, New York.

hen were collected before and after treatment. Pooled egg yolks from individual hens were analyzed.

In experiment 2, the treatment period was one month for most dietary groups. The low and high vitamin A groups were treated for the extended period of 8 weeks. The group given D-thyroxine in experiment 2 was first injected with 40μg/100 g body weight/day for 4 weeks, and the dosage was doubled for 3 additional weeks. Hens were treated with CPIB for 7 weeks. The second blood sample was taken after 4 weeks in all cases. In experiment 3, the treatment period was 3 weeks. In experiments 2 and 3, the egg yolks were pooled from 3 or 4 hens at a time using 2 eggs from each hen. In both these experiments, the hens were fasted 3 to 4 hours before blood samples were taken.

Egg yolk extracts from hens fed the low fat control diet and diets containing either 30% safflower oil or 5% lecithin and 1% β-sitosterol were analyzed for β-sitosterol. The nonsaponifiable material was purified by thin-layer chromatography (2). The sterol fraction was analyzed using a Barber-Colman, Model 20 gas chromatograph equipped with a hydrogen-flame detector and a glass column (182 cm by 4 mm) packed with 3% by weight QF-1 on Gas Chrom Q, 100/120 mesh.[4] The carrier gas was nitrogen at 70 ml/minute and the column was operated at 240°.

RESULTS AND DISCUSSION

Egg and plasma cholesterol concentrations are shown in table 3. The analysis of variance (43) was used to determine whether the treatments in the 3 experiments affected egg cholesterol. No statistical analysis of the plasma cholesterol values was performed because of the large variation in individual values. Basal egg cholesterol concentrations are considerably more constant than the basal plasma cholesterol levels. The wide variability in hen blood cholesterol levels, which may be a result of the rapid fat metabolism, differences in ovulation periods, and other factors, have been discussed (10, 44). The laying hen is probably not a very effective experimental animal to use for the determination of the effect of hypocholesterolemic agents on blood cholesterol levels, alone. It is useful, however, to compare plasma cholesterol levels with egg cholesterol levels in studying lipid metabolism in the hen. The actively laying hen "excretes" approximately 4 g of fat daily over a period of time, and because of this egg-laying capacity, the lipid metabolism of the hen must be considered distinct from that of other experimental animals, including immature female birds and male birds of various ages. In general, it can be assumed that when a hen is fed its usual low fat diet, the mechanisms for lipid biosynthesis in the tissues of the hen are regulated to meet the demands of egg production.

Dietary fats. Diets containing safflower oil at a level of either 7.5% or 15% had little effect on the egg cholesterol concentration. This may explain why some investigators, who have fed diets containing fats at levels less than 30%, have not observed increases in egg yolk cholesterol. The importance of using a colorimetric method for the determination of egg cholesterol that eliminates interfering chromogens has been noted (2). Significant increases in the egg yolk cholesterol concentration were obtained by feeding diets containing the following fats at levels of 30%: safflower oil, characterized by a high linoleic acid content; safflower oil, which had been hydrogenated to one-half its original iodine value containing a higher level of 18:1 and 18:0 acids; and coconut oil, characterizel by a high lauric and myristic acid content. There were small, nonsignificant increases when either animal fat or corn oil was fed. In experiment 2 there was little difference due to treatment in the average plasma cholesterol concentration of the groups fed diets containing fat. In experiment 1, when the plasma values of individual hens were compared with their egg values before and after treatment, the changes were not always parallel.

The hens fed menhaden oil ceased egg production after 2 weeks. This diet was consumed poorly, and either low consumption of the diet or some property of

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

TABLE 3

Effect of dietary factors and drugs on plasma and egg cholesterol

Treatment [1]	Exp. no.	No. hens/ treatment	Plasma values		Egg values	
			Before	After	Before	After
			mg/100 ml	*mg/100 ml*	*mg/g yolk*	*mg/g yolk*
LF	1	3	208 ± 72 [2]	146 ± 13	13.1 ± 0.4	12.7 ± 1.3
	2	6	110 ± 21	114 ± 25	11.9 ± 0.5	12.5 ± 0.9
	2	8			11.6 ± 0.1	12.3 ± 0.3
	3	8	117 ± 21	131 ± 30	12.1 ± 0.0	11.9 ± 0.1
7.5 SO	2	6	125 ± 31	121 ± 28	12.2 ± 0.4	12.3 ± 0.6
15 SO	2	6	124 ± 38	89 ± 31	12.5 ± 0.7	12.5 ± 0.0
30 SO	1	3	133 ± 17	111 ± 16	13.0 ± 0.8	15.6 ± 0.2 [3]
	2	6	122 ± 47	111 ± 24	11.6 ± 0.4	13.7 ± 0.6 [3]
HSO	1	3	128 ± 17	158 ± 18	13.1 ± 0.2	17.0 ± 1.8 [3]
CCO	1	3	144 ± 43	228 ± 71	12.1 ± 0.2	14.9 ± 0.2 [3]
AF	1	3	155 ± 38	189 ± 52	12.7 ± 0.3	13.7 ± 0.3
	2	6	113 ± 19	114 ± 20	11.5 ± 0.2	12.3 ± 0.1
CO	2	6	109 ± 9	106 ± 38	11.7 ± 0.0	12.8 ± 0.2
C	1	3	125 ± 21	184 ± 14	12.2 ± 0.2	19.9 ± 2.6 [3]
C/SO	1	3	82 ± 20	257 ± 24	12.2 ± 0.4	30.2 ± 2.1 [3]
L	1	3	116 ± 2	114 ± 59	13.2 ± 0.6	12.7 ± 0.4
C/L	1	3	141 ± 38	279 ± 65	13.7 ± 0.2	27.6 ± 1.5 [3]
T	1	3	132 ± 68	103 ± 44	12.4 ± 0.6	12.5 ± 0.6
C/T	1	3	104 ± 23	286 ± 113	11.8 ± 0.1	22.2 ± 0.2 [3]
S	1	3	114 ± 29	130 ± 66	12.4 ± 0.5	12.0 ± 0.2
C/S	1	3	137 ± 8	107 ± 22	12.5 ± 0.6	15.0 ± 0.3 [3]
S/SO	1	3	133 ± 72	103 ± 32	12.9 ± 1.7	18.1 ± 2.2 [3]
C/S/SO	1	3	101 ± 22	154 ± 71	12.5 ± 0.7	25.7 ± 0.9 [3]
S/L	2	4	95 ± 16	76 ± 8	12.2	10.9
A −	1	3	132 ± 47	91 ± 15	13.4 ± 0.6	13.5 ± 0.7
	2	4	127 ± 47	138 ± 15	11.6	12.8
A +	1	3	140 ± 39	86 ± 30	12.7 ± 0.1	12.7 ± 0.6
	2	4	111 ± 11	107 ± 14	12.1	12.5
SO/A −	1	3	121 ± 40	179 ± 96	12.6 ± 0.8	16.0 ± 0.9 [3]
	2	4	112 ± 32	115 ± 28	11.3	13.2
SO/A +	1	3	102 ± 6	86 ± 20	12.6 ± 0.6	14.9 ± 0.6
	2	4	114 ± 14	130 ± 13	11.2	12.7
N	1	3	135 ± 40	135 ± 37	12.5 ± 0.7	12.5 ± 0.8
N/SO	1	3	120 ± 35	131 ± 8	12.9 ± 1.1	15.8 ± 0.4 [3]
D-t 20 μg	1	3	137 ± 37	109 ± 66	12.1 ± 0.2	13.6 ± 0.8
40 μg	2	8	114 ± 30	68 ± 17	11.8 ± 0.5	15.4 ± 0.6 [3]
80 μg	2					15.4 ± 3.3 [3]
NaD-t 0.8 mg	3	8	118 ± 73	87 ± 11	12.1 ± 0.1	14.0 ± 0.4
1.6 mg	3	8	118 ± 26	94 ± 54	12.2 ± 0.2	15.3 ± 0.8 [3]
D-t/SO	1	3	137 ± 37	103 ± 34	13.2 ± 0.6	16.8 ± 0.6 [3]
CPIB 0.1%	2	8			11.9 ± 0.1	13.5 ± 0.7
0.2%	2	8			12.3 ± 0.3	12.9 ± 0.0
0.4%	2	8			11.4 ± 0.6	13.7
0.2%	3	8	74 ± 19	58 ± 3	11.8 ± 0.8	12.5 ± 0.3
0.4%	3 [4]	8	131 ± 21	111 ± 43	12.3 ± 0.5	12.8 ± 0.6

[1] These abbreviated designations of the treatments are explained in table 2.
[2] Average values ± SD.
[3] Differences due to treatment are statistically significant ($P < 0.05$).
[4] Cholesterol determined in last egg laid by each hen; plasma values from hens that had ceased egg production.

the oil may have been responsible for the failure in egg production.

A number of hypotheses can be proposed to explain the increase in egg cholesterol that was observed in the absence of any consistent effect on the cholesterol concentration in the blood of the hens that were fed various high fat diets. The increase in egg cholesterol, observed for example when safflower oil was fed, would be equivalent to an increase in cholesterol excretion of approximately 30 mg/day. In a preliminary study, this increase in egg cholesterol was maintained for long

periods of time. There was no appreciable change in egg production or egg yolk weight (approx. 20 g) when the high fat diets, other than the diet containing menhaden oil, were fed. The diets containing unsaturated fat were essentially free of cholesterol. A possible explanation for the increase in egg cholesterol content would be the increased elimination of cholesterol from the total labile body pool. It is difficult to conceive of a loss of 30 mg/day from the body pool after the hens had been fed the diet for three or four weeks.

Another explanation for the increase in egg cholesterol would be a change in the method of excretion of body cholesterol, i.e., decreased conversion of cholesterol to bile acids or decreased fecal excretion of cholesterol. There is evidence from work with other experimental animals, however, that feeding unsaturated fat increases the biliary excretion of cholesterol and fecal excretion of bile acids derived from cholesterol catabolism (45).

A number of reports have indicated that cholesterol synthesis is increased when various fats are fed to rats. Some investigators have related the decrease in lipogenesis that occurs upon fat feeding (46) to the increase in cholesterol synthesis. Possibly the acetoacetate pool is diverted to cholesterol synthesis when lipogenesis is blocked (47,48) or the relationship may be more complex (49,50). A high caloric intake per se has been related to increased sterol synthesis in the rat (51). In the present study, the caloric intake of hens fed the high fat diets increased approximately 5% above that of the hens fed the low fat diets. It is doubtful whether the metabolizable energy derived from the high fat diets is 5% higher than that derived from the low fat diet. The possibility that increased cholesterol synthesis in the liver or ovaries of the hen results in the increase in egg cholesterol has been investigated in subsequent studies.

It is not known why the feeding of fats such as corn oil and animal fat did not increase the egg cholesterol content as much as did safflower oil, hydrogenated safflower oil and coconut oil. Saturated fatty acids are not utilized well by the hen for energy (52). The absorbability of palmitic and stearic acids by the hen is low. The distribution of fatty acids in chicken fat and egg yolk fat resembles more closely the distribution of fatty acids in animal fat and corn oil than the distribution of fatty acids in the other fats that were fed. If the depression of lipogenesis following the ingestion of fat is a homeostatic mechanism, it is possible that different dietary fatty acids may affect the lipogenic control mechanisms to varying degrees depending on the needs of the organism. Some fatty acids may be deposited unchanged in the tissues and egg yolk of the chicken. It is possible that some of the high fat diets would provide more energy or substrate for cholesterol synthesis than others. Dietary fatty acids could also affect the transport of newly synthesized cholesterol from the liver to the developing ova, if the speculated increase in cholesterol synthesis is true.

Cholesterol-containing diets. The ability of hens to eliminate dietary cholesterol via the egg, reported by others, was confirmed in experiment 1. The addition of either safflower oil at a level of 29%, or the surface-active agents, Tween 80 and lecithin at a level of 5%, to a diet containing cholesterol increased the absorption of cholesterol, with the result that the plasma and egg cholesterol levels were increased to a greater degree.

When cholesterol was added to the diet at a level of 1%, the approximate percentage increase in the egg cholesterol content was 60%. With added Tween 80, the increase was 90%, with lecithin it was about 100%, and with safflower oil it was 150%. Much of the dietary cholesterol can be accounted for in the egg, depending upon the nature of other dietary components which can aid in cholesterol absorption. Diets containing either Tween 80 or lecithin without added cholesterol did not affect the egg cholesterol content. The increase observed upon feeding cholesterol with safflower oil does not appear to have resulted from the added effects of safflower oil and dietary cholesterol but was probably caused by an increased absorption or transport of cholesterol. Other investigators have also reported a synergistic effect of dietary fat on the increase

in egg cholesterol content caused by dietary cholesterol (8,21,22).

In the cases where hypercholesterolemia was observed, the increased egg cholesterol levels paralleled the increased blood levels. It has been suggested that increases in egg cholesterol must be preceded by increases in blood cholesterol concentrations, as occurs when diets containing both fat and cholesterol are fed to hens (21, 22). The present study indicates that this is not always the case (e.g., diets containing either 30% safflower oil or 1% cholesterol and 1% sitosterol can increase the egg cholesterol content without increasing the concentration of blood cholesterol).

β-*Sitosterol.* The question arose as to whether the increase in egg cholesterol observed when oils were fed was caused by plant sterols in the oils. The diet containing 1% β-sitosterol had little effect on plasma or egg sterol concentrations. The colorimetric method used for the determination of cholesterol does not differentiate between cholesterol and sitosterol, and if considerable absorption of sitosterol had occurred it should have been detected by the method used. The addition of safflower oil or lecithin to the diet containing sitosterol did not appear to increase its absorption. The amount of plant sterols contained in the oils was small compared with the 1% level fed in this study and probably had very little effect, if any, on the sterol content of the egg. When 1% sitosterol was added to the diets containing cholesterol, however, it appeared to decrease the absorption of cholesterol. The hypercholesterolemia and high egg cholesterol levels resulting from feeding cholesterol or cholesterol with oil were reduced when sitosterol was included in the diet.

Little work has been published on plant sterol absorption in the chicken. Wood et al. (22) could not detect β-sitosterol by paper chromatographic analysis in the serum or eggs from hens fed diets containing corn oil at a level of 10%.

In the present study, no detectable β-sitosterol was found in the egg yolk sterols from hens fed the low fat control diet, safflower oil at a level of 30%, or the diet containing 1% sitosterol and 5% lecithin (designed to increase sitosterol absorption) by gas chromatographic analysis. It is possible, however, that a very small amount of plant sterol could have escaped detection in the presence of a large amount of cholesterol.

In Finland, Miettinen [5] provided direct evidence of plant sterol absorption in the hen by the detection of plant sterols, other than cholesterol and Δ-7-cholesterol, by gas chromatographic analysis. Campesterol, stigmasterol, and β-sitosterol totaled 1.2% of the sterol in a batch of commercial eggs. Dietary ergosterol can also be deposited in the egg yolk (53,54). In view of the poor absorption of plant sterols by humans, it might be desirable to develop an egg in which much of the cholesterol was replaced with phytosterol. This appears to be a formidable, if not impossible, task.

Vitamin A, niacin. Niacin supplementation or diets high or low in vitamin A content had little effect on plasma and egg cholesterol concentrations; nor did they counteract the effect of safflower oil feeding on the egg cholesterol concentration (table 3). The vitamin A deficiency symptoms including loss of egg production developed earlier when the hens were fed a high fat, low vitamin A diet than when the low fat, low vitamin A diet was fed.

D-*Thyroxine.* One of the most pronounced changes that occurred in blood and egg cholesterol levels resulted from subcutaneous injection of D-thyroxine. In experiment 2, after a month of administration of 40 μg/100 g body weight/day, the plasma cholesterol concentrations of all the hens in the group were decreased to a very low level. The egg cholesterol content was increased (about 30%/g yolk) and after the dosage was doubled, the increase in egg cholesterol was maintained. If elimination of cholesterol via the egg can be considered an excretory mechanism in the hen, these observations on the effects of D-thyroxine may be in agreement with the hypothesis that thyroxine stimulates cholesterol turnover (34). In experiment 3, approximately equivalent amounts of sodium D-thyroxine, as were administered in experiment 2, were fed for 3 weeks. The egg cholesterol concen-

[5] Personal communication from Dr. T. A. Miettinen, Institute of Medical Chemistry, University of Helsinki, Helsinki, Finland.

tration increased again. The higher level of Na D-thyroxine in experiment 3 caused an increase similar to that obtained when either level of D-thyroxine was injected in experiment 2. The oral treatment did not result in a decrease of plasma cholesterol to the same extent as that obtained in experiment 2. Effective potency and length of treatment may be involved. In general, hens with a greater pre-experimental plasma cholesterol level exhibited the greatest decrease.

Secondary effects may be involved in the egg cholesterol increase. In experiments 2 and 3, the higher dosages of D-thyroxine affected egg production adversely, and in experiment 3, the lower dosage also had a slight adverse effect. Usually a decrease in egg yolk weight was noted, e.g., in experiment 3, there was an average decrease of yolk weight of approximately 9% when the lower level was fed, and of approximately 14% when the higher level was fed. A decrease in yolk size also has been observed when dessicated thyroid was fed to hens (55). Even though the weight of the egg yolks was decreased, the cholesterol content in the total egg was still higher when D-thyroxine was administered. An increase in egg cholesterol is not inconsistent with small egg yolks. An inverse relationship between the size of the egg yolk and its cholesterol concentration has been observed (56,57). The cholesterol concentration in ovarian follicles increases very rapidly during the early period of development, but after the follicles reach 2.5 g, the cholesterol content increases at a slower rate (58). D-Thyroxine could conceivably cause an increase in egg cholesterol by a combination of induced premature ovulation and increased deposition of cholesterol in the ova. The individual thyroid activity in a hen with its seasonal variation probably influences the level of egg production, egg yolk size, and yolk cholesterol content.

Ethyl p-*chlorophenoxyisobutyrate.* CPIB ester was first tested at a level or 0.6% in experiment 2 and egg production ceased after 3 days, but resumed after the drug was withdrawn. The data for egg cholesterol suggest that feeding lower dosages for 7 weeks may result in an increased egg cholesterol concentration. Levels of 0.2% CPIB ester and higher affected egg production adversely. In experiment 3, treatment with CPIB ester resulted in a greater detrimental effect on egg production. When 0.2% CPIB ester was fed, one-half of the hens were still in egg production after 3 weeks. It is difficult to interpret the effect of the drug on plasma cholesterol levels, since the pre-experimental levels were low in this group. When 0.4% CPIB ester was fed, in experiment 3, egg production ceased within a week. Often when there is a cessation in egg production for a short period of time, an increase in the blood cholesterol of the hen is observed. Plasma cholesterol values obtained after the hens in the 0.4% CPIB ester group had ceased laying are included in table 3, since they show that the plasma cholesterol level may approach a basal value when the hen ceases egg production.

It may be useful to study the qualitative and quantitative changes which might occur in egg sterols due to treatment of hens with hypocholesterolemic drugs, as well as any effect on egg production, since the latter is a sensitive indicator of the reproductive processes. Triparanol affects ova maturation and the cessation of egg production results, as well as causing the accumulation of desmosterol in the egg (59). If one of the actions of CPIB ester is inhibition of cholesterol synthesis, although at a different stage than triparanol, one possibility is that sex hormone production also is inhibited.

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Free Choice Consumption of Spiced Diets by Rats [1]

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ABSTRACT A series of experiments was conducted in which rats were fed various spices in concentrations ranging from 5 to 0.05%. Weanling rats fed a spiced diet and then given a choice with an unspiced diet ate little of the spiced diet during the free-choice situation. A group of mature rats consumed approximately equal amounts of a spiced and an unspiced diet when given a choice, whereas a group of young rats consumed significantly less of the spiced diet. These results indicate that rats have a change in taste sensitivity with respect to spice with advancing age.

Studies of food preferences by rats have dealt largely with sugars and alcohol. Richter and Campbell (1) demonstrated that rats given a choice had the greatest preference for solutions of maltose, next for glucose and sucrose and only a slight preference for galactose and none or an actual aversion for lactose. Rats restricted to alcohol for a period of time preferred alcohol when given a choice between alcohol and water (2). No similar studies have been made with spices.

In a review of the nutritional aspects of spices and flavorings, Mukerji (3) stated that spices may have an effect on appetite stimulation and gastrointestinal physiology, although few scientific studies have been made. It is well known that in many parts of the world significant amounts of spices are added to foodstuffs and an unspiced diet is considered to be bland and unappealing. The present study was designed to test the relative acceptability of diets containing various spices to rats, and also to determine whether rats, as humans, would prefer a spiced diet to an unspiced one after being restricted for a period of time to the spiced diet.

MATERIALS AND METHODS

Experiment 1. Male weanling rats of our departmental strain were divided at random into groups of 4 and fed diets containing a well-blended mixture of a control diet [3] and one of the following spices: cloves, cinnamon, or ginger in a 5% concentration. A fourth group received the unspiced control diet. The rats fed spiced diets were restricted to these diets for 3 weeks, after which another food cup containing the control diet was placed in each cage. The 2 food cups were available to the rats for the next 3 weeks and the position of the food cups in the cage was altered frequently to prevent habitual eating from one cup.

Experiment 2. Weanling rats, 6 per group, were fed spiced diets as in experiment 1. The spices included black pepper, cloves and cinnamon, each in a 0.5% concentration. The spiced diets were fed for 5 weeks followed by a 2-week free-choice period.

Experiment 3. Six groups of weanling rats, 5 per group, were fed spiced diets containing cinnamon, curry powder or black pepper in a 0.5% or 0.05% concentration for a period of 4 months. Another group of 10 rats was given the unspiced control diet. After the 4-month restricted period the rats fed the spiced diets were given a free-choice period of 2 weeks as in previous experiments. Five of the rats fed the unspiced diet were given a choice between the unspiced diet and the 0.5%

Received for publication June 30, 1966.

[1] Published with the approval of the Director of the Hawaii Agricultural Experiment Station as Technical Paper no. 813.

[2] National Science Foundation Junior Science Apprentice.

[3] Control diet: (g/kg) powdered skim milk, 212; whole wheat flour, 39.4; white flour, 91; cornmeal, 91; brown ricemeal, 91; soybean flour, 61; brewer's yeast, 18; cottonseed oil, 28; cod liver oil, 2; iodized salt, 6; and salt mixture, 6. The salt mixture contained: (in %) Ca carbonate, 89; ferric citrate, 10; and manganous sulfate, 1.

cinnamon diet, and the remaining 5 rats continued to be fed the unspiced diet and served as controls.

Experiment 4. A group of 5 rats, 8 weeks of age, weighing approximately 250 g, and a group of 5 young rats, 2 weeks of age, weighing approximately 80 g, were given a choice between the unspiced diet and a 0.5% cinnamon diet. Both groups had previously been fed the unspiced diet. The free-choice period was 4 weeks.

RESULTS AND DISCUSSION

The rats ate and grew well with the 5% ginger, cloves, and cinnamon. However, when given a choice between the spiced diet and the unspiced diet, they ate little or none of the spiced diet (table 1). These rats had a definite preference for the unspiced diet, although the apparent dislike for the spice did not prevent them from consuming sufficient food when only the spiced diet was available. There appeared to be no pronounced preference for any of the individual spices.

In the second experiment in which the spice concentrations had been reduced to 0.5%, the results were similar to those in experiment 1 in that the rats ate very little of the spiced diet in the free-choice situation (table 1). This indicated that the rats were able to detect the spice at these concentrations but had a marked preference for the unspiced diet.

To test the possibility that the restricted period in experiments 1 and 2 had not been long enough to build up a preference for the spice, a 4-month restriction period was used in experiment 3. Table 2 shows the food intake of the various spices during the restricted and free-choice periods. In this experiment the rats ate considerably more of the spiced diet than in previous experiments. It might appear, therefore, that the longer period in which the rats were restricted to the spice had caused them to have less aversion for the spice. However, the rats restricted to the unspiced diet and then given a free choice with a spiced diet ate approximately the same amount of the spiced diet as those

TABLE 1
Free-choice consumption of spiced and unspiced diets

	Spiced diet	Unspiced diet	% of total as spiced diet
	g/day	*g/day*	
	Experiment 1 [1]		
Spice, 5%			
Ginger	1.0	13.7	6.8
Cloves	0.8	13.8	5.5
Cinnamon	0.6	14.6	4.0
	Experiment 2 [2]		
Spice, 0.5%			
Black pepper	0.5	13.1	3.7
Cloves	1.8	11.4	13.6
Cinnamon	0.8	12.6	6.0

[1] Restricted period, 3 weeks.
[2] Restricted period, 5 weeks.

TABLE 2
Food intake during free-choice period in experiment 3 [1]

	Food consumed		% of total as spiced diet	
	0.5% spice	0.05 % spice	0.5% spice	0.05 % spice
	g/day			
Curry	18.4	16.6	22.4	24.0
Cinnamon	18.6	17.5	29.5	27.1
Black pepper	18.8	16.5	22.3	18.2
Unspiced diet [2]	18.6		22.0	

[1] Restricted period, 4 months.
[2] Rats had unspiced diet during restricted period and free choice with 0.5% cinnamon.

TABLE 3

Free-choice food intake by young and mature rats

	Unspiced diet	Spiced diet	% of total as spiced diet
	g/day	*g/day*	
Young rats	11.6 ± 1.5 [1]	7.0 ± 0.6 [1,2]	37.1
Mature rats	9.7 ± 3.8	10.7 ± 2.8 [3]	52.4

[1] Mean ± SE of mean.
[2] Significantly different from unspiced diet at the 1% level of confidence.
[3] Not significantly different from unspiced diet.

restricted to the spiced diets. It seemed possible that the age of the rats had influenced the consumption of the spiced diets. Wallgren and Forsander (2) observed that older rats had a greater preference for alcohol than younger rats. The possibility that the taste preference was different in young and older rats was tested in experiment 4. Table 3 shows that the young rats ate less of the spiced diet than the mature rats. The difference between the mean intake values of the spiced and unspiced diets was significant ($P < 0.01$) for the young rats but not significant for the mature rats. Since the mature rats ate approximately equal amounts of the spiced and unspiced diets, they may have been unable to detect the spice in the diet. Olefaction may also play a role in the preference aversion of the rats for the spiced diets. Strehler (4) has stated that with advancing age there is a decreased sensitivity to both taste and smell. It may well be that both odor and taste are involved in the acceptance of the spiced diets by the rats.

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gram(s)	g
international unit(s)	IU (*to be used only when weight can not be given*)
kilogram(s)	kg
liter(s)	(spell out)
meter(s)	m
microgram(s)	μg (not γ)
micromicrogram(s)	μμg
microcurie(s)	μCi
micron(s)	μ
micromicron(s)	μμ
micromolar (unit of concn)	μM
micromole (unit of mass)	μmole
milligram(s)	mg
milligrams %	(*never use*)
milliliter(s)	ml
millimeter(s)	mm
millimicrogram(s)	mμg
millimicron(s)	mμ
millimole(s)	mmole
molar (mole per liter)	M
parts per million	ppm
per cent	%
probability (in statistics)	P
square centimeter	cm^2

[1] Style Manual for Biological Journals 1960 American Institute of Biological Sciences, 2000 P street, N. W., Washington 6, D. C.

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standard deviation	SD
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t (Fisher's test)	*t*
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[2] See footnote 1.

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